Chinese Journal of Physiology 54(2): 118-123, 2011
DOI: 10.4077/CJP.2011.AMM047

Effect of Delta-Opioid Receptor Over-Expression on Cortical Expression of $GABA_A$ Receptor α1-Subunit in Hypoxia

Yuan Feng[1, 2], Xiaozhou He[3], Yilin Yang[3], Jingshan Chen[4], Kaisheng Yin[1], and Ying Xia[2, 5]

[1]*First Affiliated Hospital with Nanjing Medical University, Nanjing, Jiangsu, People's Republic of China*
[2]*Yale University School of Medicine, New Haven, CT, USA*
[3]*Third Clinical College of Soochow University, Changzhou, Jiangsu, People's Republic of China*
[4]*National Institute of Mental Health, National Institutes of Health, Bethesda, MD, USA*
and
[5]*Vivian L. Smith Department of Neurosurgery, The University of Texas Medical School, Houston, TX 77030, USA*

Abstract

Recent studies show that both delta-opioid receptors (DOR) and GABA receptors play a neuroprotective role in the mature cortex. Since we have observed that DOR over-expression renders the cortex more tolerant to hypoxic stress, we asked whether DOR over-expression affects GABA receptors expression in the cortex under hypoxia. As the first step, we investigated the expression of $GABA_A$ receptor α1-subunit ($GABA_A$ Rα1, the most abundant α-subunit of GABA receptors in the adult brain) in the mouse cortex with transgenic DOR over-expression after hypoxia. The results showed that $GABA_A$ Rα1 expression was lower in the transgenic than wild-type cortex, suggesting that DOR over-expression induces an inhibitory effect on $GABA_A$ receptor expression. Hypoxia for 1-3 days significantly increased $GABA_A$ Rα1 expression in the wild-type cortex, which may be an adaptive strategy for protecting the cortex against hypoxic stress. Interestingly, such increase was not found in the transgenic cortex with DOR over-expression. This may represent an interactive regulation in the transgenic cortex to efficiently balance energy production and consumption for better adaptation to hypoxic environment. Since DOR over-expression increases cortical tolerance to hypoxia, an increase in GABA receptors expression (an energy-costing process) may not be necessary in the cortex with DOR over-expression.

Key Words: GABA receptor, delta-opioid receptor, cortex, hypoxia

Introduction

Our recent studies have shown that delta-opioid receptors (DOR) play a neuroprotective role in the cortex (3, 4, 6, 16). DOR activation protects cortical neurons against hypoxic or excitotoxic insults, while transgenic DOR over-expression increases cortical tolerance to hypoxia (3, 4, 6, 11, 13, 16, 21-23). Indeed, the role of DOR in neuroprotection has been well demonstrated by many laboratories (1, 5, 7-10, 13, 15, 19, 25). On the other side, we observed that GABA is protective to mature, though toxic to immature, cortical neurons in hypoxic stress with differential expression of $GABA_A$ receptor α1-subunit (GAB_A Rα1) between ages (14, 24).

Since both DOR and GABA receptors are

Corresponding author: Dr. Ying Xia, Vivian L. Smith Department of Neurosurgery, The University of Texas Medical School, Houston, TX 77030, USA. Tel: 713-500-6288, E-mail: ying.xia@uth.tmc.edu
Received: May 10, 2010; Accepted: May 31, 2010.
 ISSN : 0304-4920. http://www.cps.org.tw

neuroprotective in mature cortex, we wonder if there is any interaction between these two membrane proteins under hypoxic condition. In specific, we asked whether DOR up-regulation affects GABA receptor expression in the mature cortex. Because $GABA_A$ Rα1 is the most abundant α-subunit of GABA receptors in the adult brain (12), as the first step, we investigated the expression of $GABA_A$ Rα1 in the mouse cortex with transgenic DOR over-expression in response to hypoxic stress.

Materials and Methods

Animals

Wild-type C57BL/6 mice (WT) were purchased from Charles River Laboratories (Wilmington, MA, USA). The transgenic mice with over-expression of delta-opioid receptors (tDOR mice) (3) were produced in our laboratory and subjected to strict genotyping with PCR before the experiments. The animals were housed and cared for, until appropriate ages for the present studies, in facilities operated by the Division of Animal Care at our institution. All animal procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Yale University School of Medicine, which is accredited by the American Association for Accreditation for Laboratory Animal Care. WT and tDOR mice at postnatal 30 days (P30) were divided into hypoxia and normoxia subgroups respectively.

Hypoxia Induction

The methods of hypoxia induction were similar as in our previous work (17, 18). In brief, the mice at postnatal day 30 were placed in an isobaric chamber in which the levels of O_2 and CO_2 were automatically controlled. All mice had free access to food and water. The inside environment was cleaned every other day. The level and duration of hypoxia were chosen based on the following two considerations: [i] the hypoxic stress should be severe enough to produce significant effects without jeopardizing the animal's life, and [ii] the resulting pO_2 level is such that it is clinically relevant and not infrequently encountered. Based on the literature and our previous work, we chose to expose the mice to a fractional inspired O_2 of 9.5 ± 0.5 % for a relatively short (1-3 days) or long (5-7 days) period. The age-matched control mice were kept in room air for the same period of time.

Tissue Collection and Preparation

Immediately following hypoxic exposure, the animals were removed from the hypoxic chamber and placed in a sealed glass desiccator for inhalational anesthesia before decapitation. The brain was rapidly removed and rinsed in cold phosphated buffered saline solution. The cerebral cortex was dissected out and stored in -80°C until use.

Western Blots

Protein samples (30 μg) were subjected to electrophoresis in 10% SDS-containing polyacrylamide gels and then transferred to a polyvinylidene fluoride (PVDF)-membrane (Amersham, Piscataway, NJ, USA). The membrane was pre-incubated with 5% non-fat milk in TBS for 1 h at room temperature and then incubated overnight at 4°C with rabbit polyclonal anti-$GABA_A$ Rα1 and/or anti-β-actin (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) was used as a second antibody for 1 h. After washing in Tris-Buffered Saline with 0.1% Tween (TTBS), the protein was visualized with chemiluminescence detection ECL (Amersham). The signal density was quantified by Alpha Imager 2200 (Alpha Innotech, San Leandro, CA, USA). Light signal bands of β-actin after short-term exposure were used for quantification to avoid potential inaccuracy resulting from signal saturation.

Statistical Analysis

In all samples measured, the density of $GABA_A$ Rα1 was normalized to that of β-actin. The data (Means ± SE) were expressed as signal ratios ($GABA_A$ Rα1 *vs*. β-actin) and subjected to non-paired Student's *t*-test. The difference was considered significant if *P* value was less than 0.05.

Results

Effect of DOR Over-Expression on $GABA_A$ Rα1 Expression in the Cortex

In all transgenic mice, the expression of $GABA_A$ Rα1 was lower in the cortex. As shown in Fig. 1, the density of $GABA_A$ Rα1 was decreased by 38% in the cortex of the transgenic mice at age of P31-33 as compared to that of the age-matched wild-type mice ($P < 0.01$, n = 6). These results suggest that DOR over-expression induces an inhibitory effect on $GABA_A$ receptor expression.

Effect of Hypoxia on $GABA_A$ Rα1 Expression in the Wild-Type Cortex

Unlike in the *in vivo* neurons in which an increased density of $GABA_A$ Rα1 was found in the

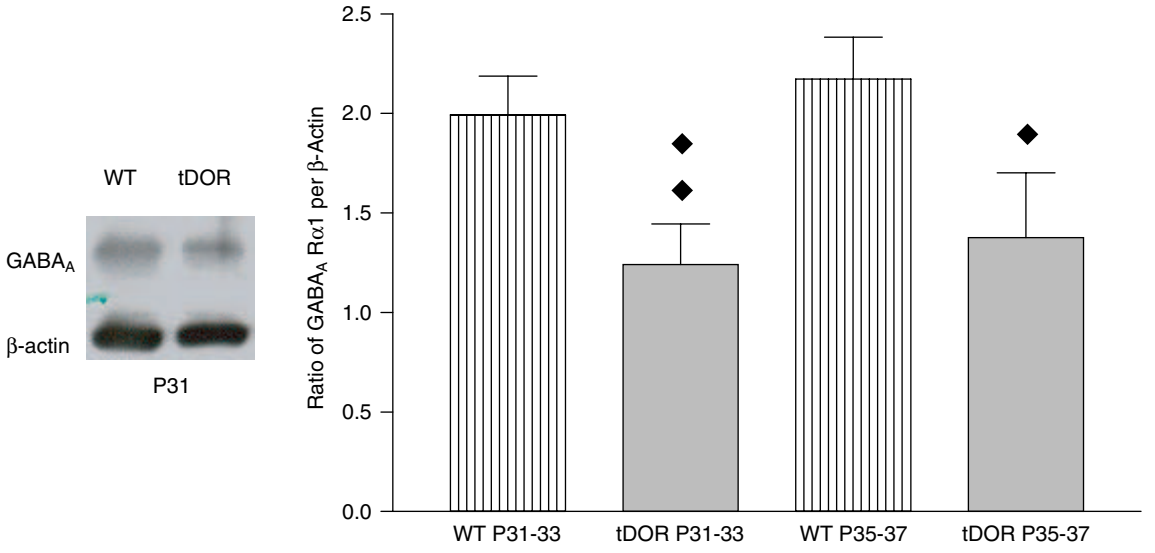

Fig. 1. Differential expression of $GABA_A$ Rα1 in wild-type cortex (WT) and transgenic cortex with DOR over-expression (tDOR). P31-33 and P35-37, postnatal 31-33 days and 35-37 days. n = 6 (all groups). ◆, $P < 0.05$. ◆◆, $P < 0.01$. In the left panel, the β-actin bands were darker than those of $GABA_A$ Rα1 because of stronger signals of β-actin on the same blot. In the right panel, the quantification of β-actin was made on lighter signal bands after a shorter term of film exposure to avoid potential inaccuracy due to signal saturation on the film. The same is true for Figs. 2-4 below. Note that the transgenic mice had lower expression of $GABA_A$ Rα1 than the control.

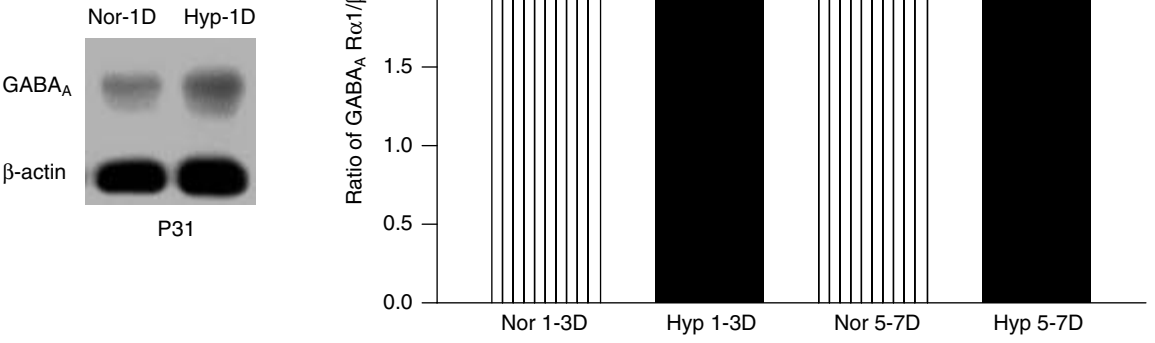

Fig. 2. Effect of hypoxia on $GABA_A$ Rα1 expression in wild-type cortex. The mice at postnatal 30 days were used. P31, postnatal 31 days. Nor 1D, 1-3D or 5-7D, normoxia for 1, 1-3 or 5-7 days. Hyp 1D, 1-3D or 5-7D, hypoxia for 1, 1-3 or 5-7 days. n = 6 (all groups). ◆, $P < 0.05$. Also refer to Fig. 1 legend for the quantification. Note that 1-3 days of hypoxia increased $GABA_A$ Rα1 expression in the cortex.

immature, but not in the mature after hypoxia (14), short-term (1-3 days) hypoxia significantly increased $GABA_A$ Rα1 expression in the cortex (Fig. 2). After an extended period (5-7 days) of hypoxia, however, $GABA_A$ Rα1 expression was not statistically different from that of the control (normoxic) cortex, suggesting that short-term, but not long-term, hypoxia increase $GABA_A$ receptor expression.

Effect of Hypoxia on $GABA_A$ Rα1 Expression in the Cortex with Transgenic DOR Over-Expression

The hypoxia-induced increase in $GABA_A$ Rα1 expression was not observed in the transgenic cortex. As shown in Fig. 3, the similar density of $GABA_A$ Rα1 was found in the wild-type and transgenic cortex after 1-3 days of hypoxia. In the group exposed

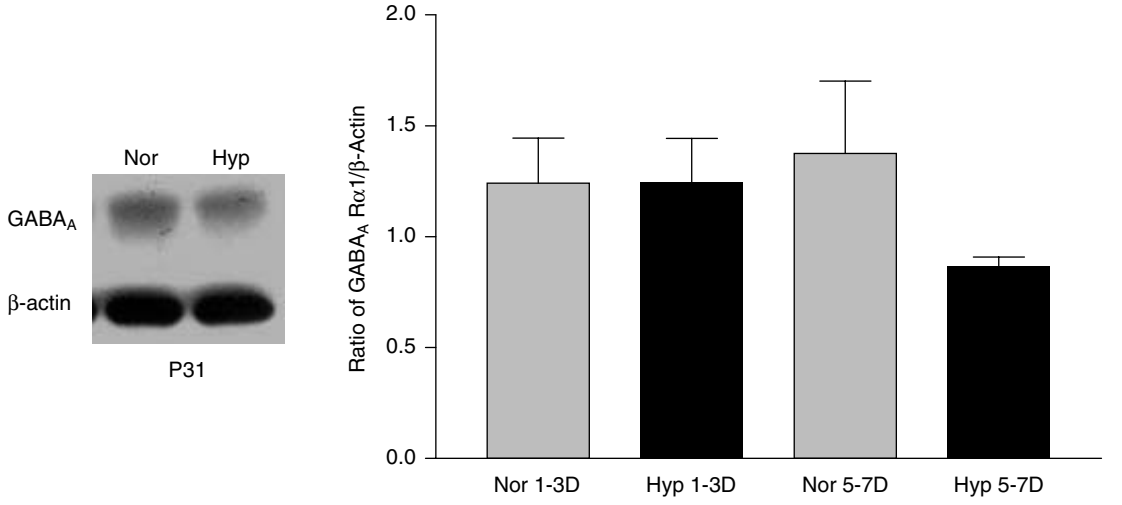

Fig. 3. Effect of hypoxia on $GABA_A$ Rα1 expression in the cortex with transgenic DOR over-expression. Nor, normoxia. Hyp, hypoxia. Other abbreviations are the same as in Figs. 1 and 2. n = 6 (all groups). Also refer to Fig. 1 legend for the quantification. Note that unlike the wild-type cortex, the transgenic cortex had no increase in $GABA_A$ Rα1 expression in the cortex.

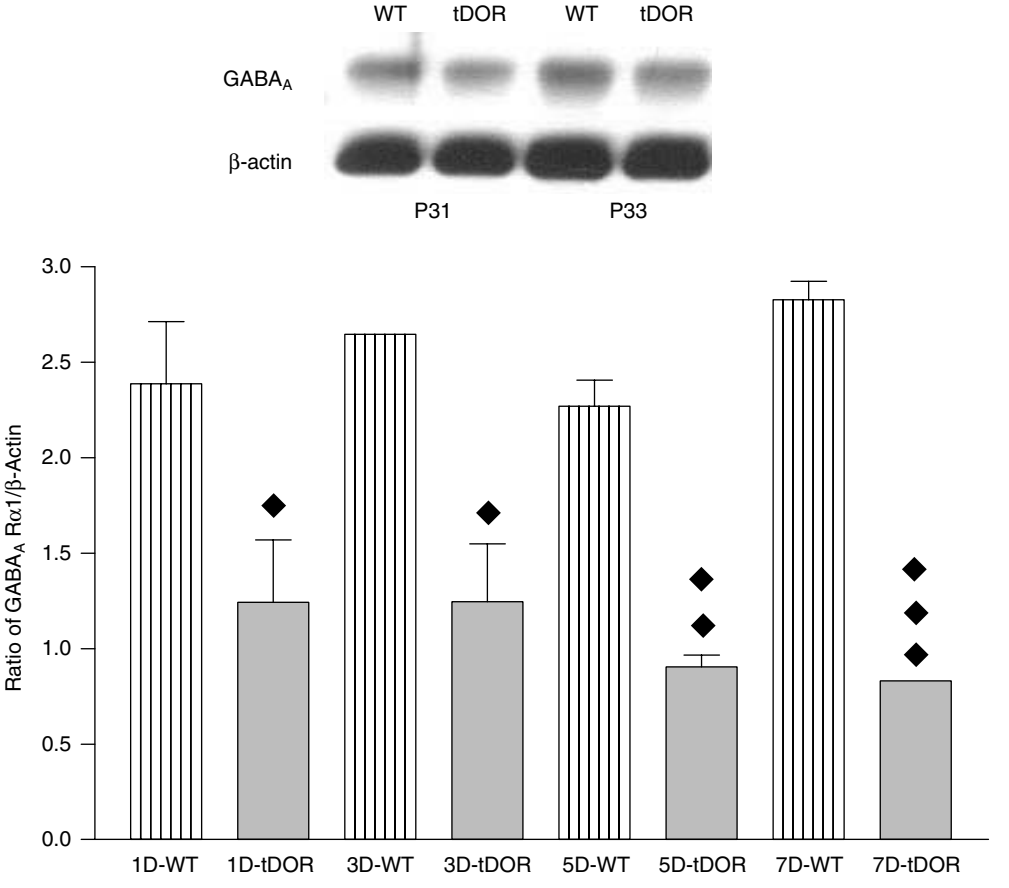

Fig. 4. Major difference in cortical $GABA_A$ Rα1 expression between the transgenic mice with DOR over-expression and wild-type mice in response to hypoxia. Both transgenic and wild-type mice were exposed to hypoxia starting from postnatal 30 days (P30). 1D, 3D, 5D, 7D: 1, 3, 5, 7 days of hypoxia. Other abbreviations are the same as in Figs. 1-3. n = 3 in all groups. ◆, $P < 0.05$. ◆◆, $P < 0.01$. ◆◆◆, $P < 0.001$. Also refer to Fig. 1 legend for the quantification. Note that $GABA_A$ Rα1 density was much lower in the transgenic cortex, as compared to that of the transgenic cortex, after 1-7 days hypoxia.

to long-term hypoxia (5-7 days), the density of $GABA_A$ Rα1 tended to decrease in the transgenic cortex though no statistical significance. This tendency is clearly evident when directly comparing the transgenic and wild-type cortex in hypoxic condition. As demonstrated in Fig. 4, all age groups showed a lower density of $GABA_A$ Rα1 in the transgenic than wild-type cortex. For example, the density of $GABA_A$ Rα1 was 60-70% lower in the transgenic than wild-type cortex after 7 days of hypoxia.

Discussion

We have made three interesting findings in this work. First, DOR over-expression down-regulated $GABA_A$ Rα1 expression in the cortex. Second, unlike the *in vitro* cortical neurons, *in vivo* wild-type cortex increased $GABA_A$ Rα1 expression after 1-3 days of hypoxia. Third, the same hypoxic stress did not increase $GABA_A$ Rα1 expression in the transgenic cortex with DOR over-expression. To our knowledge, these are the first observations on the interaction between DOR and GABA receptors.

There is no clear clue regarding the effect of DOR on GABA receptor expression in the past. Our data suggest that an increase in DOR expression may have an inhibitory effect on GABA receptor expression. In consistent with this viewpoint, a recent study (20) shows that DOR expression reduces the activity of GAT-1, a prominent GABA transporter. Taken together, it is likely that DOR activity inhibits GABA function and/or expression through an unknown mechanism.

Our previous work has shown that hypoxia differentially affects $GABA_A$ Rα1 expression in immature and mature cortical neurons *in vitro* (14). We therefore asked in this study if this is true in the *in vivo* cortex. Unlike the observations made in the *in vitro* cortical neurons that showed no significant increase in $GABA_A$ Rα1 expression in the mature after hypoxia, hypoxia for 1-3 days significantly increased $GABA_A$ Rα1 expression in the cortex. The difference between *in vitro* and *in vivo* cortical samples may result mainly from the hypoxic severity. The *in vitro* cortical neurons were subjected to severe hypoxia (1% of oxygen) (14), while the animals in this study were exposed to a fractional inspired O_2 of $9.5 \pm 0.5\%$. Taking into consideration the fact that a longer term of hypoxia could not increase $GABA_A$ expression in this study, it is possible that mild and/or short-term hypoxia can stimulate GABA receptor expression, while severe and/or long-term hypoxia can not increase its expression in the cortex. Since GABA activity is neuroprotective in the mature brain, an increase in GABA receptor expression may represent a protective strategy in the mature cortex in the early stage of hypoxic stress.

Our current data suggest that hypoxia, even with a short period, could not increase (or even decrease) $GABA_A$ Rα1 expression in the transgenic cortex. This may represent an interactive regulation in the transgenic cortex to efficiently balance the energy production and consumption in hypoxia. Since DOR is neuroprotective and DOR over-expression increases cortical tolerance to hypoxia (3, 4, 6, 11, 13, 16, 21-23), an increase in GABA receptor expression may not be necessary in the transgenic cortex with DOR over-expression because an increase in GABA receptor expression is an energy-costing process. The reduction of GABA receptor expression may reduce energy consumption, which may better help the cortex in adaptation to hypoxic stress.

In conclusion, our first data suggest that DOR over-expression reduces GABA receptor expression and abolishes the hypoxia-induced increase in GABA receptor expression in the cortex.

Acknowledgments

This work was supported by NIH (HD-34852, AT-004422) and American Heart Association (AHA-0755993T), USA.

References

1. Borlongan, C.V., Hayashi, T., Oeltgen, P.R., Su, T.P. and Wang, Y. Hibernation-like state induced by an opioid peptide protects against experimental stroke. *BMC Biol.* 7: 31, 2009.
2. Chao, D., Bazzy-Asaad, A., Balboni, G. and Xia, Y. δ-, But not μ-, opioid receptor stabilizes K^+ homeostasis by reducing Ca^{2+} influx in the cortex during acute hypoxia. *J. Cell. Physiol.* 212: 60-67, 2007.
3. Chao, D., Qian, H., Ghassemi, F., Chen, J.S. and Xia, Y. Transgenic overexpression of δ-opioid receptors protects the cortex from anoxic disruption of ionic homeostasis. *Soc. Neurosci. Abstract*, Program No. 87.19/MM68, 2006.
4. Chao, D. and Xia, Y. Ionic storm in hypoxic/ischemic stress: can opioid receptors subside it? *Prog. Neurobiol.* 90: 439-470, 2010.
5. Charron, C., Messier, C. and Plamondon, H. Neuroprotection and functional recovery conferred by administration of kappa- and delta-opioid agonists in a rat model of global ischemia. *Physiol. Behav.* 93: 502-511, 2008.
6. Feng, Y., Chao, D., He, X., Yang, Y., Kang, X., Lazarus, L.H. and Xia, Y. A novel insight into neuroprotection against hypoxic/ischemic stress. *Acta Physiol. Sin.* 61: 585-592, 2009.
7. Govindaswami, M., Brown, S.A., Yu, J., Zhu, H., Bishop, P.D., Kindy, M.S. and Oeltgen, P.R. Delta 2-specific opioid receptor agonist and hibernating woodchuck plasma fraction provide ischemic neuroprotection. *Acad. Emerg. Med.* 15: 250-257, 2008.
8. Horiuchi, T., Kawaguchi, M., Kurita, N., Inoue, S., Sakamoto, T., Nakamura, M., Konishi. N. and Furuya, H. Effects of delta-opioid agonist SNC80 on white matter injury following spinal cord ischemia in normothermic and mildly hypothermic rats. *J. Anesth.* 22: 32-37, 2008.
9. Iwata, M., Inoue, S., Kawaguchi, M., Nakamura, M., Konishi, N.

and Furuya, H. Effects of delta-opioid receptor stimulation and inhibition on hippocampal survival in a rat model of forebrain ischaemia. *Br. J. Anaesth.* 99: 538-546, 2007.

10. Kao, T.K., Ou, Y.C., Liao, S.L., Chen, W.Y., Wang, C.C., Chen, S.Y., Chiang, A.N. and Chen, C.J. Opioids modulate post-ischemic progression in a rat model of stroke. *Neurochem. Int.* 52: 1256-1265, 2008.
11. Ma, M.C., Qian, H., Ghassemi, F., Zhao, P. and Xia, Y. Oxygen sensitive δ-opioid receptor-regulated survival and death signals: Novel insights into neuronal preconditioning and protection. *J. Biol. Chem.* 280: 16208-16218, 2005.
12. McKernan, R.M. and Whiting, P.J. Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci.* 19: 139-143, 1996.
13. Pamenter, M.E. and Buck, L.T. δ-opioid receptor antagonism induces NMDA receptor-dependent excitotoxicity in anoxic turtle cortex. *J. Exp. Biol.* 211(Pt 21): 3512-3517, 2008.
14. Qian, H., Banasiak, K., Zhao, P. and Xia, Y. Effects of inhibitory neurotransmitters on GABA and glycine receptor expression: Age-difference in cortical neurons during hypoxia. *Soc. Neurosci. Abstract*, 2004.
15. Su, D.S., Wang, Z.H., Zheng, Y.J., Zhao, Y.H. and Wang, X.R. Dose-dependent neuroprotection of delta opioid peptide [D-Ala2, D-Leu5] enkephalin in neuronal death and retarded behavior induced by forebrain ischemia in rats. *Neurosci. Lett.* 423: 113-117, 2007.
16. Sung, J.H., Chao, D.M. and Xia, Y. Neuronal responses to hypoxia. In Wang and Ying (eds): *New Frontiers in Neurological Research*. Kerala: Research Signpost, pp. 73-153. 2008.
17. Xia, Y., Eisenman, D. and Haddad, G.G. Sulfonylurea receptor expression in rat brain: effect of chronic hypoxia during development. *Pediatr. Res.* 34: 634-641, 1993.
18. Xia, Y., Warshaw, J.B. and Haddad, G.G. Effect of chronic hypoxia on glucose transporters in heart and skeletal muscle of immature and adult rats. *Am. J. Physiol.* 273 (5 Pt 2): R1734-R1741, 1997.
19. Xiong, L.Z., Yang, J., Wang, Q. and Lu, Z.H. Involvement of delta- and mu-opioid receptors in the delayed cerebral ischemic tolerance induced by repeated electroacupuncture preconditioning in rats. *Chinese Med. J.* 120: 394-399, 2007.
20. Yang, Z.J., Bao, G.B., Deng, H.P., Du, H.M., Gu, Q.B., Pei, G., Pu, L., Schwarz, W. and Xia, P. Interaction of δ-opioid receptor with membrane transporters: Possible mechanisms in pain suppression by acupuncture. *J. Acupuncture Tuina Sci.* 6: 298-300, 2008.
21. Zhang, J.H., Xia, Y. and Haddad, G.G. Activation of α-opioid receptors protects cortical neurons from glutamate excitotoxic injury. *Soc. Neurosci. Abstract* 28: 736, 1999.
22. Zhang, H., Gibney, G.T. and Xia, Y. Neuroprotective role of delta-opioid receptors in cortical neurons. *Am. J. Physiol. (Cell Physiol)* 282: C1225-C1234, 2002.
23. Zhang, J., Qian, H., Zhao, P., Hong, S.S. and Xia, Y. Rapid hypoxia preconditioning protects cortical neurons from glutamate toxicity through δ-opioid receptor. *Stroke* 37: 1094-1099, 2006.
24. Zhao, P., Qan, H. and Xia, Y. GABA and glycine are protective to mature but toxic to immature rat cortical neurons under hypoxia. *Eur. J. Neurosci.* 22: 289-300, 2005.
25. Zhu, M., Li, M., Tian, X., Ou, X., Zhu, C. and Guo, J. Neuroprotective role of δ-opioid receptors against mitochondrial respiratory chain injury. *Brain Res.* 1252: 183-191, 2009.