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Hot Reports on Actual Condition and
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Hazards : Medical Study / Toxicity : S2-4

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Arsenic affects AMPA receptor trafficking in primary cultured neurons

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Abstract

High concentration of arsenic contamination in ground water causes serious concern in many areas in the world, especially South Asian region. The University of Miyazaki has been extensively working on prevention and rehabilitation works of arsenic affected people in Nepal, India and Bangladesh. In an effort to test if drinking of arsenic contaminated water may cause psycho-behavioral symptoms through AMPA type glutamate receptor function: major excitatory neuronal transmitter receptor in central nervous system, we initiated study of arsenic toxicity and neuronal malfunctions in neurons. The present study was aimed to evaluate the effects of 4 different types of arsenic, such as standard arsenic (As-V, As-III) and their metabolites Di-methylarsonous acid (DMA), Mono-methylarsonous acid (MMA) on synaptic localization of AMPA type glutamate receptor, particularly focusing on GluA1/A2 subunits using primary cultured neurons. Time and dose dependency of all four arsenic types did not show any significant changes in total GluA1 while cell surface GluA1/A2 expressions showed significant ($p < 0.1$) decreased in 0.15 μ M of As (III) and DMA at 60 min period. It is reported that regulation of GluA1/A2 complex cell surface expression is partially governed by phosphorylation at Ser-831 and Ser-845 sites of GluA1 cytoplasmic domain. All of the As compounds decreased Phos-GluA1-845 at 60 min duration. Live staining of surface GluA1 of 21 DIV (date *in vitro*) exhibited decrease in individual synaptic GluA1 during As (III) and DMA treatment, showing GluA1 internalization at synaptic sites. This phenomenon was confirmed using double staining with pre-synaptic and post-synaptic markers: PSD-95 and Synapsin. Electrophysiological recording of miniature excitatory post synaptic currents (mEPSC's) was conducted in order to understand the changes in synaptic AMPARs. Cumulative distribution plot of mEPSC amplitude confirm that AS (III) and DMA disturbs synaptic GluA1 cell surface expression. Arsenic wash out studies showed that inhibited GluA1 expressions were recovered in 24 hours, indicating synaptic homeostasis. In summary our study suggests that arsenic toxicity is involved in AMPA receptor trafficking, resulting in neuronal functions

Keywords: Arsenic, Toxicity, Neurons, AMPARs, Trafficking

1. Introduction

In periodic table, Arsenic (As) is having atomic number of 33 and having elemental atomic weight of 74.92. Arsenic compounds are used in making special types of glass, paints and semiconductor chip industries. Recently, a number of arsenic compounds have been used as medicines especially in treatment of Leukemia. Arsenic is one of the most toxic elements that can be found. Humans may be exposed to

arsenic through food, water and air. Exposure may also occur through skin contact with soil or water that contains arsenic. But levels of arsenic in fish and seafood that contain significant amounts of inorganic arsenic may be a danger to human health. Exposure to inorganic arsenic can cause various health effects, such as irritation of the stomach and intestines, decreased production of red and white blood cells, skin blisters and lung suffocation. It is suggested that the

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uptake of significant amounts of inorganic arsenic can intensify the chances of cancer development, especially skin cancer, lung cancer, liver cancer and lymphatic cancer. A very high exposure to inorganic arsenic can also cause infertility and miscarriages in women, and it can cause skin problem, declined resistance to infections, heart disruptions and brain damage (WHO, 2001). Despite its notoriety as a deadly poison, arsenic is an essential trace element for some animals, and maybe even for humans, although the necessary intake may be as low as 0.01 mg/day.

In spite of all adverse effect to human, As toxicity research on central neuronal system is still not progressed. Areas of neurotoxicological impairments include several higher brain functions, such as poor cognitive performance, disturbances in visual perception, retardation of psychomotor speed, attention problem, speech, and memory loss (Florea and Busselberg 2006). The lower intellectual functions and deficiencies in learning and memory is also reported in arsenic epidemiological studies (Rodriguez et al. 2003), given neuronal network in the brain may be affected due to disturbed glutamate receptor functions, such as N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors. We have intensively studied in this area, in order to understand the mechanism of arsenic action because AMPAs are the primary excitatory neurotransmitter receptors in the central nervous system.

2. Materials and method

All chemicals and mediums for culture are of commercial grade

2.1. Neuronal cell culture: Cultures were prepared from cortices or hippocampi of rat embryos as per standard procedure. After dissection and removal of meninges followed by enzymatic and mechanical dissociation, primary neurons are plated on to poly-L-lysine coated culture vessel and subsequently cultured with neuronal growth medium (Gibco, Paisley, UK). 21 DIV (date *in vitro*) cultures are used for biochemical (cortical cells) and cell biology (hippocampal cells) assay.

2.2. Arsenic type and treatments: We selected four types of arsenic, standard pentavalent arsenic (As-V) and trivalent arsenic (As-III) and its metabolites: Di-methylarsonous acid (DMA), Mono-methylarsonous acid (MMA). Selected doses were 0, 0.01, 0.05, 0.1, 0.15 and 0.2 mM and duration of exposer was 0, 30 and 60 min.

2.3. The surface and total AMPA receptors are measured in Biotynalation assay. Briefly, after arsenic treatment for stipulated time and duration, neurons are solubilized with detergents containing buffer. After spin, a little supernatant was kept for total AMPA-R measurement. The surface

AMPA-R was isolated by neutravidin agarose beads.

2.4. Immunoblotting and Immunostaining: We focused on GluA1 and its two phosphorylated subunits like GluA1-S845 and GluA1-S831. Primary antibodies used were anti-GluA1 (JH-4214), anti-GluA2 (JH 4297) for western blot. For surface live staining we used GluA1 (9094) as primary antibody and Alexa red flour-488 poly rabbit IgG as second antibody (Invitrogen, Life technologies. Corp. NY, USA). All antibodies were kindly provided from Dr. Haganir, Johns Hopkins University (USA). For synaptic staining, we used anti-PSD-95 (Antibodies, Inc, Davis, CA, USA) and anti-Synapsin (Chemicon, Millipore, Billerica, MA, USA) as pre and post synaptic markers, respectively.

2.5. Recordings of Miniature Excitatory postsynaptic Currents: Whole cell recordings were carried out in DIV-21 cultured neurons after arsenic. Recordings of each neurons lasted for 60 min and data was analyzed by Axopatch software (Molecular Devices, CA, USA)

3. Results and Discussion

In order to determine the neurotoxicological potential of arsenic and its metabolites on neuronal functions, we

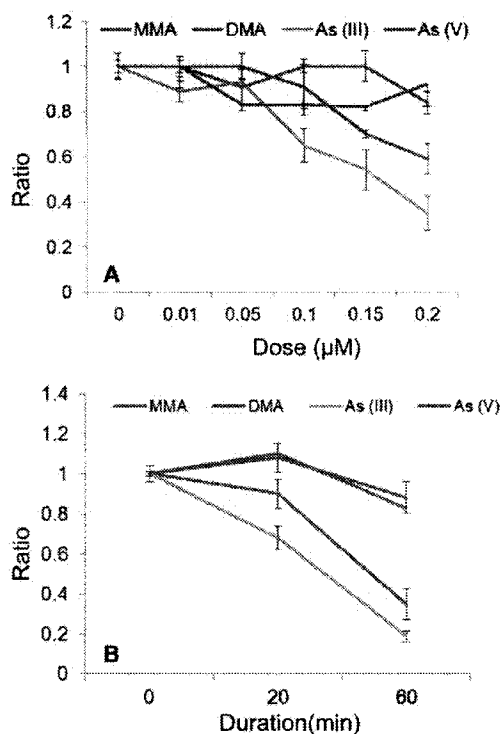


Figure 1. A. Fluctuation of surface GluA1 expression with different doses and time (B) duration after treatment with different kinds of Arsenic: As (V), As (III), MMA and DMA

investigated two arsenites, pentavalent (As -V), trivalent (As-III) arsenic, and metabolites, such as DMA and MMA in DIV 21 primary rat neurons. To assess the exact dose and

exposure time, we did dose and time dependency test on measurement of surface GluA1. Results were depicted in figure 1. A significant decrease of GluA1 expression was noted in As (III) and DMA treatment at 60 min exposure time. This shows that pentavalent organic arsenic which is metabolite of inorganic arsenic and MMA form are less toxic to neurons may be because of the organic nature of the compound. To ensure that above observed results were occurring on GluR1 at synaptic sites, we exposed primary culture neurons to As (III) exposer (0.15 μ M) and MMA (0.15 μ M), followed by fixation and immunostaining of surface GluA1 (Figure 2) and found significant decrease.

It has been hypothesized that regulation of GluA1 cell surface expression was governed by phosphorylation at Ser-831 and Ser-845 positions of GluA1 cytoplasmic domain. Figure 3 shows the relative ratio of Phospo-S831 (Upper panel) and phopo-S845 (lower panel) of GluA1 following exposure to various arsenic compounds at two points time durations, 20 and 60 minutes.

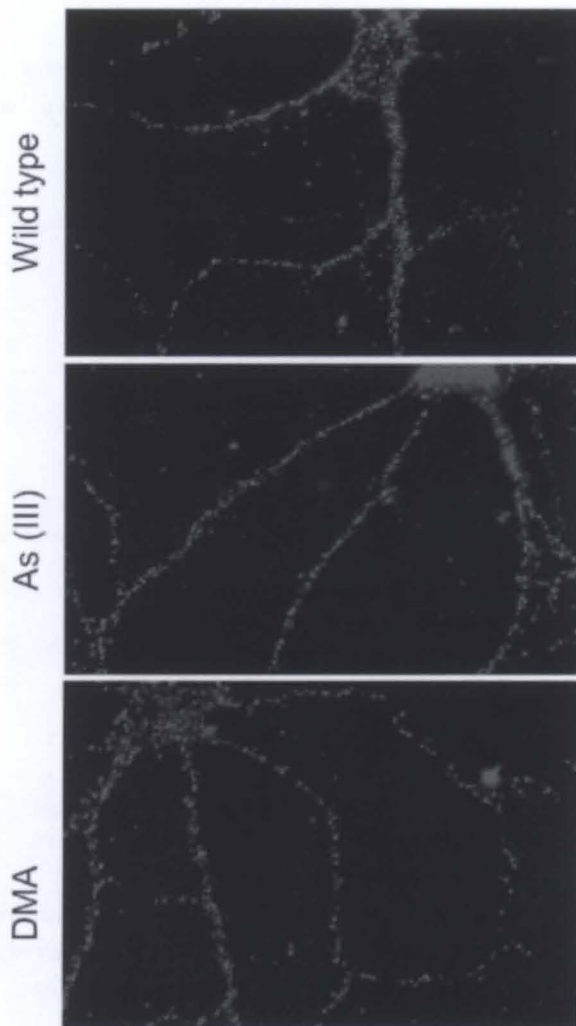


Figure 2. Immunostaining of surface GluA1 after treating with two different arsenic and followed by FITC conjugated GluA1 specific secondary antibody.

It is reported that phosphorylation of GluA1 regulates

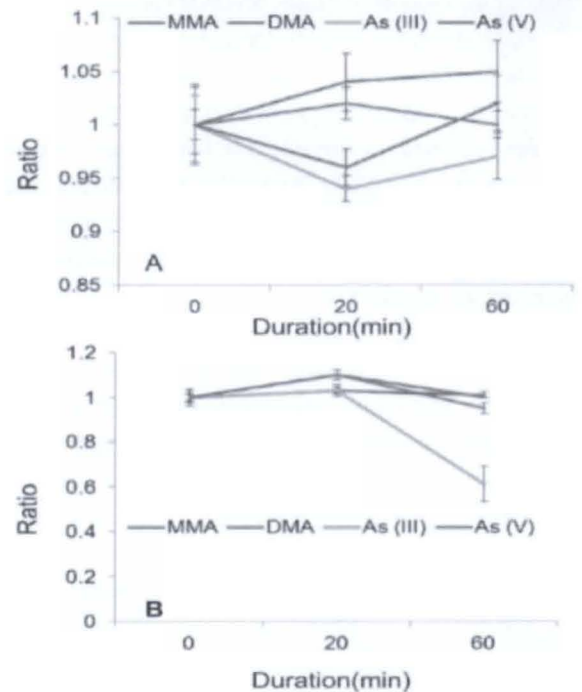
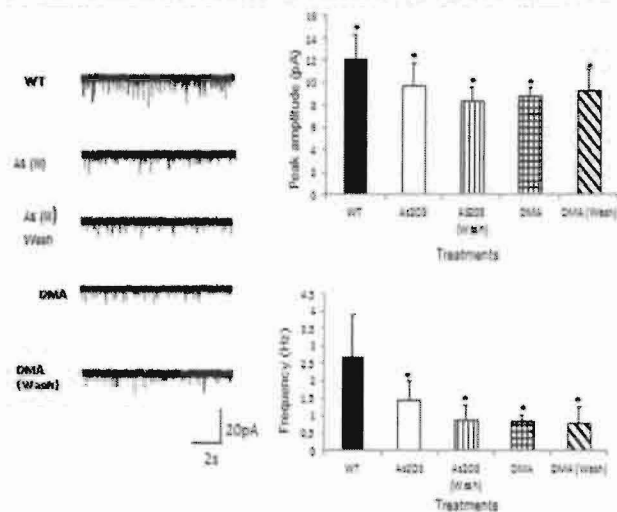


Figure 3. Fluctuation of surface GluA1 -Phos-831 (A) and GluA1-Phos-845(B) after treating with different As compounds at different time duration

channel properties and trafficking (He, et.al. 2011). None of the four As compounds elicited any change in the Phos-GluA1-S831 in mature neurons. Interestingly, only As-III significantly ($p < 0.01$) decreased Phos-GluA1-S845 in mature neurons than the other As compounds, indicating the differential regulation of As on AMPA-R function. These results may support the idea that phosphorylation of the GluA1-S845 site "primes" the AMPARs for synaptic plasticity and is associated with memory loss as observed by Seol and others (Seol et.al. 2007). As shown in this study As (III) due to its high toxic nature must have decreased the Phos-GluA1-S845 and may affect memory formation.

The strength of excitatory synaptic transmission depends partly on the number of AMPA receptors (AMPA-R) on the synaptic surface and, thus, can be modulated by membrane trafficking events. These processes are critical for some forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD). In next step, we analyzed As effects for AMPAR function using electrophysiological techniques: single cell patch clamp experiments to measure mEPSCs currents with two types of As, As(III) and DMA; because these two substances showed severe toxic and a significant decrease in AMPAR as shown in Figure 1.

Representative traces of mEPSCs in wild type (control), As treatment and As wash out group over 60 min duration were demonstrated in Figure 4. Quantified data showing that both types of As decreased the peak amplitude and frequency than the control group and this decrease in peak amplitude is



partially recovered while As washout experiments conducted

Figure 4. Long term potentiation of synaptic function induced by As (III) and DMA in single cell neuron and wash out experiments. Recordings were plotted as mean frequency (Hz) and peak amplitude (pA)

but mean frequency failed to recover even after washout. The changes in AMPARs by AS may explain the process of impairment connected with learning and memory, as recent studies revealed significant correlation between children's intellectual and memory functions and arsenic exposer (Wright et.al. 2006). The depressant effect was not reversible after a 60-min washout of the DMA and As (III) and were more pronounced than in those taken wild types confirms the nature of neurotoxic potential

It is now widely accepted that AMPAR regulation is a key component in the expression of postsynaptic forms of LTP and LTD, as well as homeostatic synaptic plasticity of excitatory synapses. There are largely 2 modes of AMPAR regulation that contribute to synaptic plasticity: One is via regulation of its synaptic trafficking and the other is via alterations in phosphorylation of its subunits (Lee, 2006). We then continued our study on synaptic homeostatic behavior while treating with As (III) and DMA in long term recovery experiments like 2 hrs. and 24 hrs. time duration. It is clear from the result (Figure 5) that As significantly inhibited the both GluA1 and A2 at 60 min exposure time.

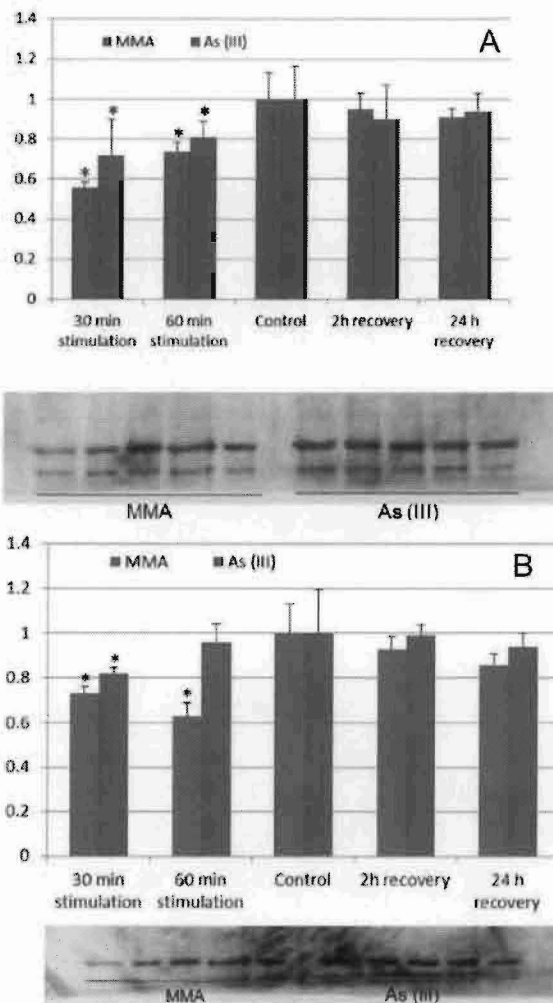


Figure 5. Fluctuation of GluA1 (A) and GluA2 after treating with As (III) and MMA and recovery after 2 hrs and 24 hrs with normal medium

The activity of both AMPARs are recovered back to the normal condition, showing the synaptic haemostasis. However, a observed slight deviation from mEPSCs experiment (Figure 4) remains to be solved by further experiments. We consistently observed the decrease in surface GluA1 that was similar to that observed in biotinylation western blot experiments (Figure 1). There is a strong correlation between neuronal surface AMPARs and synaptic AMPARs levels (Kessels et.al. 2009). AMPARs recruited to synapses from preexisting surface AMPARs population (Patterson et.al, 2010). Hence changes and entire process in LTP is managed and governed by endo and exocytosis of AMPARs. In order to observe surface AMPARs trafficking we conducted co-immunostaining of cell surface GluA1 with post synaptic density marker, PSD95. Results are depicted in figure 6

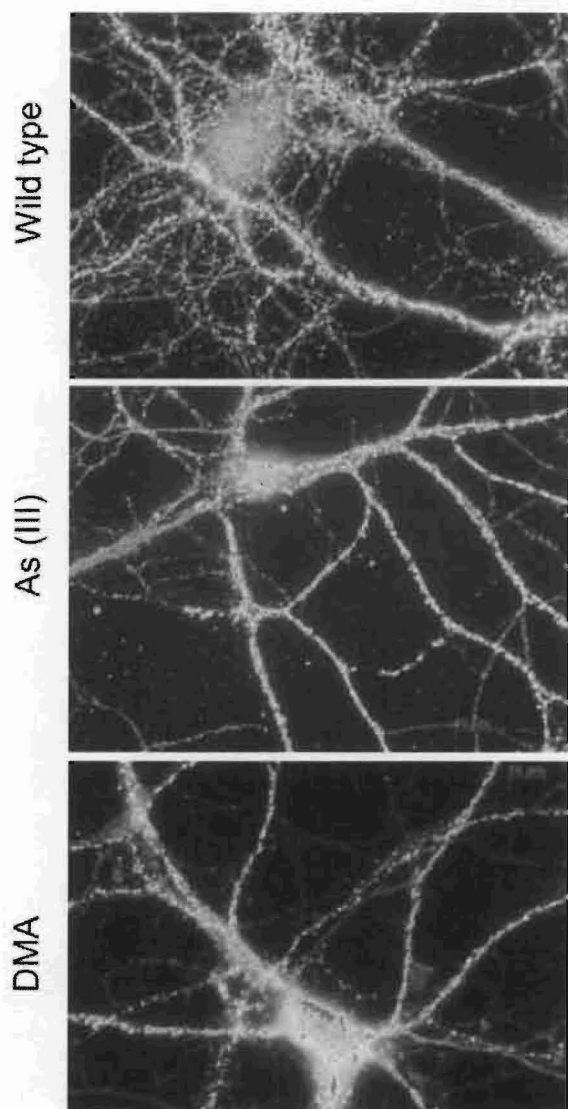


Figure 6. Immunostaining of Post synaptic AMPARs by PSD-951 after treating with two different arsenic and followed by GFP-GluA1 conjugated with Anti mouse green Alexa-488 as secondary antibody.

It is very clear from the present observation (Figure 6) that As did change the post synaptic AMPARs. From the previous results that As changes synaptic AMPA-R response (Figure 4) and decreased synaptic AMPARs (Figure 2) cumulatively suggests the coordinated events of AMPARs internalization and confirms that two forms of arsenic systematically affect the AMPARs trafficking. A close proximal perisynaptic endocytosis zone to the PSD is maintained by protein-protein interaction in involving the long forms Homer and Shank (Lu et al. 2007). To find out process of trafficking, we continued immunolocalisation of presynaptic markers by using synapsin and present study showed AS (III) and DMA significantly disturbed the AMPARs (results not shown)

4 Summary

5 In end, among all four As studied, AS (III) and DMA found to be potent neurotoxic. Preliminary result so far accumulated from this study indicates arsenic disturbs the synaptic plasticity. Overall, present study also indicates that arsenic toxicity may be involved in higher neuronal functions through AMPA receptor channel function and perhaps deteriorate synaptic plasticity.

5 Acknowledgement

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