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THE EFFECTS OF METABOTROPIC GLUTAMATE RECEPTOR 7 ALLOSTERIC AGONIST *N,N′*-DIBENZHYDRYLETHANE-1,2-DIAMINE DIHYDROCHLORIDE ON DEVELOPMENTAL SEVOFLURANE NEUROTOXICITY: ROLE OF EXTRACELLULAR SIGNAL-REGULATED KINASE 1 AND 2 MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY

W.-Y. WANG,[a] H. WANG,[b] Y. LUO,[a] L.-J. JIA,[a] J.-N. ZHAO,[c] H.-H. ZHANG,[a] Z.-W. MA,[d] Q.-S. XUE[a] AND B.-W. YU[a]*

[a]*Department of Anesthesiology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Ruijin Er Road 197, 200025 Shanghai, China*

[b]*Department of Anesthesiology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China*

[c]*Department of Anesthesiology, Sir Run Run Shaw Hospital, Medical College, Zhejiang University, Hangzhou, China*

[d]*Department of Neurobiology, Shanghai Jiao Tong University School of Medicine, Shanghai, China*

Abstract—The present study was designed to evaluate the possible neuroprotective effects of metabotropic glutamate receptor (mGluR7) allosteric agonist *N,N′*-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082) on developmental sevoflurane neurotoxicity. To achieve the objective, hippocampal cultures (7 DIV, 7 day *in vitro*) were treated with different doses of L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4, an agonist of group III mGluRs), (RS)-α-Methylserine-O-phosphate (MSOP, an antagonist of group III mGluRs), AMN082 or *cis*-2-[[(3,5-Dichlorophenyl)amino]carbonyl]cyclohexanecarboxylic acid (VU0155041, an agonist of mGluR4) before exposed to sevoflurane. Cell apoptosis were determined by flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL)-staining. For *in vivo* study, rat pups (7 PND, 7 postnatal day) were injected with AMN082, L-AP4 or saline before sevoflurane exposure. Extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38, caspase-3, Bcl-2, and Bax were detected by Western blot. The locomotor activity and cognitive functions were evaluated by open-field test and Morris water maze (MWM), respectively. We found that L-AP4 prevented sevoflurane-induced cell apoptosis, but MSOP promoted. Specially, application of AMN082 contributed to the relief of sevoflurane-induced apoptosis *in vitro*, whereas VU0155041 did not. In addition, sevoflurane treatment led to a decrease of Bcl-2 and an increase of caspase-3 and Bax, which were mitigated by AMNO82 *in vivo*. Moreover, we showed that sevoflurane treatment resulted in a remarkable suppression of phospho-ERK1/2, which was restored by AMN082. Application of U0126 (an inhibitor of MEK) abolished the neuroprotective effects of AMN082 on sevoflurane neurotoxicity both *in vitro* and *in vivo*. In addition, sevoflurane exposure also led to an increase of phospho-JNK, but SP600125 (an inhibitor of JNK) did not attenuate sevoflurane-induced apoptosis. The total and phosphorylated p38 remained unchanged in sevoflurane-treated rat pups. Finally, AMN082 improved the learning and memory defects caused by postnatal sevoflurane exposure without alternations in emotion or locomotor activity. These preliminary data indicate that AMN082 may protect immature brain against sevoflurane neurotoxicity, and the ERK1/2 MAP kinase signaling is likely to be involved. Further studies are needed to fully assess the neuroprotective role of mGluR7 agonist AMN082 in developmental anesthetic neurotoxicity.

Key words: mGluRs, sevoflurane, neurotoxicity, MAPK, learning and memory, hippocampus.

Recently, both rodent and primate studies have demonstrated the potential deleterious effects of anesthetic exposure to the developing brain with regard to the increasing neuronal apoptosis and subsequent cognitive dysfunctions (Brambrink et al., 2010; Sanders et al., 2009; Satomoto et al., 2009). A retrospective cohort study investigated more than 5000 children and found that children who had undergone anesthesia and surgery before 3 years of age were nearly twice as likely to be diagnosed with developmental and behavioral disorders compared with children who had never undergone anesthesia (DiMaggio et al., 2009). Another two pilot studies also showed that children who had early exposure to anesthesia were at an increased risk for developing a learning and memory disability (Kalkman et al., 2009; Wilder et al., 2009). Furthermore, Wilder's team also found the risk for the development of a learning disability increases with repeated exposure of anesthetics. These findings suggest that developmental anesthetic exposure may be an independent

*Corresponding author. Tel: +86-021-64370045-666223; fax: +86-021-64333548.
E-mail address: Buweiyu@yahoo.com.cn (B.-W. Yu).

Abbreviations: AMN082, *N,N′*-dibenzhydrylethane-1,2-diamine dihydrochloride; EDTA, ethylenediaminetetraacetic acid; ERK1/2, extracellular signal-regulated kinase 1 and 2; FCM, Flow cytometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; JNK, cJun N-terminal kinase; L-AP4, L-(+)-2-Amino-4-phosphonobutyric acid; MAPK, mitogen-activated protein kinase; mGluR, metabotropic glutamate receptor; MSOP, (RS)-α-Methylserine-O-phosphate; MTT, 3-(4,5dimethylthiazol-2-yl)-2, 5diphenyl tetrazolium bromide; MWM, Morris water maze; Sevoflurane, 2,2,2-trifluoro-1-[trifluoromethyl]-ethyl fluoromethyl ether; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling; U0126, 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; VU0155041, *cis*-2-[[(3,5-Dichlorophenyl)amino]carbonyl]cyclohexanecarboxylic acid.

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risk factor for subsequent abnormal social behavior. Commonly used anesthetics (e.g. sevoflurane) are generally believed to antagonize *N*-methyl-D-aspartic acid receptor and activate γ-aminobutyric acid-A receptor. Mounting evidence suggested that anesthetics act directly on a variety of proteins such as G protein-coupled receptors (Franks and Lieb, 1994; Ishizawa et al., 2002). For example, inhalational anesthetic could inhibit the functions of metabotropic glutamate receptors (mGluRs) (Minami et al., 1998). However, whether mGluRs modulate developmental anesthetic neurotoxicity and which subtype is involved remain unclear.

The mGluRs consist of at least eight subtypes that are classified into three groups (I–III) according to their sequence homologous characteristics, signal transduction mechanisms, and pharmacological profiles. Group III mGluRs are composed of mGluR4, mGluR6, mGluR7, and mGluR8, which are selectively activated by L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4; Schoepp et al., 1999). For subtypes of group III mGluRs, both mGluR4 and mGluR7 are abundantly expressed throughout hippocampus (Corti et al., 2002; Shigemoto, 2003). In contrast, the expression of mGluR6 is restricted in retina (Nakajima et al., 1993) and mGluR8 mainly in olfactory bulb, pontine, and piriform cortex (Saugstad et al., 1997). In primary hippocampal neuronal cultures, activation of group III mGluRs has been reported to prevent nitric oxide-induced apoptosis and maintain DNA integrity (Vincent and Maiese, 2000; Vincent et al., 1999). The neuroprotective effects of group III mGluRs were also elegantly demonstrated in excitotoxic and rotenone toxicity model (Bruno et al., 2000; Jiang et al., 2006). In addition, a large body of studies suggested that ligands for specific mGluRs subtypes have potential for the treatment of multiple neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Conn et al., 2005; Lee et al., 2004). Based on this evidence, we postulated that developmental anesthetic-induced neuronal apoptosis and cognitive disorders may be alleviated by activation of group III mGluRs.

Mitogen-activated protein kinase (MAPK) cascades play a critical role in regulating cell survival and death. Among the MAPK families, extracellular signal-regulated kinase 1 and 2 (ERK1/2) has been widely associated with cell survival, whereas c-Jun N-terminal kinase (JNK) and p38 are often implicated in cell death. Activation of group III mGluRs with L-AP4 increased the phosphorylation and activity of ERK1/2 and was necessary for neuroprotection mediated by this class of receptors (Iacovelli et al., 2002). We then speculated that ERK1/2 MAPK signaling might be involved in the neuroprotective effects of certain subtype of mGluRs. Research on this topic has been hampered by lack of selective group III mGluRs subtypes modulators. The recent developments of *cis*-2-[[(3,5-Dichlorophenyl) amino]carbonyl]cyclohexanecarboxylic acid (VU0155041; Niswender et al., 2008) and *N*,*N*′-dibenzhydrylethane-1,2-diamine dihydrochloride (AMN082; Mitsukawa et al., 2005), selective positive allosteric modulator of mGluR4 and mGluR7 respectively, have made the neuropharmacological and neurochemical studies of their functions feasible. In the current study, we evaluated the possible neuroprotective effects of VU0155041 and AMN082 on developmental anesthetic neurotoxicity induced by sevoflurane, one of the most commonly used inhalational anesthetics, especially in pediatric patients. Moreover, we also examined the role of ERK1/2, JNK, and p38 MAPK signaling in sevoflurane neurotoxicity using hippocampal neuronal cultures and rat pups.

EXPERIMENTAL PROCEDURES

Animals and agents

Sprague–Dawley rats were gained from China Academy of Chinese Medical Sciences (Shanghai, China). All studies performed on animals were approved by the Institutional Animal Care and Use Committee (Shanghai Jiao Tong University School of Medicine, Shanghai, China). Animals were housed with food and water available *ad libitum* and maintained throughout the experiments on a 12-h light-dark cycle. Every effort was made to minimize the suffering and number of animals.

All chemicals and antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA), Tocris BioScience (Ellisville, MO, USA), Gibco Invitrogen (Carlsbad, CA, USA), Cell Signaling Technology (Beverly, MA, USA), and Abcam (Hong Kong, China) unless specified in the text.

Preparation of primary hippocampal neuronal cultures

Hippocampal neurons were derived from embryonic day 18 Sprague–Dawley rat embryos (Vincent and Maiese, 2000). Briefly, hippocampal tissues were dissected in 1× HBSS, gently minced and trypsinized (trypsin 0.05%; 37 °C, 5% CO_2 for 10 min), and the digestion was stopped by DMEM plus 10% heat-inactivated FBS. Cells were plated at the concentration of 4×10^5/ml onto dishes, 96-well plates or coverslips, which were coated with 100 μg/ml poly-L-lysine. Neurons were maintained in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C and fed with neurobasal media supplemented with 2% B27, 0.5 mM L-glutamine, and 10 mM HEPES. Half of the medium was replaced with fresh medium every 2–3 days. Cells were used at 7 day *in vitro* (DIV).

Sevoflurane exposure

Sevoflurane exposure was performed as described previously (Satomoto et al., 2009). Neuronal cultures and rat pups were placed in a tightly sealed plastic chamber at 37 °C. Three percent sevoflurane was delivered from a calibrated vaporizer. For neuronal cultures, sevoflurane was flushed in a gas mixture of 95% O_2 and 5% CO_2. For *in vivo* study, seven postnatal day (PND) rat pups were exposed to sevoflurane in a gas mixture of 30% O_2 and 70% N_2. Control experiments were performed in the same manner, except no sevoflurane was added when flushing the chamber. The gas mixture was delivered at a flow rate of 2 L/min. The concentrations of sevoflurane, O_2, and CO_2 in the chamber were continuously monitored using a Datex Capnomac Ultima gas analyzer (Datex Ohmeda, USA). After sevoflurane exposure, rat pups were euthanized by overdose pentobarbital (100 mg/kg, i.p.) for Western blot, others were transferred to their cages for behavior study 4 weeks later.

Western blot

Thirty micrograms of lysates from neuronal cultures or hippocampal tissues were loaded and separated by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane,