

Chronic exposure to low levels of aluminum alters cerebral cell signaling in response to acute MPTP administration

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Two-month-old male B/6C3F1 mice were treated for 10 weeks with 100 μ M aluminum lactate (Al) in drinking water. This dose of Al did not alter body weight, and there was no evidence of systemic toxicity. The degree of phosphorylation of several kinases which lead to transcription factor activation (reflecting the extent of their activation) was studied. The proportion of extracellular signal-regulated kinase (ERK) that was activated was depressed in cortex but not in the hippocampus following treatment but c-Jun N-terminal kinase (JNK), p38, I κ B phosphorylation was unaltered in either tissue. Treatment of mice with 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) alone produced no significant changes in the degree of activation of any transcription factor studied. When MPTP dosing had been preceded by extended exposure to low levels of Al in drinking water, ERK activation was profoundly depressed in cortex and hippocampus, whereas JNK in hippocampus and I κ B in cortex were greatly elevated. These changes consequent to exposure to both Al and MPTP were accompanied by an increase in NF- κ B in both regions, whereas AP-1 was elevated in the hippocampus alone. Neither agent alone modulated AP-1 or NF- κ B. Thus a synergistic interaction occurred between the toxicants. This interaction tended to promote the functioning of a kinase largely associated with inflammation and to depress that of ERK, which is associated with maintenance of cell survival. It is concluded that exposure to levels of Al with no evident toxicity can worsen the response to an acute challenge with MPTP. Al treatment alone was able to increase striatal 3,4-dihydroxyphenylacetic acid levels, suggesting an elevation of the rate of dopamine turnover in the striatum. However, no interaction in alteration of monoamine levels was found between Al and MPTP. *Toxicology and Industrial Health* 2007; 22: 515–524.

Key words: aluminum; cell signaling; inflammation; MPTP

Introduction

The concentration of aluminum lactate (Al) in drinking water has been reported to vary from

10 μ M to 25–230 μ M in Ontario and Texas (Nieboer, *et al.*, 1995; Forbes and Hill, 1998; Cech and Monterera, 2000). Because an elevated concentration of aluminum in the drinking water has been correlated with a higher risk of developing Alzheimer's disease (AD), in certain districts, there may be a potential for harmful exposure to the metal. Indeed, nine out of thirteen published

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epidemiological studies have shown a statistically significant positive relationship between concentration of aluminum in drinking water and occurrence of AD (Flaten, 2001). Epidemiologic studies have also shown a link between Al exposure and Parkinson's disease (PD) (Altschuler, 1999).

Our laboratory has previously reported that Al present in drinking water of mice elevated the proinflammatory cytokines, TNF- α and IL-1 α , in a dose-dependent manner at concentrations of 100 μ M Al and above (Campbell, *et al.*, 2004). Al also stimulated the secretion of other parameters related to oxidative stress, such as nNOS and malondialdehyde (Becaria, *et al.*, 2006). These changes parallel events associated with neurodegenerative diseases, such as AD and PD (Hald and Lotharius, 2005). Similar changes have also been observed in animal models of PD involving use of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) (Nishimura, *et al.*, 2001; Tieu, *et al.*, 2003; Dalfo and Ferrer, 2006). An agent which can produce lesions similar to those that are observed in PD, also induces parkinsonism-like symptoms (Saporito, *et al.*, 2000).

Aluminum lactate and MPTP share other common properties. Both agents are able to induce oxidative stress (Becaria, *et al.*, 2006; Franco, *et al.*, 2007). In addition, inflammatory changes have been reported in the brain of animals exposed to either Al at low levels for an extended period (Campbell, *et al.*, 2004), or treated acutely with MPTP (Pattarini, *et al.*, 2007). Thus both Al salts and MPTP can separately effect the brain in a manner which has been associated with neurodegenerative diseases. The hypothesis underlying the current study is that exposure to both agents may result in a synergistic interaction between the two compounds.

Inflammatory and oxidative responses can be enhanced through mitogen-activated protein kinases (MAPK) (Rahman and Adcock, 2006) acting on key transcription factors, such as NF- κ B and AP-1. At a molecular level, the immune system can be partly controlled by a series of regulatory proteins through protein phosphorylation. The MAPK family is one of the major regulatory proteins that play a role in cell function by transducing extracellular signals into cellular responses (Pinsky, *et al.*, 2004). We have elected to concentrate on these upstream events in inflammation-related sig-

nal pathways. There are three kinases included in the MAPK family: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Each of these exists in an inactive form and in an activated phosphorylated form. We measured the levels of phosphorylated ERK (pERK)/ERK, phosphorylated JNK (pJNK)/JNK, and pp38/p38 in the brain. This ratio is used to evaluate the proportion of each MAP kinase that exists as the activated form. In addition, NF- κ B and AP-1, and glial fibrillary acidic protein (GFAP) (an index of astrocyte activation) were also assayed.

Experimental methods

Animal treatment

Two-month-old male B/6C3F1 mice (a hybrid between C57BL/6 and C3H) were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA). They were housed four per cage and maintained on a 12-h light/dark cycle in a temperature controlled ($20 \pm 1^\circ\text{C}$) room. Food and water were provided *ad libitum*. For the experimental group ($n = 6$), the drinking water was supplemented with 100 μ M of Al. This salt has a greater potential for increasing the aluminum content in the cerebral cortex than other aluminum salts (Testolin, *et al.*, 1996). The dose of Al chosen was based on our previous experimental results showing that, at this level, Al induced increases in levels of proinflammatory cytokines and oxidative parameters in the mouse brain. Epidemiological evidence suggests that long-term exposure to Al is necessary to lead to an adverse effect (McLachlan, *et al.*, 1996). Therefore, a 10-week exposure period was selected, which is approximately equivalent to 8 years of human life.

Control mice received double-distilled water. The amount of water consumed was recorded twice a week and the weight of the animals was determined each week. After 10 weeks of Al treatment, mice were injected (i.p.) four times with 10 mg MPTP per mouse at 2-h intervals. The dose of MPTP used in this study is nontoxic but sufficient to sensitize the dopaminergic system to other insults (Shepherd, *et al.*, 2006). Animals were killed 72 h later. One half of each brain was divided into three regions – cortex, hippocampus,

and striatum, and quickly frozen on dry ice. The other half of the each brain was fixed with 4% paraformaldehyde. In this study, only cortex and hippocampus were assayed for signaling events. Striatum was too small and there was not sufficient amount available for the protein studies, but dopamine and its metabolites were measured here.

Preparation of samples

Cytoplasmic and nuclear fractions were prepared using the method of Lahiri and Ge (2000). The brain tissue from each animal was weighed and homogenized in an ice-cold buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.5% NP-40). The samples were incubated for 10 min and centrifuged (1500 *g*) at 4 °C for 1 min. The supernatant containing the cytoplasmic constituents was collected and protease inhibitor cocktail was added. The samples were aliquoted and stored at -80 °C. The nuclear pellet was resuspended in a buffer composed of 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF. The samples were then centrifuged at 11 000 *g* for 5 min at 4 °C. The supernatant that contained nuclear extract was aliquoted and stored at -80 °C.

Electrophoretic mobility shift assay

The extent of NF- κ B and AP-1 activation was determined in the nuclear fraction of brain tissue using a protocol developed by Promega (Madison, Wisconsin, USA). The amount of protein in 2 μ L of the nuclear extract was determined by the BCA protein assay kit (Pierce, Rockford, Illinois, USA) and 50 μ g of each sample, incubated with ³²P-labeled oligonucleotides containing either the NF- κ B or the AP-1 consensus sequence was loaded onto a gel. A negative control containing no cell extract, as well as competitor reactions were included. The specific competitor contained unlabeled NF- κ B or AP-1 consensus nucleotide, whereas the nonspecific competitor contained unlabeled SP-1 consensus oligonucleotide. The competitor reactions also contained 50 μ g of nuclear fraction derived from Al-treated mouse brains. X-ray films were manually developed and the intensity of each band was measured and quantitated using

the image analyzer, Eagle Eye, from Strategene (San Diego, California, USA).

Western blots

The levels of ERK, pERK, JNK, pJNK, p38 and p-p38 were determined using Western blotting. Protein content was determined using the BCA protein assay kit (Pierce). 25 μ g of each sample was resolved on a SDS-10% PAGE and transferred onto a nitrocellulose membrane (Biorad, Hercules, California, USA). After blocking overnight in TBST (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20) containing 5% nonfat milk, membranes were washed 4 \times and incubated for 1 h with primary antibodies [mouse monoclonal antibody against pERK (1:500); rabbit polyclonal antibody against ERK (1:1500); goat polyclonal antibody against pJNK (1:500); rabbit polyclonal antibody against JNK-1 (1:500); rabbit polyclonal antibody against p38 (1:500); and rabbit polyclonal antibody against p-p38 (1:500)], all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA. After washing the membranes 4 \times with TBST, they were incubated with appropriate secondary HRP-conjugated antibodies (1:10 000). Bands were detected with ECL reagents (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) following the manufacturer's protocol. An antibody against actin (mouse monoclonal, 1:1500 dilution) purchased from Chemicon International (Temecula, California, USA) was used to insure equal loading of protein for each sample. The intensity of specific bands was measured and quantitated using the image analyzer, Eagle Eye, from Strategene (San Diego, California, USA).

Immunohistochemistry

The brains were excised and placed in 4% paraformaldehyde. The paraffin tissue slides were prepared by Histo-Scientific Research Laboratories, Inc (Mt. Jackson, Virginia, USA). Sections were deparaffinized and then rehydrated. Sections were incubated in hydrogen peroxide for 5 min at room temperature to block endogenous peroxidase activity. Samples were blocked in serum for 1 h to minimize the nonspecific background staining, and then immunostained with a 1:1000 dilution of primary mouse anti-GFAP (BD, Biosciences, San Jose, CA, USA). Incubation times were 24 h for the

primary antibody, 1 h for the secondary antibody, and 1 h for ABC kit reagents (Vector, Laboratories, Burlingame, CA 94010, USA). Sections were washed and visualized using 3,3'-Diaminobenzidine (DAB) staining. The GFAP images were captured by a Nikon DXM1200F HIQE Color CCD camera attached to an Eclipse TE2000 fluorescent microscope (Nikon Inc, Melville, NY, USA).

Determination of dopaminergic markers

Concentrations of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum were quantitated by a method of high performance liquid chromatography combined with electrochemical detection as described previously (Ali, *et al.*, 1993).

Statistical analysis

The differences among these groups were tested using one-way analysis of variance followed by the Tukey test. Values were accepted as significant if $P < 0.05$ level using a two-tailed criterion.

Results

Activation of NF- κ B and AP-1

In cortical tissue, levels of NF- κ B were significantly increased following treatment with both MPTP and Al. However, Al or MPTP alone did not lead to significant changes in activation of NF- κ B compared with control (Figure 1A,B).

The level of NF- κ B in hippocampus region was also elevated in Al and MPTP groups, but no significant changes were apparent after exposure to either Al or MPTP alone compared with control (Figure 1C). The transcription factor AP-1 also responded to various treatments. In cortex, no significant difference in the levels of AP-1 was detected among four groups (Figure 2A). However, in the hippocampus of animals treated with Al and MPTP, the level of AP-1 was significantly higher relative to control values or to values from groups treated with either Al or MPTP alone (Figure 2B).

Changes in GFAP levels

Increases in the levels of GFAP represent the activation of astrocytes. Treatment with both Al and

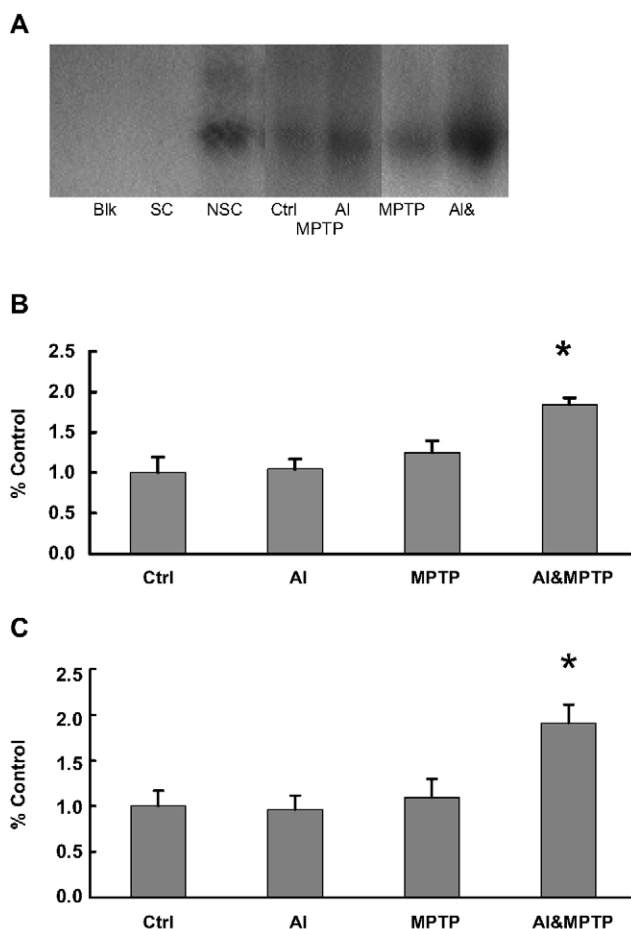


Figure 1 Levels of NF- κ B. (A) Typical gel showing the migrating NF- κ B bands in cortex. Blk, blank; SC, specific competitor; NSC, nonspecific competitor; Ctrl, control; Al, aluminum lactate. (B) Integrated density of the shifted NF- κ B bands in cortex. (C) Integrated density of the shifted NF- κ B bands in hippocampus. *Value differs ($P \leq 0.05$) from control. †Value differs ($P \leq 0.05$) from MPTP group. Bars represent mean of 6–7 samples \pm SE

MPTP led to a 148% increase in cortical GFAP levels. Neither Al nor MPTP alone caused a significant change in the level of GFAP compared with control. This observation was confirmed with both immunohistochemical staining and Western blot (Figure 3). Neither treatment with MPTP and Al separately or together led to changes in levels of GFAP in the hippocampus. Both Western blot and immunohistochemistry staining showed no significant variations among groups (Figure 4).

Proportion of activated kinases in mouse brain ERK (*pERK/ERK*)

The ratio of phosphorylated ERK to ERK indicates the proportion of total ERK that has been

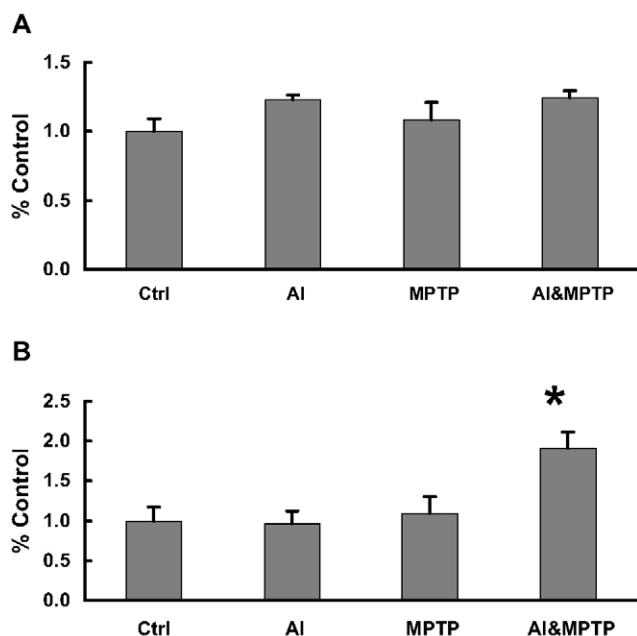


Figure 2 Levels of AP-1. Integrated density of the shifted AP-1 bands in cortex (A) and hippocampus (B). *Value differs ($P \leq 0.05$) from control. †Value differs ($P \leq 0.05$) from MPTP group. Bars represent mean of 6–7 samples \pm SE

activated in the brain. AI treatment suppressed the activation of ERK by 68% in the cortex. MPTP treatment did not induce any significant change in the activation of this kinase. However, when animals were given both AI and MPTP, the proportion of activated ERK in cortex was further decreased to 45% (Figure 5A).

In hippocampus, neither AI nor MPTP separately caused any obvious alteration in the pERK/ERK ratio (Figure 5B). However, when mice were treated with both AI and MPTP, the level of pERK was significantly reduced and level of ERK itself was increased in hippocampal tissue. The ratio of pERK/ERK was decreased to 15% of the original control value.

JNK (*pJNK/JNK*)

Phosphorylated JNK is the main upstream kinase that enables the activation of AP-1. In cortical tissue, there was no perceptible change in the proportion of pJNK in AI, MPTP, or AI MPTP groups relative to control values (Figure 6A).

In the hippocampus, co-treatment with AI and MPTP caused a significant elevation of JNK. Neither AI nor MPTP alone altered the proportion of JNK that was phosphorylated (Figure 6B).

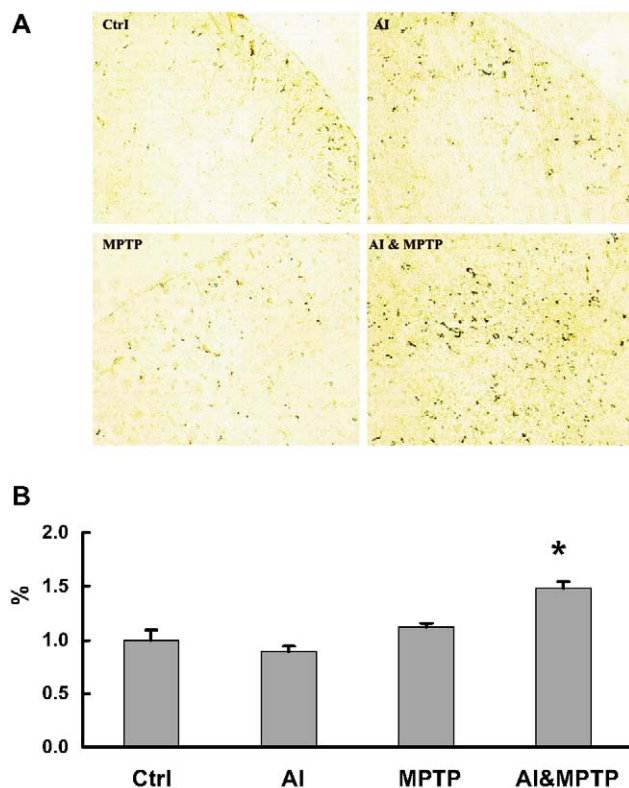


Figure 3 Levels of GFAP in cortex. (A) GFAP immunohistochemical staining. Dark staining material reveals activated astrocytes. (B) Integrated density of the GFAP bands from Western blot. *Value differs ($P \leq 0.05$) from the control value. Bars represent mean of six samples \pm SE

p38 (*p-p38/p38*)

The ratio p-p38/p38 did not change in either cortex or hippocampus following treatment with AI, MPTP, or AI and MPTP compared with control values (Figure 7A,B).

I κ B (*pI κ B/I κ B*)

The activation level of the inhibitor of κ B (I κ B) was also measured by Western blot. In cortex, a significant synergistic effect between AI and MPTP was found. AI and MPTP provoked the activation of I κ B to pI κ B by 174% over the control value (Figure 8). MPTP alone increased the ration pI κ B/I κ B by 138%, but the difference was not statistically significant. pI κ B was not detected in the hippocampus (data not shown).

Levels of dopamine and its metabolite DOPAC

MPTP treatment alone elevated striatal dopamine levels while elevating those of DOPAC (Figure 9A, B). This implied increased degradation of dopamine. AI treatment alone was also able to increase

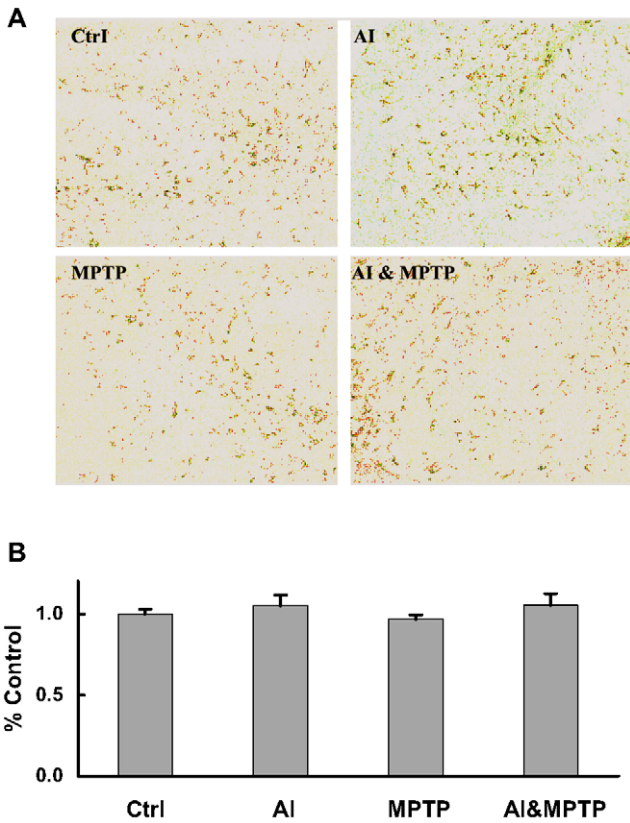


Figure 4 Levels of GFAP in hippocampus. (A) GFAP immunohistochemistry. Dark staining material reveals astrocytic activation. (B) Integrated density of the GFAP bands. Bars represent mean of six samples \pm SE

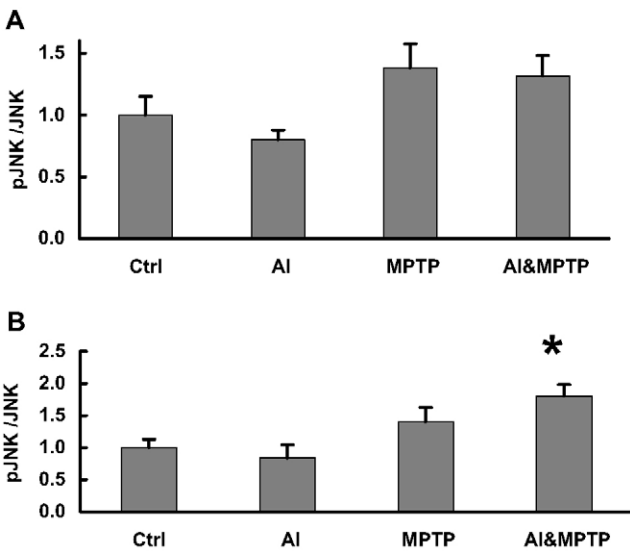


Figure 6 Proportion of JNK in activated form. (A) Integrated density of the bands for hippocampus. (B) Integrated density of the bands for cortex. *Value is significantly different ($P \leq 0.05$) from the control. †Value differs ($P \leq 0.05$) from MPTP group. Bars represent mean of six samples \pm SE

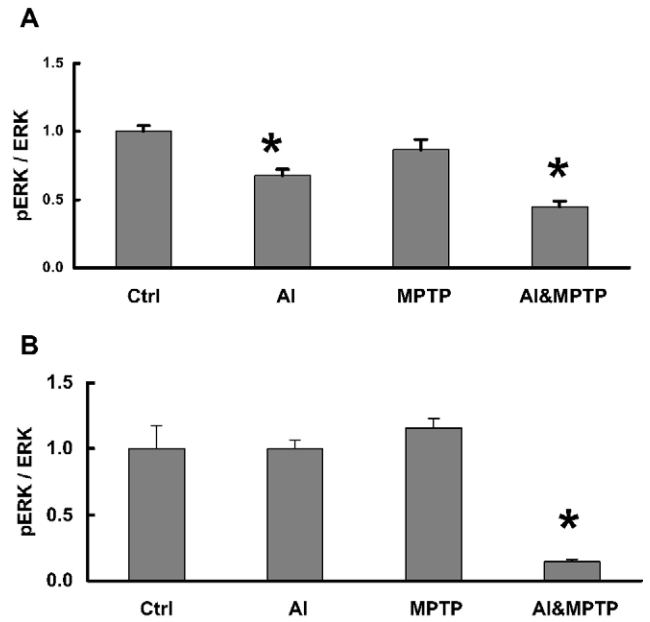


Figure 5 Proportion of ERK in activated form. (A) Integrated density of the bands for hippocampus. (B) Integrated density of the bands for cortex. *Value differs ($P \leq 0.05$) from the control. †Value differs ($P \leq 0.05$) from MPTP group. Bars represent mean of six samples \pm SE

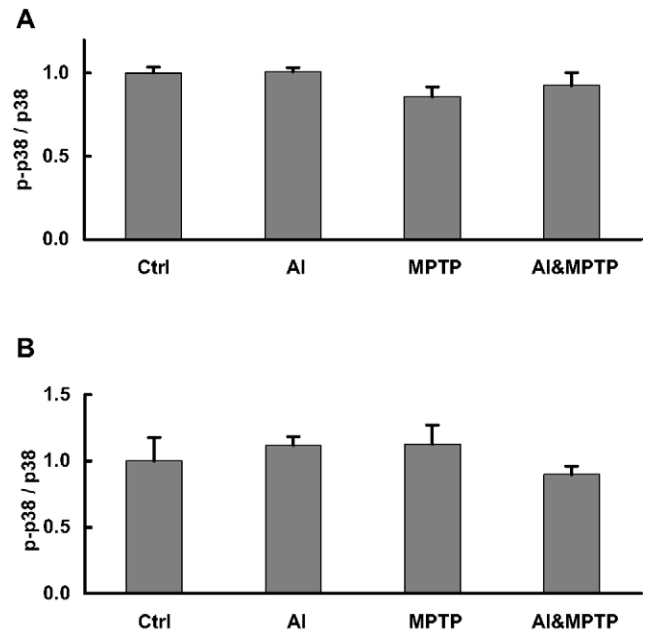


Figure 7 Proportion of p38 in activated form. (A) Integrated density of the bands for hippocampus. (B) Integrated density of the bands for cortex. Bars represent mean of six samples \pm SE

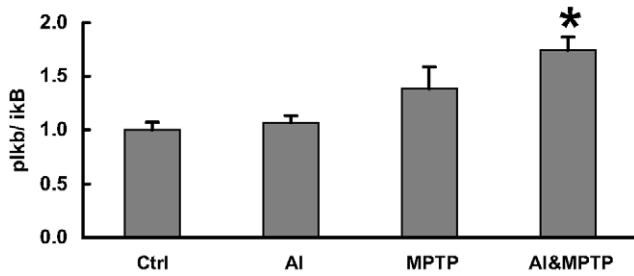


Figure 8 Proportion of IκB in cortex in activated form. Integrated density of bands. *Value differs ($P \leq 0.05$) from the control. Bars represent mean of 6–7 samples \pm SE

DOPAC levels, suggesting an elevation of the rate of dopamine turnover in the striatum. However, no synergistic interaction in alteration of monoamine levels was found between AI and MPTP. On the contrary, both agents together caused a diminution of the magnitude of changes caused by MPTP alone.

Discussion

The finding that MPTP, at a dose that did not cause overt effects at the time-point evaluated, could cause changes in the brain when treated together with AI suggests a synergistic interaction between these toxicants. There was some difference between the cortical and hippocampal responses, suggesting that signaling pathways were differentially effected. The relative levels of activated ERK were suppressed and activated JNK were increased in the hippocampus of the AI and MPTP-treated group only.

As AI treatment alone only induced a decrease in pERK in cortex but no change with any other kinases, this kinase may be involved in the signaling pathway that triggered the elevation of cortical inflammatory cytokines and oxidative parameters with chronic exposure to AI in drinking water. Inhibition (dephosphorylation) of ERK, and activation of JNK are implicated in neuronal death (Davis, 2000; Mielke and Herdegen, 2000; Ballif and Blenis, 2001). Thus, the suppression in pERK in cortex and hippocampus, and up-regulation in pJNK in hippocampus observed in this study may eventually lead to neuronal cell death. The consistent levels of p-p38/p38 throughout the groups suggest that p38 was not involved in AI or MPTP-induced changes.

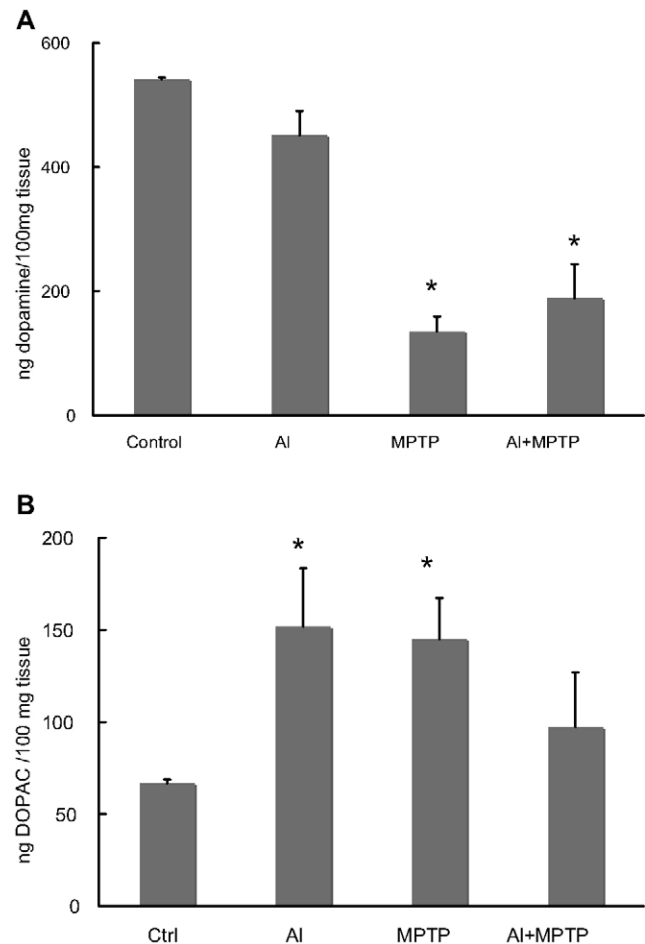


Figure 9 Striatal levels of (A) dopamine and (B) its metabolite, DOPAC. *Value differs ($P \leq 0.05$) from the control. Bars represent mean of 6 values \pm SE

Several reports from other laboratories parallel the findings pertaining to MPTP described here. *In vivo*, MPTP treatment of intact animals stimulated a threefold increase in JNK phosphorylation (Saporito, *et al.*, 2000). Prior treatment with a JNK inhibitor, SP600125, reduced the MPTP-induced JNK phosphorylation and apoptosis of dopaminergic neurons, and restored dopamine concentration in mouse brains (Wang, *et al.*, 2004). In isolated cell cultures, an acute dose of MPTP rapidly elevated JNK phosphorylation and stimulated ERK dephosphorylation, accompanied by cell death. In addition, the ERK inhibitor, PD98059, exacerbated MPTP toxicity reducing cell viability, while the JNK inhibitor, CEP-11004, alleviated MPTP-induced axon degeneration and cell death (Giro-lamo and Billett, 2006). These results indicate that

ERK and JNK play essential roles in cell survival and differentiation.

Changes in MAPK could lead to an altered degree of activation of NF- κ B and AP-1. In the bulk of the cortex, NF- κ B was only increased in the Al and MPTP-treated group, and the pI κ B level was elevated in this group. Al or MPTP alone did not lead to clear changes in levels of NF- κ B or pI κ B. Our previous data also found that Al did not lead to noticeable changes at the concentration studied here (100 μ M), although NF- κ B activation was observed at lower levels of Al (10 μ M) (Campbell, *et al.*, 2004). There were no obvious changes observed in AP-1 levels in cortical tissue, following any treatment.

In hippocampal tissue, while Al or MPTP separately did not induce any evident alteration in levels of NF- κ B or AP-1, levels of NF- κ B and AP-1 were increased following treatment with Al and MPTP together. pI κ B was not detectable in hippocampus. Many studies have indicated that an increase of pERK may phosphorylate the I κ B and consequently activates NF- κ B (Hatada, *et al.*, 2000; Wang, *et al.*, 2004). However, an increased level of NF- κ B has been reported to reciprocally inhibit the phosphorylation of ERK (Ahmed, *et al.*, 2006). We observed a selective suppression of pERK and up-regulation of pI κ B in cortex or hippocampus in animals treated with both MPTP and Al. This suggested that other kinases, such as the inhibitor of κ B kinase, might be involved in signaling pathways activating NF- κ B (Figure 10). Al and MPTP together may have triggered the activation of NF- κ B through signaling pathways that phosphorylated I κ B, and the increased level of NF- κ B led to suppression of pERK in both cortex and hippocampus. However, additional studies need to be conducted to clarify this.

The up-regulation of pJNK has been shown to play an important role in activation of AP-1 (Cuschieri and Maier, 2005). In our study, the activation of transcription factor AP-1 specifically in hippocampal tissue after treatment with both Al and MPTP may be attributable to the observed increase in the proportion of phosphorylated JNK in this region.

Aluminum lactate and MPTP together stimulated astrocyte activation in cortex, the nuclear translocation of AP-1 in hippocampus, and activa-

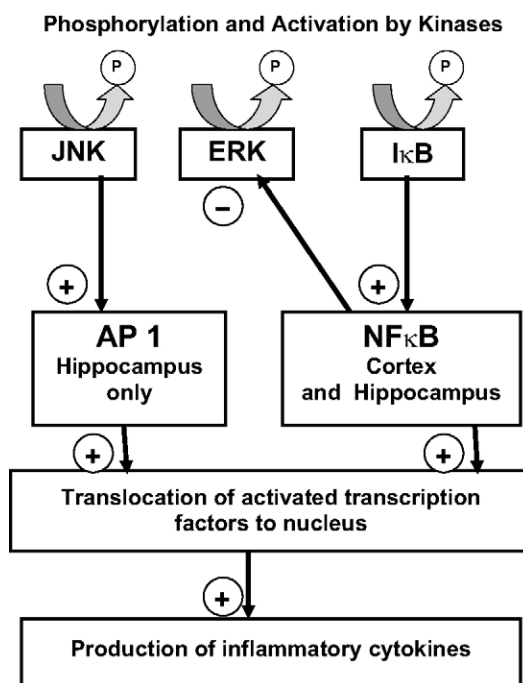


Figure 10 MAP kinase pathways related to experimental results. ⊕ represents positive feedback, and ⊖ indicates negative feedback postulated to be accentuated after Al + MPTP treatment

tion of NF- κ B in both regions. These different responses in cortex and hippocampus suggest that different regions of brain might be selectively vulnerable to a toxic insult. Al or MPTP alone did not induce any major changes in these parameters. As we have not performed a time course study, it might be that a delayed response was missed. However, when treated with both agents, a synergistic effect was observed. Al may thus either enhance or accelerate the MPTP-induced responses in the brain.

Glial cells, MAPK and transcription factors, such as NF- κ B and AP-1, are involved in neurotoxicity associated with PD. The changes in these parameters are predominantly reported from the striatum (Teismann, *et al.*, 2003; Kuan and Burke, 2005; Sarkar and Fisher, 2006). Our findings imply that joint exposure to Al and MPTP may potentiate a similar neurotoxicity in cortex and hippocampus. Although we were not able to examine striatal tissue, it is likely that similar but more severe changes would have been found. However, Al treatment together with MPTP did not further alter changes in striatal dopamine or DOPAC levels effected by MPTP alone. Thus the effect of MPTP upon striatal dopamine was not potentiated

by Al. This suggests that the effects of MPTP upon cell signaling pathways may not be mediated by dopaminergic mechanisms.

A limitation of this study is that no toxic endpoints, such as cell death or behavioral disorders, were measured. To demonstrate that the molecular changes observed could underlie acceleration or enhancement of progression of PD by Al, additional studies are needed.

In conclusion, the studies presented here imply that repeated exposure to Al enhances or accelerates MPTP effects in mouse brain. This may be due to modulation of astrocyte activation, or MAPK-related signaling pathways. NF- κ B was activated and this may have led to inhibition of the activation of ERK by negative feedback control. Additionally, in hippocampus, increased phosphorylation of JNK may be causally related to activation of AP-1.

Chronic exposure to some environmental factors, such as Al, at very low concentrations might not have any noticeable toxicity to humans. However, such an exposure could play a role in increasing the sensitivity or the vulnerability of the CNS common age-related neurodegenerative disease. The cause of PD has not been determined, but environmental exposure is likely to play a significant role in its onset and development. The possibility of developing PD may thus be enhanced for those who have a history of chronic exposure to low levels of Al.

Acknowledgments

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