BPB Reports

Report

Involvement of Rho in Methylmercury-Induced Morphological Change in Cultured Astrocytes Obtained from Rat Cerebral Hemisphere

Hideaki Shimizu, Wataru Aoki, Takaaki Fujieda, and Tatsumi Adachi*

Faculty of Pharmacy, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan
Received November 2, 2023; Accepted November 27, 2023

We recently found that the morphology of dibutyryl cyclic AMP (dbcAMP)-stimulated cultured cerebral hemisphere astrocytes changed from stellate to polygonal within 3 h after methylmercury (MeHg) exposure at 2 and 3 μM. To elucidate the mechanism of this change (spreading) in astrocytes induced by MeHg, the effects of inhibitors of Rho and its downstream effector, Rho kinase, and the expression levels of total and active RhoA were investigated under serum-free conditions in the presence of dbcAMP. Pretreatment with C3 transferase (a Rho inhibitor) completely inhibited the spreading of astrocytes induced by MeHg at both doses for 3 h. However, pretreatment with Y-27632 (a Rho kinase inhibitor) inhibited the spreading induced by MeHg at 2 μM, but not by that at 3 μM. Expression levels of total RhoA were similar under all conditions examined, including in the presence of MeHg. In contrast, the expression level of active RhoA in astrocytes exposed to MeHg at 2 or 3 μM for 30 min was markedly higher than that in astrocytes exposed to solvent alone, although no difference was observed in the level between astrocytes exposed to MeHg at either dose. In addition, the active RhoA levels in MeHg-exposed astrocytes at both doses were similar to the level in astrocytes maintained in 15% serum-containing medium. These results suggest that RhoA activation is involved in the change in shape of astrocytes induced by MeHg at 2-3 μM, and that Rho kinase is, at least partly, related to the shape change in its downstream signal transduction.

Key words methylmercury, astrocyte, morphology, Rho-dependent signaling pathway

INTRODUCTION

Mercury is a hazardous metal, that is ubiquitous in the environment.[1] One typical mercury compound, methylmercury (MeHg), passes through the blood-brain barrier and damages the central nervous system (CNS).[2] Astrocytes play many important roles in the CNS, including maintaining neuronal survival and regulating neurotransmitters.[3] For example, a majority of the uptake of glutamate (a major excitatory transmitter), which produces neuronal injury,[4] in the CNS is assumed to be due to its transporters, L-glutamate/L-aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1), in glial cells, particularly astrocytes.[5] It is well known that glutamate uptake into astrocytes is inhibited by MeHg.[6,7] In addition, MeHg-induced neuronal cell damage is suppressed by MK-801, a non-competitive antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, particularly in the cerebral cortex.[8,9] Therefore, neuronal cell degeneration caused by MeHg likely involves both glutamate and astrocyte dysfunction in that brain region.

We have examined the influence of mercury compounds including MeHg on cell death and morphology in cultured astrocytes under several culture conditions. Susceptibility to cell death from MeHg is similar in the cerebral hemisphere and in cerebellar astrocytes,[10] whereas a region-dependent difference is observed in morphological change induced by MeHg exposure at 3 μM in astrocytes maintained in a serum-free defined medium (SFDM) containing 0.5 mM dibutyryl cyclic AMP (dbcAMP).[11] In cerebral hemisphere astrocytes, the morphology of almost all astrocytes changed from stellate to polygonal within 3 h after MeHg exposure at more than 2 μM.[12] It has been demonstrated that spreading of cortical astrocytes is induced by lysophosphatidic acid (LPA)[13,14] and endothelins[15] in the presence of cAMP analogs. The fact that the glutamate uptake and expression levels of GLAST are lower in endothelin-caused polygonal astrocytes than in stellate astrocytes in the presence of dbcAMP[15] led us to speculate that dysfunction of astrocytes caused by MeHg-induced spreading may enhance glutamate-related neuronal cell damage. However, neither the mechanism nor significance of the MeHg-induced astrocyte shape change has been elucidated, although the influence of MeHg on astrocyte shape is suggested to be similar to that of LPA, including stress fiber formation and a region-dependent difference.[11]

It has been demonstrated that the spreading of astrocytes induced by LPA is inhibited by pretreatment with C3 transferase (a Rho inhibitor), which specifically inhibits the Rho subfamily (RhoA, B and C) of small GTP-binding proteins by ADP-ribosylation of small GTPases of these proteins.[13,14] Since these results suggest that LPA-induced shape changes in astrocytes are regulated by Rho, the spreading of astrocytes caused by MeHg might also be related to this molecule.

In the present study, the effects of C3 transferase or Y-27632, an inhibitor of Rho kinase (Rho-associated coiled-

*To whom correspondence should be addressed. e-mail: tadachi@cis.ac.jp

coil forming kinase, ROCK), a downstream effector of Rho,[16,17] on MeHg-induced spreading in dbcAMP-stimulated astrocytes prepared from the cerebral hemisphere were investigated to elucidate the mechanism of the shape change. The expression levels of total and active RhoA were also determined in astrocytes under several conditions, including in the presence of MeHg.

MATERIALS AND METHODS

Animals Wistar rats obtained from CLEA Japan Co. (Tokyo, Japan) were maintained at 22.5 ± 2.5°C and 55 ± 10% relative humidity under a 12-h light/dark cycle, and were given standard laboratory chow and tap water *ad libitum*. Pregnant rats were prepared as previously described,[10,18] and housed individually until birth. The protocol for animal experiments was approved by the animal experimentation committee of Chiba Institute of Science. All care and experimental procedures were carried out according to the fundamental guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan (Notice No. 71 of 2006).

Cell Culture and Treatment Astrocyte cultures were prepared from the cerebral hemispheres of newborn rats (within 24 h after birth) as previously described.[12] Astrocytes were plated on poly-L-lysine (PLL; Sigma, St. Louis, MO, USA)-coated culture plates (BD Bioscience, Billerica, MA, USA) or dishes (BD Bioscience), and maintained with 15% fetal calf serum (FCS; Invitrogen Co., Carlsbad, CA, USA)-containing medium (FCScM), the composition of which was previously described.[10-12] Cells on 24-well or 6-well plates or 10 cm dishes were used for the experiment using inhibitors or for determining expression levels of total or active RhoA, respectively.

After the cells reached 85-95% confluence, the FCScM was changed to a 0.5 mM dbcAMP (Sigma)-containing SFDM (DcSFDM). The composition of SFDM was previously described.[10] Some cultures were pretreated with cell permeable C3 transferase (Rho Inhibitor I; Cytoskeleton Inc., Denver, CO, USA) or Y-27632 (Calbiochem, San Diego, CA, USA), both of which dissolved in distilled water, for 3.5 h or 2 h, 0.5 h or 2 h after the medium change, respectively. Final concentrations of C3 transferase and Y-27632 were 1 μg/ml and 10 μM, respectively. Four hours after the medium change to DcSFDM, astrocytes were exposed to methylmercuric chloride (Tokyo Chemical Industry Co., Tokyo, Japan) at final concentrations of 2 or 3 μM or solvent (ethanol) alone, according to the method previously described.[10] Three hours or 30 min after their exposures, cells were fixed with 1% glutaraldehyde or lysed with applicable solutions, respectively.

Western Blotting and Immunoprecipitation Analysis for RhoA Western blot analysis of total RhoA was performed according to the modified method used for glial fibrillary acidic protein (GFAP) described previously.[18] Briefly, the cell lysates in Laemmli's buffer heated at 65°C for 15 min were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and resolved proteins were electrophoretically transferred onto nitrocellulose blotting membranes (GE Healthcare, Chicago, IL, USA). The membranes were blocked in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS (-)] containing 4% bovine serum albumin (BSA; Sigma) and 0.05% Triton X-100, and probed with antibodies against RhoA (1:500; Cytoskeleton Inc.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:500; Chemicon International Inc., Temecula, CA, USA). Detection was performed using the ECL Western blot detection system (Amersham, Buckinghamshire, UK). Band intensities were analyzed by densitometry using Multi Gauge (ver. 3.0) software (Fujifilm, Tokyo, Japan). The expression of total RhoA was normalized to that of GAPDH. For analysis of active RhoA, immunoprecipitation analysis for GTP-bound RhoA was performed using the Rho activation assay biochem kit (Cytoskeleton Inc.) according to the assay protocol in the instruction manual.

Statistical Analysis Significant differences between individual means were determined by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Considering the similarity in the influence of MeHg and LPA on astrocyte morphology,[11] the spreading of astrocytes induced by MeHg as well as LPA may also be related to Rho. To elucidate the involvement of Rho in the MeHg-induced spreading of astrocytes, the effect of C3 transferase (a Rho inhibitor) was investigated. In the absence of C3 transferase, the astrocyte shape changed from stellate to polygonal after exposure to MeHg at 2-3 μM for 3 h, although the stellate shape was maintained after exposure to solvent alone (Fig. 1), as previously described.[12] In the absence of MeHg exposure, astrocyte shape was stellate regardless of the presence or absence of the inhibitor (Fig. 1). It was seen that pretreatment with C3 transferase could completely inhib-

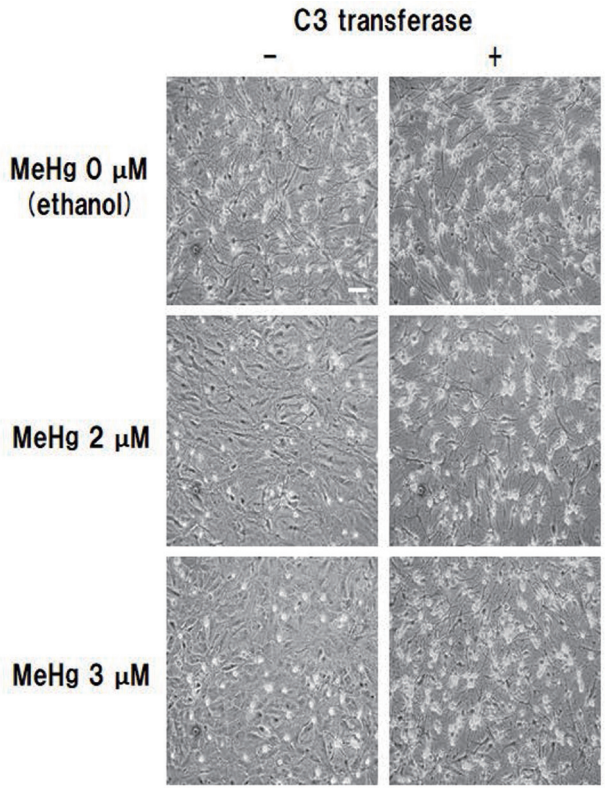

Fig. 1. Effect of C3 Transferase on MeHg-Induced Morphological Changes in Cultured Astrocytes

Cells on 24-well plates were maintained in DcSFDM for 4 h with or without pretreatment with C3 transferase (1 μg/ml, 3.5 h), and then were exposed to MeHg (2-3 μM) or ethanol for 3 h. Bar = 50 μm.

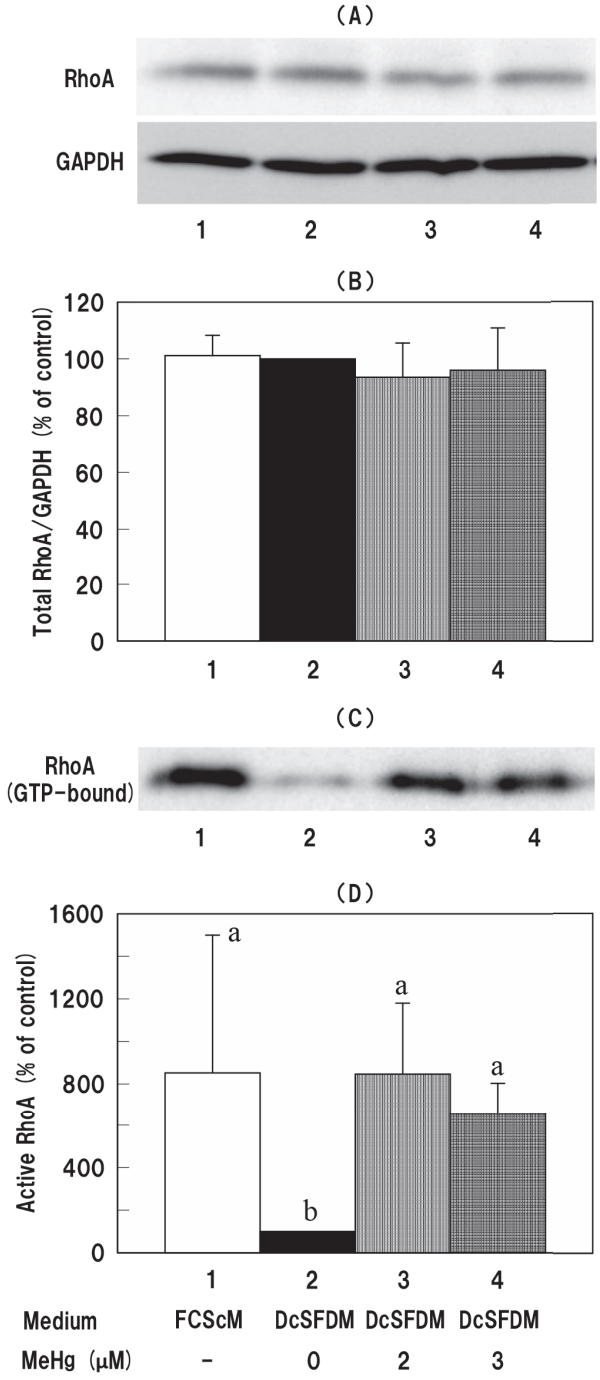

Fig. 2. Influence of MeHg on Expression Levels of RhoA in Cultured Astrocytes.

Some cells were maintained in FCScM. Other cells were maintained in DcSFDM for 4 h, and then exposed to MeHg (2-3 μM) or ethanol for 30 min. A: Representative Western blot data of total RhoA and GAPDH. B: Percentages of the expression levels of total RhoA in relation to GAPDH in ethanol-exposed control cells. Values represent the mean ± S.D. obtained from 4-5 independent experiments. No significant difference is observed among the 4 groups. C: Representative Western blot data of active RhoA. D: Percentages of the expression levels of active RhoA in ethanol-exposed control cells. Values represent the mean ± S.D. obtained from 3-4 independent experiments. Values with different letters (a, b) are significantly different ($p < 0.05$).

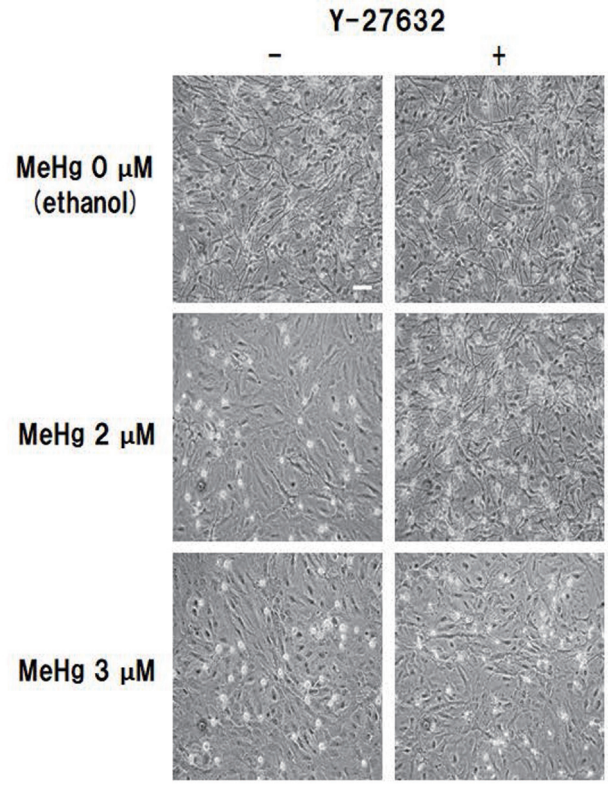

Fig. 3. Effect of Y-27632 on MeHg-Induced Morphological Changes in Cultured Astrocytes.

Cells on 24-well plates were maintained in DcSFDM for 4 h with or without pretreatment with Y-27632 (10 μM, 2 h), and then were exposed to MeHg (2-3 μM) or ethanol for 3 h. Bar = 50 μm.

it the spreading of astrocytes induced by MeHg at both doses (Fig. 1). Therefore, Rho definitely plays critical roles in MeHg-induced shape changes. To determine whether Rho was activated by MeHg exposure before the shape changed to polygonal in astrocytes maintained in DcSFDM, the expression levels of total and active RhoA were examined under several conditions. The expression levels of total RhoA were similar regardless of the culture medium and the presence or absence of MeHg (Fig. 2A and B). In contrast, the expression level of GTP-bound active RhoA in astrocytes exposed to MeHg at 2 or 3 μM for 30 min was markedly higher than that in astrocytes exposed to solvent alone, although no difference was observed in the expression level between astrocytes exposed to MeHg at either dose (Fig. 2C and D). In addition, the active RhoA levels in MeHg-exposed astrocytes, which showed a stellate shape (data not shown), at both doses were similar to the level in astrocytes maintained in FCScM that had a polygonal shape (data not shown), which were used as a positive control (Fig. 2C and D). Thus, RhoA activation caused by MeHg exposure at 2-3 μM was observed before spreading in astrocytes.

It is well-established that Rho kinase is a downstream effector of Rho.[16,17] To estimate the signal transduction after RhoA activation, and perhaps other Rho proteins, by MeHg exposure, the effect of Y-27632 (a Rho kinase inhibitor) on MeHg-induced spreading of astrocytes was investigated. The results for astrocyte shape in the absence of Y-27632 (Fig. 3) were similar to those in the absence of C3 transferase (Fig. 1), and astrocyte shape remained stellate with pretreatment with Y-27632 in the absence of MeHg exposure (Fig. 3). In contrast to C3 transferase, pretreatment with Y-27632 inhibited the astrocyte spreading induced by MeHg exposure at 2 μM but not at 3 μM (Fig. 3). These results suggest that Rho kinase would play important roles in MeHg-induced shape changes, at least at a lower dose, whereas other Rho effectors

might be related to that change at least at higher doses. Thus, the mechanism for MeHg-induced shape change has been partly revealed in this study. However, further study is needed to completely clarify the mechanism, including involvement of Rho effectors other than Rho kinase.

A relationship between morphology, which is regulated by Rho and related proteins such as Rho kinase, and glutamate transporters has been reported in astrocytes. Endothelin-1, which can activate Rho,[19] downregulates GLAST and suppresses glutamate uptake, without influencing the polygonal shape and stress fibers, in dbcAMP-stimulated astrocytes.[15] Fasudil (a Rho kinase inhibitor), which causes a shape change to stellate the same as Y-27632, increases the expression of GLAST and GLT-1 on cell surfaces and the uptake of D-aspartate, with the loss of stress fibers in astrocytes.[20] MeHg has also been demonstrated to suppress the expression of both glutamate transporters, and to increase the glutamate level, in rat cerebral cortex.[21] In the present study, MeHg is revealed to influence Rho, and to cause a change in shape to polygonal in dbcAMP-stimulated astrocytes. If the spreading of astrocytes induced by MeHg is accompanied with downregulation of glutamate transporters, as observed in astrocytes treated with endothelin-1,[15] Rho and related proteins in astrocytes may be involved in the excitotoxicity caused by MeHg.

In conclusion, the stellate shape of dbcAMP-stimulated cerebral hemisphere astrocytes changes to a polygonal shape with MeHg exposure at 2-3 μM through a Rho-dependent signaling pathway. In addition, Rho kinase is, at least partly, related to the shape change in the downstream signal transduction.

Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) World Health Organization (WHO). *Environmental Health Criteria 1, Mercury*, WHO, Geneva (1976).
2) WHO. *Environmental Health Criteria 101, Methylmercury*, WHO, Geneva (1990).
3) Kirchhoff F, Dringen R, Giaume C. Pathways of neuron-astrocyte interactions and their possible role in neuroprotection. *Eur. Arch. Psychiatry Clin. Neurosci.*, **251**, 159–169 (2001).
4) Cheung NS, Pascoe CJ, Giardina SF, John CA, Beart PM. Micromolar L-glutamate induces extensive apoptosis in an apoptotic-necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. *Neuropharmacology*, **37**, 1419–1429 (1998).
5) Beart PM, O'Shea RD. Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol.*, **150**, 5–17 (2007).
6) Brookes N, Kristt DA. Inhibition of amino acid transport and protein synthesis by $HgCl_2$ and methylmercury in astrocytes: selectivity and reversibility. *J. Neurochem.*, **53**, 1228–1237 (1989).
7) Aschner M, Eberle NB, Miller K, Kimelberg HK. Interactions of methylmercury with rat primary astrocyte cultures: inhibition of rubidium and glutamate uptake and induction of swelling. *Brain Res.*, **530**, 245–250 (1990).
8) Miyamoto K, Nakanishi H, Moriguchi S, Fukuyama N, Eto K, Murao K, Wakamiya J, Arimura K, Osame M. Involvement of enhanced sensitivity of *N*-methyl-D-aspartate receptor in vulnerability of developing cortical neurons to methylmercury neurotoxicity. *Brain Res.*, **901**, 252–258 (2001).
9) Park ST, Lim KT, Chung YT, Kim SU. Methylmercury-induced neurotoxicity in cerebral neuron culture is blocked by antioxidants and NMDA receptor antagonists. *Neurotoxicology*, **17**, 37–45 (1996).
10) Adachi T, Kunimoto M. Acute cytotoxic effects of mercuric compounds in cultured astrocytes prepared from cerebral hemisphere and cerebellum of newborn rats. *Biol. Pharm. Bull.*, **28**, 2308–2311 (2005); corrected in *Biol. Pharm. Bull.*, **33**, 930 (2010).
11) Adachi T, Katamoto Y, Iwatate T, Yanagisawa Y, Yasuda I. Region-dependent differences in morphological changes induced by mercury compounds and lysophosphatidic acid in cultured rat astrocytes. *Univ. Bull. Chiba Inst. Sci.*, **12**, 1–10 (2019).
12) Adachi T, Shimizu H, Kawasumi K, Suzuki Y, Hayashi T, Tsurusaki R, Watanabe Y, Iwasaki M, Yanagisawa Y, Yasuda I. Dose-related difference and involvement of tyrosine phosphorylation in methylmercury-induced morphological change in cultured astrocytes obtained from rat cerebral hemisphere. *BPB Reports*, **6**, 16–20 (2023).
13) Suidan HS, Nobes CD, Hall A, Monard D. Astrocyte spreading in response to thrombin and lysophosphatidic acid is dependent on the Rho GTPase. *Glia*, **21**, 244–252 (1997).
14) Ramakers GJA, Moolenaar WH. Regulation of astrocyte morphology by RhoA and lysophosphatidic acid. *Exp. Cell Res.*, **245**, 252–262 (1998).
15) Matsuura S, Ikegaya Y, Yamada MK, Nishiyama N, Matsuki N. Endothelin downregulates the glutamate transporter GLAST in cAMP-differentiated astrocytes *in vitro*. *Glia*, **37**, 178–182 (2002).
16) Narumiya S, Ishizaki T, Watanabe N. Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett.*, **410**, 68–72 (1997).
17) Wheeler AP, Ridley AJ. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp. Cell Res.*, **301**, 43–49 (2004).
18) Adachi T, Takanaga H, Sakurai Y, Ishido M, Kunimoto M, Asou H. Influence of cell density and thyroid hormone on glial cell development in primary cultures of embryonic rat cerebral hemisphere. *J. Neurosci. Res.*, **79**, 608–615 (2002).
19) Koyama Y. Endothelin ET_B receptor-mediated astrocytic activation: pathological roles in brain disorders. *Int. J. Mol. Sci.*, **22**, 4333 (2021).
20) Lau CL, O'Shea RD, Broberg BV, Bischof L, Beart PM. The Rho kinase inhibitor Fasudil up-regulates astrocytic glutamate transport subsequent to actin remodelling in murine cultured astrocytes. *Br. J. Pharmacol.*, **163**, 533–545 (2011).
21) Deng Y, Xu Z, Xu B, Liu W, Wei Y, Li Y, Feng S, Yang T. Exploring cross-talk between oxidative damage and excitotoxicity and the effects of riluzole in the rat cortex after exposure to methylmercury. *Neurotox. Res.*, **26**, 40–51 (2014).