Effects of sodium arsenite on neurite outgrowth and glutamate AMPA receptor expression in mouse cortical neurons

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There has been broad concern that arsenic in the environment exerts neurotoxicity. To determine the mechanism by which arsenic disrupts neuronal development, primary cultured neurons obtained from the cerebral cortex of mouse embryos were exposed to sodium arsenite (NaAsO2) at concentrations between 0 and 2 μM from days 2 to 4 in vitro and cell survival, neurite outgrowth and expression of glutamate AMPA receptor subunits were assessed at day 4 in vitro. Cell survival was significantly decreased by exposure to 2 μM NaAsO2, whereas 0.5 μM NaAsO2 increased cell survival instead. The assessment of neurite outgrowth showed that total neurite length was significantly suppressed by 1 μM and 2 μM NaAsO2, indicating that the lower concentration of NaAsO2 impairs neurogenesis before inducing cell death. Immunoblot analysis of AMPA receptor subunit expression showed that the protein level of GluA1, a specific subunit of the AMPA receptor, was significantly decreased by 1 μM and 2 μM NaAsO2. When immunocytochemistry was used to confirm this effect by staining for GluA1 expression in neuropeptide Y neurons, most of which contain GluA1, GluA1 expression in neuropeptide Y neurons was found to be significantly suppressed by 1 μM and 2 μM NaAsO2 but to be increased at the concentration of 0.5 μM. Finally, to determine whether neurons could be rescued from the NaAsO2-induced impairment of neurogenesis by compensatory overexpression of GluA1, we used primary cultures of neurons transfected with a plasmid vector to overexpress either GluA1 or GluA2, and the results showed that GluA1/2 overexpression protected against the deleterious effects of NaAsO2 on neurite outgrowth. These results suggest that the NaAsO2 concentration inducing neurite suppression is lower than the concentration that induces cell death and is the same as the concentration that suppresses GluA1 expression. Consequently, the suppression of GluA1 expression by NaAsO2 seems at least partly responsible for neurite suppression induced by NaAsO2.

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1. Introduction
The developing brain is vulnerable to toxic chemical compounds such as heavy metals and persistent organic pollutants during gestation and early childhood, and exposure to them permanently affects brain functions (Costa et al., 2004; Grandjean and Landrigan, 2006; Winneke, 2011; Goulet et al., 2003; Costa et al., 2004; Farina et al., 2011; Winneke, 2011). Among those toxic chemicals, arsenic has been known to have neurotoxic effects during development. Follow-up studies of the victims of arsenic poisoning in the Morinaga milk incident in Japan have shown that oral exposure to arsenic during infancy increases the prevalence of brain disorders (Dakeishi et al., 2006). Epidemiological studies from Mexico, Bangladesh, China and Taiwan have shown that chronic exposure to arsenic decreased cognitive performances of children (Calderon et al., 2001; Wang et al., 2007; Hamadani et al., 2011; Wasserman et al., 2004, 2007; Tsai et al., 2003).

Studies have been performed on experimental animals to determine how arsenic exposure impairs normal brain development. Rats and mice exposed to arsenic during gestation and early childhood exhibit behavioral deficits such as changes in locomotor activity, learning, memory, depression-like behavior and neuromotor reflex (Rodriguez et al., 2002; Xi et al., 2009; Martinez-Finley et al., 2009). The behavioral changes caused by arsenic seem to be accompanied by neurochemical abnormalities and loss of nerve fibers (Nagaraja and Desiraju, 1993; Martinez et al., 2008;
The inhibitory effects of arsenic on neuritogenesis have been monitored in vitro experiments, and the results have been consistent with the results observed in animals in vivo. Studies conducted on immortalized cell lines have shown that arsenic impairs neurite outgrowth and complexity (Frankel et al., 2009). Arsenic has been shown to exert its neurotoxic effects by inducing apoptotic cell death as well as by inhibiting neuritogenesis (Koike-Kuroda et al., 2010). We recently reported the finding that neurite elongation in the Neuro2A cell line was suppressed by sodium arsenite at a lower concentration than the concentration that induced apoptosis (Aung et al., 2013). Thus, neuritogenesis would seem to be more sensitive to arsenic toxicity than cell viability. Arsenic may exert its toxic effects through several mechanisms, including some distinct mechanisms that are responsible for either the suppression of neurite growth or for the induction of apoptosis. One of the possible mechanisms underlying arsenic-induced neurite suppression is thought to be oxidative stress. It has been reported that treatments with an antioxidant, a superoxide scavenger or a H2O2 scavenger effectively reverse the inhibitory effect of arsenic on neurite outgrowth of Neuro2A cell line (Wang et al., 2010). Based on the results that the treatment with an AMPK activator or the overexpression of a constitutively active AMPKα1 reversed arsenic-induced inhibition of neurite outgrowth (Wang et al., 2010), the authors suggested that the LKB1-AMPK signaling pathway is involved in the arsenic-induced neurite suppression. However, the precise mechanisms underlying neurite suppression by arsenic are not completely understood.

Glutamate transmission plays essential roles in a variety of brain functions and in development. AMPA receptors, a major subtype of glutamate receptors, are composed of four types of subunits, i.e., GluA1, 2, 3 and 4, and play a critical role in excitatory glutamate transmission (Kumar and Mayer, 2013). AMPA receptors are also involved in the formation of neuronal networks and connectivity. Pharmacological potentiation of AMPA receptors and overexpression of either AMPA receptor subunit GluA1 or GluA2 by transient transfection promotes neurite outgrowth (Voss et al., 2007; Prithviraj et al., 2008; Chen et al., 2009). These results suggest that modifications of AMPA receptor subunit expression and subsequent increases in excitability due to enhanced glutamate transmission are closely related to the regulation of neuritogenesis.

In the present study, we investigated the effects of arsenic on AMPA receptors in primary cultures of mouse cortical neurons to determine the mechanism by which arsenic exposure suppresses neuritogenesis. Immunoblot and immunocytochemistry analyses showed that arsenic reduced the level of GluA1 expression in parallel with the suppression of neurite outgrowth. We also transfected GluA1/2 into primary neurons with plasmid vectors to determine whether overexpression of AMPA receptors would rescue neurite outgrowth suppressed by arsenic.

2. Materials and methods

2.1. Mice

Male and female C57BL/6j mice were purchased from CLEA Japan (Tokyo, Japan) and were bred at the National Institute for Environmental Studies. They were acclimatized to the environment for about 1 week prior to use. Throughout the experiments, animals were maintained in a controlled environment at a temperature of 24 ± 1 °C and humidity of 50 ± 10% and under a 12/12 h light/dark cycle (light, ZT0–12; dark, ZT12–24). Food (CE-2, CLEA) and water were available ad libitum unless otherwise indicated. Adult males and females were kept in couples to obtain pregnant females. Vaginal plug was checked each day to estimate the gestational age.

For the experiment focusing on neuropeptide Y (NPY) neurons, NPY-hrGFP transgenic mice generated by Prof. Bradford B. Lowell (Beth Israel Deaconess Medical Center) were purchased from the Jackson Laboratory (B6.FVB-Tg[Npy-hrGFP]1Lowj, Stock#006417, Bar Harbor, ME) (van den Pol et al., 2009). For culture of primary NPY-hrGFP neurons, the brain at embryonic day 17 was used and green fluorescence in the cerebral cortex observed with a IX70 fluorescence microscope (Olympus, Tokyo, Japan).

2.2. Ethics statement

Mice were handled in a humane manner in accordance with the National Institute for Environmental Studies guidelines.

2.3. Primary culture of neurons and transfection of plasmids

Primary cultures of mouse cortical neurons were prepared following the method described previously (Chenal and Pellerin, 2007) with slight modifications. Briefly, the pregnant mouse at embryonic day 17 was sacrificed by decapitation and the cerebral cortices of embryos were collected under dissection microscope. The cortices were washed with 10 mM HEPES-buffered Krebs–Ringer bicarbonate buffer (HKRB) containing 10 mM glucose. They were incubated in HKRB supplemented with 20 μM papain (Sigma Chemical Co., St. Louis, MO), 0.015 mg/ml deoxyribonuclease (Sigma), 0.75 mg/ml bovine serum albumin (Sigma) and 1 mM cysteine (Sigma) for 10 min at 37 °C in a shaking water bath, followed by gentle mechanical trituration. The obtained cell suspension was centrifuged at 100 × g for 5 min and the cell pellet collected. In the case that transfection of plasmids was required, we used the Amaxa Nucleofector with mouse neuron nucleofector® kit (#VPG-1001, Lonza, Basel, Switzerland) following manufacturer’s instruction. For tracing neurites, we used the pmStrawberry plasmid expressing red fluorescent protein mStrawberry under the control of CMV promoter (Maekawa et al., 2009) in Figs. 1C–J, 4, and Supplementary Fig. 1. For overexpressions of GluA1 and 2, we used pGFP-GluA1 expressing GFP-fused GluA1 under the control of CMV promoter (kindly provided by Dr. Jeremy M. Henley, University of Bristol) and pVenus-GluA2 expressing GluA2-fused with Venus, a GFP variant, under the control of CMV promoter. Correct band sizes and surface expressions of fused proteins in primary cultures of neurons have been already determined previously (Perestenko and Henley, 2003; Maekawa et al., 2009). We used pEGFP (Clontech, Takara, Japan) expressing GFP as a control instead of pGFP-GluA1 and pVenus-GluA2. Cells undergoing or not transfection were resuspended in Neurobasal medium (Invitrogen, Carlsbad, CA) containing B-27 supplement (50×), Invitrogen, 0.5 mM l-glutamine (Invitrogen) and antibiotics (Pen/Strep, #15140-148, 100×, Invitrogen). Cells were seeded onto poly-d-ornithine (15 mg/L)-precoated coverslips (12 mm, Matsunami glass, Osaka, Japan) in 24 wells cell culture plates (Falcon, Becton Dickinson AG, Basel, Switzerland). The cell density of primary cultures of neurons was 478 ± 41 cells/mm2 (mean ± S.E.M.). Sodium arsenite (NaAsO2) was added on the 2nd day in vitro (DIV2) and maintained up to DIV4 at different concentrations. Transfection efficiency was determined by counting the cells positive for both mStrawberry and 4,6-diamidino-2-phenylindole (DAPI), a nucleus marker, in the images of fixed and DAPI-stained samples, which were obtained by confocal fluorescence microscopy (details are described below). The transfection efficiency was found to be 25.9%.

2.4. Cell survival assay by the WST-1 method

The effect of NaAsO2 on cell survival of neurons was determined using a colorimetric assay based on the reduction of tetrazolium
salt to formazan by mitochondrial dehydrogenase activity. Ten microliters of Tetrazolium reagent WST-1 (Roche, 
Burlingame, CA) was then reacted with the cells at DIV4 for 1 h at 37 °C, and absorbance at 450 nm was then measured using a reference wavelength of 650 nm. The absorbance at 650 nm was subtracted from the absorbance at 450 nm, and this value was then expressed as a percentage of the value obtained for the neurons without NaAsO₂ exposure, whose viability was set at 100%.

2.5. Quantification of neurite outgrowth and cell numbers

At DIV4, cells were fixed with ice-cold 4% paraformaldehyde–phosphate buffered saline (PBS) for 30 min and washed with PBS. Coverslips were mounted with Vectashield Hard set containing DAPI (H-1500, Vector Laboratories, Burlingame, CA). Images were taken with a Leica TCS SP5 AOBS filter free spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). In Figs. 1C–J and Supplementary Fig. 1, to trace neurites, the fluorescence of pmStrawberry was detected as described in a previous report (Maekawa et al., 2009). Concerning the NPY-hrGFP neurons in Fig. 3A–G, the fluorescence of hrGFP was detected following the setting of GFP (Maekawa et al., 2009). Full optical sections of cells were made by capturing Z-series with one micrometer steps. The integrated image constructed from optical sections in order to define the details of neurites was made with Leica Application Suite-Advanced Fluorescence (LAS-AF, Version 1.8.2 build 14655, Leica). The images of either red or green channel from the integrated image were extracted to visualize either pmStrawberry or hrGFP, respectively, and was converted to gray scale for further analysis. The total length of neurites and the number of branches were examined by using the software NeuronJ (Meijering et al., 2004) which is a plug-in of ImageJ (NIH, Bethesda, MD). Neurites were categorized as “primary”, which consist of neurites directly extending from the cell body or “secondary and higher order”, which consist of all neurites except “primary”. The number of branches was counted following this categorization. For representative presentations in figures, inverted black with white images were used for legibility.

To count cell numbers, the blue channel image from the integrated image was extracted to visualize DAPI. The number of DAPI-positive nuclei in each image was automatically counted by using the command “Analyze Particle” (setting: Size (80–Infinity)) in ImageJ after thresholding (background (29–170)). The number of cells positive for mStrawberry was counted manually.

Fig. 1. Effects of NaAsO₂ on cell viability and neurite outgrowth in primary cultures of neurons. (A) Cell viability was assessed using the WST-1 assay. Cell viability was significantly reduced by exposure to 2 μM NaAsO₂ (b, p < 0.01 vs 0, 0.125, 0.25, 0.5, 1 μM). No significant decrease of cell viability was found between the exposures to 0 μM and 1 μM NaAsO₂. The exposure to 0.5 μM NaAsO₂ rather increased cell viability when compared to 0, 1 and 2 μM (a, p < 0.01). (B) Cell viability was assessed by counting DAPI-labeled nuclei in the images obtained by confocal microscopy in groups exposed to NaAsO₂ concentrations of 0, 0.5, 1, and 2 μM from DIV2 to DIV4. NaAsO₂ significantly reduced cell numbers at the concentration of 2 μM and increased it at the concentration of 0.5 μM (a, p < 0.01 vs other groups, b, p < 0.01 vs 0 and 1 μM). (C–F) Confocal images of neurons exposed to 0, 0.5, 1 and 2 μM NaAsO₂ from DIV2 to 4. (C) Total neurite length of neurons exposed to 0, 0.5, 1 and 2 μM NaAsO₂ from DIV2 to 4. A significant reduction of total neurite length per cell was found at 1 μM (a, p < 0.05 vs 0 μM and p < 0.01 vs 0.5 μM) and the degree of reduction was more severe at 2 μM (b, p < 0.01 vs 0 and 0.5 μM). (H) Total number of primary branches. (J) The number of secondary and higher order branches. The number of secondary and higher order branches was significantly decreased in groups exposed to 1 μM or 2 μM NaAsO₂ when compared to the group exposed to 0.5 μM NaAsO₂ (a, p < 0.01 vs 1.0 and 2.0 μM), but there was no significant difference when compared to the group at 0 μM. The experiment was repeated at least three times on different cell preparations and the data taken from different experiments which share similar sample size were pooled together. The number shown in parenthesis represents the total number of separate determinations.
2.6. Glutamate assay

To detect glutamate in culture medium, the glutamate research ELISA kit (#BA E-2300, LDN, Nordhorn, Germany) was used following manufacturer’s instruction. In parallel, the amount of proteins in each culture well was determined with a BCA protein assay kit (Thermo Fisher Scientific, Yokohama, Japan) and glutamate levels corrected for protein level in each well.

2.7. Western blotting

Western blotting was carried out as described previously (Maekawa et al., 2009) with a minor modification. Briefly, cells were lysed in 0.5% Triton X-100 lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (1.9 mg/ml aprotinin, 10 mg/ml leupeptin, 100 mM sodium orthovanadate and 200 mM phenylmethanesulfonyl fluoride). The protein concentration of lysate was determined with a BCA protein assay kit and the value was used for equilibrating the protein level of each lane for SDS-PAGE. After boiling with SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.001% bromophenol blue), the samples were subjected to SDS-PAGE and then transferred to PVDF membranes (Hybond-P, Thermo Fisher Scientific). Membranes were blocked by 1% casein–PBS for 1 h and allowed to react with anti-GluA1 (1:1000, AB1504, Millipore), anti-GluA2/3 (1:1000, AB1506, Millipore), and anti-GluA4 (1:1000, AB1508, Millipore), and anti-β-actin (1:5000, Sigma–Aldrich) overnight at 4 °C, washed, and then allowed to react with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG1) for 1 h at room temperature. After washing, membranes were developed using the ECL Plus Western Blotting Detection System (Thermo Fisher Scientific). Band density of each well was determined with ImageJ.

2.8. Immunocytochemistry and assessment of relative intensity of fluorescence

After fixing with ice-cold 4% paraformaldehyde–PBS and rinsing in PBS, cells were incubated with 1% casein–PBS for 1 h at room temperature in order to block non-specific binding of antibody. Next, cells were incubated with rabbit anti-GluA1 (1:100), anti-GluA2/3 (1:100), and anti-GluA4 (1:100) in 1% casein–PBS for 24 h. After further washing with PBS, cells were incubated with AlexaFluor 594-labeled donkey anti-rabbit IgG (1:200, A21207, Invitrogen) for 2 h at 37 °C. After washing again with PBS, cells were examined with confocal microscope. The number of GluA1-positive neurons among NPY-hrGFP neurons was manually counted by using the integrated images of neurons without treatment with NaAsO2. Relative intensity of GluA1 immunofluorescence was calculated in the area delineating the cell body of NPY-hrGFP neurons with or without treatment with NaAsO2 by using LAS-AF.

2.9. Statistical analyses

Data are expressed as mean ± S.E.M. Replicates are indicated in parentheses. Data in Figs. 1, 2A–C, and 3E–G were analyzed with one-way
ANOVA followed by Fisher’s post hoc least significant difference using R (The R Foundation for Statistical Computing, Vienna, Austria).

Fig. 4 and Supplementary Fig. 1 were analyzed with two-way ANOVA followed by Games–Howell’s post hoc test using R. Data in Fig. 2E and Supplementary Fig. 2 were analyzed with a Student’s t-test in Microsoft Excel 2008 for Mac (Microsoft, Redmond, WA). Differences were considered significant for p < 0.05.

3. Results

3.1. Effect of NaAsO2 on cell viability and neurite outgrowth

To determine the concentrations of NaAsO2 that affect the survival of primary cultured neurons, we exposed them to NaAsO2 concentrations of 0, 0.125, 0.25, 0.5, 1, and 2 μM from DIV2 to 4. As shown with the cell survival assay using WST-1 on DIV4 neurons, NaAsO2 significantly reduced cell viability at 2 μM (Fig. 1A). However, no significant decrease in cell viability was observed at concentrations up to 1 μM (Fig. 1A). In contrast to the effect of

2 μM NaAsO2, 0.5 μM significantly increased the survival of primary neurons and a tendency to increase cell survival was also found with 0.125 μM and 0.25 μM. In order to confirm these data, determination of cell numbers by counting DAPI-labeled nuclei in the images obtained by confocal microscopy (19–25 images in each group which were pooled from four independent experiments) was also performed in groups exposed to NaAsO2 concentrations of 0, 0.5, 1, and 2 μM from DIV2 to DIV4 (Fig. 1B). We found that NaAsO2 significantly reduced cell numbers at 2 μM and increased it at 0.5 μM. No significant difference from control was observed at 1 μM.

Analysis of the computer-assisted neurite tracings showed a significant reduction in total neurite length per cell at 1 μM and 2 μM NaAsO2 (Fig. 1G), with a greater reduction found at 2 μM (Fig. 1G). A concentration of 0.5 μM NaAsO2 tended to increase total neurite length, but the difference with the untreated group did not significance.

We also counted the number of total, primary, as well as secondary and higher order branches per cell. Although total
number of branches tended to decrease as the NaAsO2 concentration increased (1.0 mM vs 0 and 0.5 mM, $p = 0.09$), there was no significant difference between the different concentrations on the total number of branches or on the number of primary branches (Fig. 1H and I). The number of secondary and higher order branches in the group exposed to 1 mM or 2 mM NaAsO2 were smaller than in the control group, but differences were not significant (Fig. 1J).

3.2. Effect of NaAsO2 on extracellular glutamate levels and on AMPA receptor expression

Since the neurotoxic effects of arsenic can be mediated by excitotoxicity, we therefore hypothesized that NaAsO2 decreased cell survival by increasing extracellular glutamate levels. Extracellular glutamate levels were measured by collecting the culture medium of primary neurons that had been exposed to NaAsO2 at concentrations of 0, 0.125, 0.25, 0.5, and 1 mM from DIV2 to 4 (Fig. 2A). However, no significant differences in glutamate levels were detected, suggesting that NaAsO2 does not affect glutamate release by primary cultured cortical neurons. Next, we performed an immunoblot analysis to assess the levels of expression of AMPA receptor subunits. Cultured neurons were exposed to NaAsO2 at concentrations of 0, 0.5, 1 and 2 mM from DIV2 to DIV4, and cell lysates were obtained at DIV4. Immunoblotting with anti-GluA1 antibody showed decreased expression of GluA1 in neurons exposed to 1 mM or 2 mM NaAsO2 in comparison with neurons exposed to 0 mM and 0.5 mM (Fig. 2B), while there was no significant difference in β-actin protein levels (Fig. 2B). Quantification of the GluA1/β-actin intensity ratio with the ImageJ software program revealed that exposure to NaAsO2 at 1 mM or 2 mM significantly reduced GluA1 expression (Fig. 2C). As far as other AMPA receptor subunits are concerned, a comparison between neurons exposed to NaAsO2 at concentrations of 0 mM and 1 mM was performed (Fig. 2D). In this experiment, we first confirmed the decreased GluA1 expression by exposure to 1 mM NaAsO2. Immunoblotting with anti-GluA2/3 and anti-GluA4 antibodies revealed no changes in GluA2/3 and GluA4 levels as a result of exposure to 1 mM NaAsO2, suggesting that NaAsO2 affects the expression of glutamate AMPA receptors in a subunit-specific manner. Since we previously showed that NaAsO2 has no significant effect on the mRNA levels of microtubule associated protein-2 (MAP2) and β-actin (Aung et al., 2013), we measured the protein levels of MAP2 and β-actin as negative controls in this study. As expected, their expression was the same, no matter if the cells had been exposed to 1 mM NaAsO2 or not (Fig. 2D). Quantification of band density confirmed that exposure to 1 mM NaAsO2 significantly reduces of GluA1 expression but does not reduce expression of any of the other subunits (Fig. 2E).

3.3. Effect of NaAsO2 on GluA1 expression in primary cultured NPY-hrGFP neurons

To confirm by a different approach the results of the immunoblot analyses showing that NaAsO2 reduces GluA1 expression levels, we used primary cultures prepared from transgenic mice that express a humanized Remilla green fluorescence protein under the control of a neuropeptide Y gene promoter (NPY-hrGFP). Immunofluorescence staining with anti-GluA1...
antibody revealed that at DIV4, 98.5% of the NPY-hrGFP neurons were GluA1-positive under our culture conditions. Before examining GluA1 levels, we checked to see whether neurite outgrowth of NPY-hrGFP neurons could be suppressed in a similar manner as that shown in Fig. 1. The results of exposure to NaAsO₂ concentrations of 0, 0.5, 1 and 2 μM showed that neurite elongation was reduced when exposed to 1 μM and that the reduction was more pronounced at 2 μM (Fig. 3A–D), indicating that the NaAsO₂ concentrations that reduced neurite outgrowth in NPY-hrGFP neurons were the same as those presented in Fig. 1G. Using the same paradigm of NaAsO₂ treatment, we measured the immunofluorescent intensity of GluA1 and GluA4 in NPY-hrGFP neurons. The relative intensity of fluorescence using anti-GluA1 antibody was significantly higher in neurons exposed to 0.5 μM NaAsO₂ compared to the control group (Fig. 3F). By contrast, 1 μM NaAsO₂ reduced the intensity of the GluA1-immunofluorescent signal and 2 μM NaAsO₂ reduced its intensity even more. These results suggest the existence of a relation between the reduction in GluA1 expression level and neurite suppression. On the other hand, the intensity of the GluA4-immunofluorescent signal increased with the concentration of NaAsO₂ (Fig. 3G), confirming that NaAsO₂ specifically reduces GluA1 expression and does not reduce GluA4 expression. We also investigated the effects of NaAsO₂ on GluA2/3 by immunofluorescent staining with an anti-GluA2/3 antibody, but the immunofluorescent signal for GluA2/3 was weak at DIV4, and there was no marked difference in GluA2/3 immunofluorescent intensity between the cells that had been exposed to NaAsO₂ vs. control (data not shown).

3.4. Effects of GluA1 and GluA2 overexpression on impaired neurite outgrowth

To determine whether overexpression of exogenous AMPA receptor subunits could compensate for the decreased GluA1 expression induced by NaAsO₂ and rescue the inhibitory effect of NaAsO₂ on neurite outgrowth, primary cultured neurons prepared from mouse cerebral cortex were transfected with a plasmid vector to express GFP-fused GluA1 (pGFP-GluA1) or Venus-fused GluA2 (pVenus–GluA2) under the control of a CMV promoter in combination with pmStrawberry and then exposed to 2 μM NaAsO₂ from DIV2 to DIV4 (Fig. 4A–D). A pEGFP plasmid vector was used as control to express GFP instead of the GluA1 and GluA2 expression vectors. Neurite outgrowth by the neurons transfected with the control vector expressing GFP was strongly inhibited by 2 μM NaAsO₂ (Fig. 4C), whereas neurite outgrowth was stimulated by GluA1 overexpression (Fig. 4B), and GluA1 overexpression mitigated the inhibitory effect of NaAsO₂ on neurite outgrowth (Fig. 4D). Quantification of total neurite length also demonstrated that 2 μM NaAsO₂ significantly reduced the total neurite length in neurons in the absence of GluA1 overexpression and that the inhibition of neuritogenesis by NaAsO₂ was prevented by GluA1 overexpression (Fig. 4E). Exposure to 2 μM NaAsO₂ tended to reduce the total number of branches in both the presence and absence of GluA1 overexpression (Fig. 4F) but there was no significant difference. No significant differences were observed in the number of primary branches (Fig. 4G). The number of secondary and higher order branches was significantly increased by GluA1 overexpression in the absence of NaAsO₂ exposure (Fig. 4H), but the number remained low after exposure to NaAsO₂ even though GluA1 was overexpressed (Fig. 4H).

GluA2 overexpression had effects on neurite length and the number of branches that were similar to the effects of GluA1 overexpression (Supplementary Fig. S1A–H). The results of GluA2 overexpression are shown in detail in the supplementary text.

4. Discussion

Neuritogenesis in our primary cultures of mouse cortical neurons was impaired by exposure to NaAsO₂ concentrations of 1 μM or above, and NaAsO₂ exposure at the same concentrations also decreased their GluA1 levels. GluA1 or GluA2 overexpression reduced the inhibition of neurite elongation by NaAsO₂. These results indicate that the suppression of neurite outgrowth by NaAsO₂ may at least be partially attributable to reduced glutamate AMPA receptor expression and thereby excitability of neurons. On the other hand, exposure to 0.5 μM NaAsO₂, a lower concentration than the concentration that induced neurite suppression, increased cell survival and GluA1 levels as revealed by immunocytochemistry.

4.1. Arsenic-induced suppression of neurite outgrowth and reduction of GluA1 expression

In this study, 1 μM NaAsO₂ did not affect cell viability, but strikingly suppressed neurite outgrowth. In a previous study we found that low concentrations of NaAsO₂ affected neuritogenesis before inducing apoptotic and necrotic cell death (Aung et al., 2013). Studies by other groups have shown that neurite damage is a cue for the induction of cell death (Berliocchi et al., 2005; Volbracht et al., 2001). Thus, the suppression of neurite outgrowth may be a primary step in the developmental neurotoxicity of arsenic.

Several hypotheses have been proposed to explain how arsenic suppresses neurite outgrowth. Excitotoxicity is caused by excess levels of neurotransmitters, such as glutamate and other excitatory transmitters, and it has been reported to be involved in several neurodegenerative diseases (Lau and Tymianski, 2010). Since we suspected that excitotoxicity could mediate the arsenic-induced neurite suppression, we measured glutamate concentrations in the culture medium after NaAsO₂ exposure. Surprisingly, the glutamate concentration in the culture medium was unaltered by NaAsO₂ (Fig. 2A) and thus excitotoxicity does not seem to be the cause of neurite suppression in our culture system. A decrease in the number of glutamate receptors is another possible cause of neurodegenerative diseases, because changes in cerebral AMPA receptor expression have been reported in neurodegenerative diseases (Opitz et al., 2000), and AMPA receptor knockdown itself is a cause and an aggravating factor of neurodegeneration (Oguro et al., 1999). We performed two experiments to test the hypothesis that NaAsO₂ suppresses AMPA receptor expression. In the first experiment, AMPA receptor protein levels in total cell lysates were measured by immunoblot. The results showed that NaAsO₂ at concentrations of 1 μM and above reduced GluA1 levels and that the reduction paralleled the suppression of neurite elongation. In the second experiment, we performed immunocytochemistry on NPY-hrGFP neurons to confirm the result of the first experiment. Since neurites of NPY-hrGFP neurons were as vulnerable to NaAsO₂ as the neurites of cortical neurons transfected with pmStrawberry (Fig. 3A–D), we concluded that NPY-hrGFP neurons could be used as a typical example of cortical neurons in regard to sensitivity to NaAsO₂. It is also advantageous to utilize NPY-hrGFP neurons when evaluating GluA1 levels, because most NPY-hrGFP neurons exhibited GluA1-immunoreactivity in our culture system. The immunocytochemistry analysis of GluA1 in NPY-hrGFP neurons confirmed that NaAsO₂ at concentrations of 1 μM and above reduced GluA1 expression. In addition, following exposure to 1 μM NaAsO₂ from DIV2 to DIV4, the intracellular Ca²⁺ increase induced by treatment with 200 μM glutamate or 50 μM AMPA was significantly reduced (Supplementary result and Supplementary Fig. 2), an observation consistent with the fact that AMPA receptor levels in primary neurons are lowered by 1 μM NaAsO₂. Based on
these results, we conclude that reduced GluA1 expression is a factor that links NaAsO₂ exposure at concentrations of 1 μM and above to impaired neurite outgrowth.

Some mechanistic explanations of how NaAsO₂ reduces GluA1 levels can be proposed. Arsenic is known to induce oxidative stress, and oxidative stress leads to the suppression of neurite outgrowth and changes in cytoskeletal proteins expression (DeFuria and Shea, 2007; Wang et al., 2010). We recently demonstrated that NaAsO₂ specifically changed gene expression of cytoskeletal proteins in Neuro2A cells (Aung et al., 2013). Exposure to NaAsO₂ dose-dependently reduced the mRNA levels of tau and tubulin in Neuro2A cells but increased the mRNA levels of the light and medium subunits of neurofilaments. Our preliminary results suggest that similar changes in mRNA expression of the same cytoskeletal proteins can be found in primary neurons after exposure to 1 μM NaAsO₂ (data not shown). Since the intracellular transport, synaptic insertion and recycling of GluA1 have been reported to be controlled by cytoskeletal proteins (Anggono and Huganir, 2012; Perestenko and Henley, 2013), reduced GluA1 levels may be caused by cytoskeletal dysfunction in response to oxidative stress induced by NaAsO₂. Apart from cytoskeletal dysfunction, epigenetic regulation of GluA1 may be responsible for the arsenic-induced suppression of neurite outgrowth. It has been reported that expression of HDAC2, a subtype of histone deacetylase sensitive to neurotoxic insults, was found to increase in response to oxidative stress and neurotoxic accumulation of β-amyloid, and that HDAC2 expression increase reduced gene expression of proteins related to synaptic plasticity including GluA1, as a result of deacetylation of histones localized at their promoters (Gräff et al., 2012). Given that arsenic affects HDAC2 expression through eliciting oxidative stress, GluA1 expression may be reduced by the activity of HDAC2. These different hypotheses should be tested in future studies.

4.2. Rescue of arsenic-induced suppression of neurite outgrowth by overexpression of GluA

AMPA receptors can be composed of any tetrameric combination of GluA1-4 subunits. They are permeable to Na⁺ after glutamate binds to them, whereas Ca²⁺ permeability depends on their subunit composition. AMPA receptors lacking GluA2 are Ca²⁺-impermeable, whereas AMPA receptors containing Q/R-edited GluA2 are Ca²⁺-permeable (Hume et al., 1991; Verdoorn et al., 1991). Based on the observation that GluA1 expression is specifically reduced by exposure to NaAsO₂ at 1 μM and above, we over-expressed GluA1 in primary neurons. Compensatory overexpression of GluA1 partially reversed the neurite suppression induced by 2 μM NaAsO₂ in this study (Fig. 4), thereby indicating that the impaired neurite outgrowth is at least partly due to the reduction of the GluA1 expression level. In addition, overexpression of GluA2 (like GluA1) also partly reversed the impaired neurite outgrowth (Supplementary Fig. 1). It has been reported that overexpression of not only GluA1 but also GluA2 increases total neurite length in primary culture of neurons (Prithviraj et al., 2008; Chen et al., 2009). Surprisingly, neurons overexpressing GluA2 showed strong increases of intracellular Ca²⁺ concentrations in response to glutamate despite the Ca²⁺-impermeable channel properties of GluA2 (Chen et al., 2009), presumably because the increased Na⁺ entry obtained by GluA2 overexpression activates Ca²⁺-permeable NMDA receptor or voltage-dependent Ca²⁺ channel. Activation of a common pathway involving Ca²⁺ following neuronal excitation downstream of GluA1 and GluA2 might be key for recovery of neurons from arsenic-induced neurite suppression.

The observation that GluA overexpression rescues neurons from arsenic neurotoxicity implies that GluA stimulation by certain drugs may have a therapeutic effect on neurodegeneration induced by arsenic. AMPA receptor agonists and modulators, such as ampakines, might be candidate drugs for the prevention and/or treatment of arsenic-induced neurodegeneration.

4.3. Effect of low arsenic concentrations on neurons

Although exposure to NaAsO₂ concentrations of 1 μM and above reduced cell survival, the concentration of 0.5 μM NaAsO₂ had the opposite effect and rather significantly increased cell survival, while immunocytochemistry measurements showed that it tended to increase neurite outgrowth and significantly increased GluA1 levels. Biphasic effects of chemicals depending on the concentration are known phenomena in toxicology (Conolly and Lutz, 2004). Moreover, exposure to arsenic concentrations below the concentration that causes cell death has been reported to stimulate keratinocyte proliferation and vascular cell proliferation through overexpression of growth factors and activation of tyrosine phosphorylation, respectively (Germolec et al., 1996; Barchowsky et al., 1999). We have not yet identified the pathway by which arsenic increases cell survival and GluA1 levels in neurons at concentrations below the threshold for suppression of neurite growth. It will constitute the aim of a future study.

The occurrence of neurodevelopmental disorders such as autism and ADHD have been suspected of being associated with exposure to environmental chemicals, including arsenic (Grandjean and Landrigan, 2006). Increases in the size of some brain regions and decreases in the size of others have been reported in neurodevelopmental diseases (Seidman et al., 2011; Bauman and Kemper, 2005), and these anomalies might be responsible for patient’s cognitive dysfunction. If arsenic affects neurons differently depending on its concentration, and since great differences in arsenic distribution among brain regions have been reported after exposure to arsenic, reduction and stimulation of cell survival and/or neurite outgrowth may occur simultaneously in different brain regions (Sánchez-Peña et al., 2010). Therefore, not only a decrease but also an increase in cell number and/or neurite outgrowth should be taken into account as a feature of arsenic adverse effects.

In addition, excitotoxicity by excess glutamate release is also of particular concern in the etiology of developmental disorders including autism (Chauhan and Chauhan, 2006). Although in our culture conditions an increase in extracellular glutamate could not be evidenced following the exposure to NaAsO₂, exposing rats to arsenic has been reported to increase their cerebral glutamate content (Nagaraja and Desiraju, 1993). Therefore, the possibility that excitotoxicity occurs following arsenic exposure in vivo still remains. We suspect that increased GluA1 expression levels by exposure to low doses of NaAsO₂ can lead to higher vulnerability of cortical neurons to glutamate, given that excess glutamate release and GluA1 expression increase might occur simultaneously. Indeed, further study will be necessary to determine whether arsenic affects glutamate AMPA receptor expression and/or glutamate release in vivo.

5. Conclusions

In conclusion, exposure to arsenic at concentrations below the concentration that caused cell death in primary cultures of cortical neurons impaired neurotogenesis and we speculate that impaired neurotogenesis is partly due to reduction of GluA1 expression. By contrast, cell survival and GluA1 expression both increased when exposed to NaAsO₂ concentrations below the concentrations that impaired neurite outgrowth. Future studies will be required to determine whether these findings in culture systems also occur in experimental animals in vivo.
Author contributions
Conceived and designed the experiments: F.M. and K.N. Performed the experiments: F.M., M.O., T.T. Analyzed the data: F.M., T.T., M.O., K.A., S.T., K.N. Contributed reagents/materials/analysis tools: T.T., K.A., S.T., L.P., K.N. Wrote the paper: F.M.

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Competing interests
The authors have declared that no competing interests exist.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2013.05.006.

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