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The influence of different sub-type delta opioid receptors in nerve growth factor-induced neuronal differentiation in rat pheochromocytoma PC12 cell

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Abstract

The impairment of neuronal differentiation for long-term opioid drug-exposed infants is a serious problem and there are several speculated mechanisms. We have previously reported that altering the endogenous delta opioid receptor by long-term interaction with its antagonist augmented the nerve growth factor (NGF)-induced neuronal differentiation of rat pheochromocytoma PC12 cells. In this study using subtype-specific antagonist, we present data showing further that type 2 delta opioid receptor (δ_2-DOR) is the receptor on the PC12 cells participating in the progression of neuronal differentiation upon NGF-stimulation. Unlike the δ_2-DOR, alteration of type 1 DOR (δ_1-DOR) activity by δ_1-DOR-specific antagonist appeared to be toxic for the PC12 cells. The different influence of the subtypes of delta opioid receptors in the neuronal differentiation of the PC12 cells suggests that each subtype of opioid receptor may trigger different biological activities *in vivo*.

Keywords: Pheochromocytoma cell; Naloxone; δ-Opioid receptor; Nerve growth factor; Neuronal differentiation

Substantial evidence had showed that alteration of opioid receptor activity disturbs the neurobehavioral development of newborns [2,18], indicating the involvement of the opiate system in neuronal differentiation. Prolonged exposure to naltrexone, an opiate antagonist, resulting in an increased brain growth in infant rats further implies a direct involvement of the opiate receptor in neuronal cell growth [17]. However, the molecular mechanism underlying these phenomena is still unclear.

The rat pheochromocytoma PC12 cells triggered by NGF to differentiate toward neuron-like cells, has been widely used in vitro to study the neuronal differentiation [6,7]. Evidences indicate that there is a variety of endogenous opioid systems present in different lines or strains of PC12 cells, and these opioid systems are reported to involve in certain degree with the neuronal function. In a previous study we assessed the effect of naloxone and morphine on the differentiation of PC12 cells induced by NGF and found a negative influence of the endogenous opioid receptor activity on the NGF-induced cell differentiation [15]. Inoue and Hatanaka demonstrated that NGF increased the expression of enkephalin specific binding sites on PC12 cells [8], and the specific binding sites were characterized pharmacologically as the delta opioid receptor [1]. We have confirmed a NGF dose-dependent, time-related increase of delta opioid receptor (DOR) transcripts but no other types of opioid receptor transcripts in the PC12 cells [15]. Such NGF-induced biochemical alterations raise the possibility that the expressed DOR is likely functionally involved in the PC12 cell differentiation induced by NGF.

We have previously shown that alteration of endogenous delta opioid system caused dedifferentiation of NGF-treated PC12 cells [15]. The function of DOR in vivo is primarily involved in analgesia, gastroparesis and antitussis [12] and DOR has been pharmacologically further characterized into δ_1- and δ_2-subtypes [10,12–14]. Analgesic difference of these two subtypes of DOR evidenced by their antagonist effect performed in mice [15] is the only available means to functionally distinguish these two receptors so far. The gene encoding the binding sites that proved to be the δ_1-DOR was cloned from rat and mouse [1,3]. However, whether the δ_2-subtype receptor is encoded from a different gene or modi-

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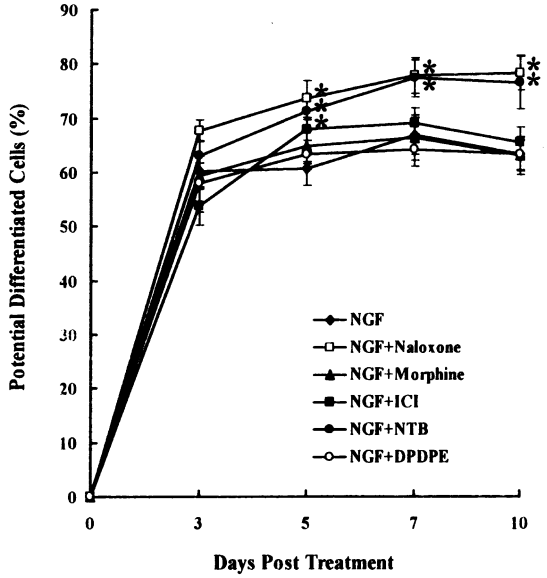

Fig. 1. Quantitative data of the influence of opioid drugs on NGF-induced PC12 cell differentiation. Cells were treated with NGF alone (◆), or NGF plus naloxone (□), NGF plus morphine (▲), NGF plus ICI-174864 (■), NGF plus NTB (●), and NGF plus DPDPE (○) for 10 days. Results are expressed as the mean ± SEM of 6–10 independent experiments. *$P < 0.05$ compared to NGF-treated group by paired Student *t*-test.

fied post-translationally to express distinct pharmacological activity from the δ_1-DOR remains unclear.

The present study was undertaken to determine which subtype of DOR is involved in this cell differentiation. PC12 cells of passages 25–30 were plated on poly-D-lysine (Sigma) (100 μg/ml) pre-coated dishes at 4×10^5 cell/cm^2 density on the day before treatment. The cells were cultured in Dulbeccos' modified Eagle's medium (Life Technologies) supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The effect of endogenous opioid system in the PC12 cells on the NGF-induced differentiation was analyzed by morphological change. We defined the cells that are flattened and have extended neurites as the potential differentiated cells. For each group, triplicate dishes were set up at 24 h before treatment, and total seven groups (A, B, C, D, E, F, and untreated cells) were analyzed. Cells in group A were treated with murine 2.5S NGF (50 ng/ml). Cells in group B were treated with NGF (50 ng/ml) plus naloxone (10 μM) (non-selective opioid receptor antagonist). Cells in group C were treated with NGF (50 ng/ml) plus morphine (10 μM). Cells in group D were treated with NGF (50 ng/ml) plus N,N-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI-174864; DOR selective antagonist) (10 μM). Cells in group E were treated with NGF (50 ng/ml) plus naltriben methanesulfonate (NTB, selective δ_2-DOR antagonist) (10 μM). Cells of group F were treated with NGF (50 ng/ml) plus 10 μM of [D-PEN ^{2,5}]-enkephalin (H-Tyr-D-Pen-Gly-Phe-D-Pen-OH; DPDPE; DOR agonist) (10 μM). To analyze the degree of cell differentiation, pictures from random fields of each cell sample were taken on day 3, 5, 7, and 10 post-treatment. The percentage of potential differentiated cells was calculated from the pictures by dividing the number of potential differentiated cells by the total cell number. At least 6–10 independent experiments were performed to obtain means and SEM. Student's *t*-test was used for statistical analysis. A *p* value less than 0.05 was considered to be statistically significant.

By morphologically comparison, there was no significant difference in the density of neurite formation and the length of extended neurites between NGF-treated cells and those treated by NGF with other opioid reagents (data not shown). Over 60% of the cells became differentiated upon 3-day exposure to NGF, and the number steadily increased after 5, and 7-day exposure, then reached a plateau on the tenth day (Figs. 1 and 3). When cells were simultaneously treated with NGF and naloxone, the percentage of potential differentiated cells was significantly increased compared to that of the NGF-treated cells on day-5, day-7 and day-10 post-treatment ($P < 0.05$) (Fig. 1). Similar to naloxone, NTB potentiated the NGF-induced cell differentiation after 5, 7 and 10 day continuous exposure (Fig. 1), indicating the involvement of the δ_2-DOR for this differentiation process. There was a transient enhancement ($P < 0.05$) of cell differentiation after 3 days of simultaneous exposure of cells to NGF and ICI-174864, however, cell differentiation gradually returned to similar level when cells were exposed to NGF alone on day 7 and day 10 post-treatment. There was no significant change on NGF-induced differentiation when cells were simultaneously treated with opioid agonist, such as morphine, or DPDPE, which further confirmed that endogenous opioid peptide altering the activity of opioid recep-

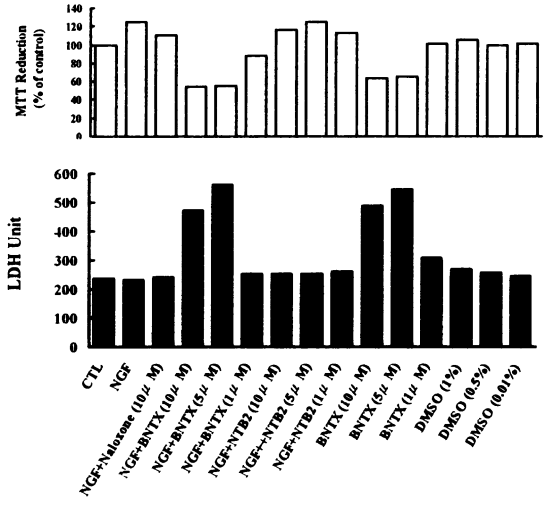

Fig. 2. The cytotoxicity of NTB and BNTX on PC12 cells. Cells were incubated with NGF, NGF plus naloxone (10 μM), NGF plus BNTX (10, 5, or 1 μM), NGF plus NTB (10, 5, or 1 μM), BNTX (10, 5, or 1 μM), DMSO (1, 0.5, or 0.01%), and untreated as control (CTL). After 3-day treatment, MTT reduction and LDH release assays were performed. Data are the mean of triplicate determination.

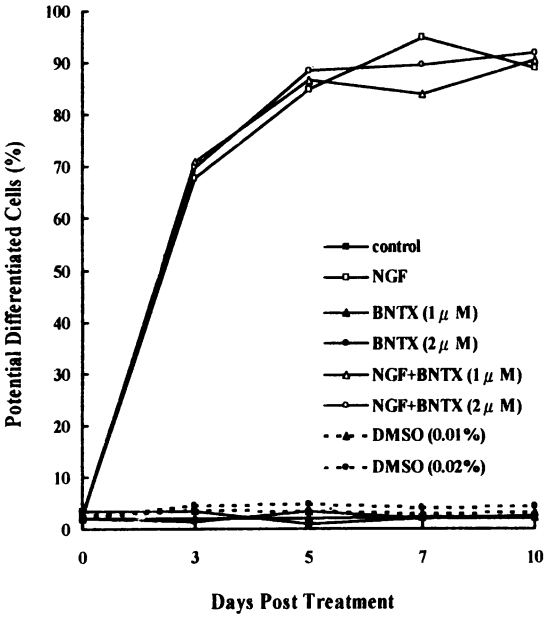

Fig. 3. Quantitative data of the influence of BNTX on NGF-induced differentiation of PC12 cells. Cells were treated with NGF alone (□), or NGF plus 1μM BNTX (△) or plus 2 μM BNTX (○), 1 μM BNTX (▲), 2 μM BNTX (●), 0.01% DMSO (▲ with dash line), 0.02% DMSO (● with dash line), and no treatment (■). Results are expressed as the mean of three independent experiments.

tor in the PC12 cells might cause cell dedifferentiation upon NGF stimulation.

On the other hand, the addition of NGF with 10 μM of 7-benzylidenenaltrexone (BNTX maleate; Tocris Cookson Inc.), a selective δ_1-DOR antagonist, caused cell death after 24 h. To further analyze the toxicity of BNTX, cells were grown at 2×10^5 cells/6-cm plate and treated for 3 days. Cell toxicity was analyzed by determining the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and the cytoplasmic lactate dehydrogenase (LDH) enzyme activity [4,9]. As shown in Fig. 2, increasing BNTX concentrations significantly decreased the MTT reduction ability, which was not due to the DMSO solvent used. At the concentration higher than 5 μM, BNTX decreased the MTT reduction ability by 40%. This was further confirmed by measuring the LDH release in culture medium. A two-fold increase of LDH release was detected at 5 and 10 μM concentrations of BNTX used (Fig. 2). All these indicate a cytotoxic signal triggered by the δ_1-DOR interacting with its antagonist, which was not invoked through the δ_2-DOR signalling pathway.

By serial dilution, we found that no cell death occurred with BNTX at 1 and 2 μM concentrations. Cells were set up as described above with the following treatments: NGF (50 ng/ml), BNTX 1 μM or 2 μM, NGF (50 ng/ml) + BNTX 1 μM or 2 μM, 0.01% or 0.02% DMSO, and no drug added as control. Unlike the result from exposure to NTB, there was no difference in the percentage of potential differentiated cell between the NGF-treated and the NGF/BNTX-treated cells (Fig. 3). However, a significant increase of neurite outgrowth appeared on 10-day post-treatment in the NGF/BNTX-treated cells compared to the NGF-treated cells (Fig. 4).

Cumulated evidences had shown the importance of endogenous opioid system on neuronal growth and death. Sakaguchi et al. reported that the addition of enkephalin with NGF increased neuronal survival through μ-type opioid receptor, but not delta or kappa opioid receptors on primary cultured dorsal root ganglion neurons from chick embryo [11]. Etorphine, a non-selective opioid agonist inhibited cell growth to different degree on PC12 and neuroblastoma SK-N-SH cells, probably through the induction of neuronal cell apoptosis [16]. Disruption of the endogenous opioid receptor activity in PC12 cells clearly affects the NGF-induced neuronal differentiation as we have shown in this study and in our previous report. Our results further indicate that alteration of the endogenous δ_2-DOR activity by chronic treatment with the type-specific antagonist, NTB, indeed increased the NGF-induced PC12 cell differentiation and a different cell response was observed from the endogenous δ_1-DOR in the same PC12 cells. Recently, Gomes et al. proposed the possibility that heterodimerization between different types of opioid receptors may contribute to distinct properties of opioid receptors [5]. This may be one of the possible mechanisms behind our observation reported in this study. However, further characterization will be needed to support this hypothesis. As far as we know this is the first report giving the direct evidence indicating a possible physiological role of specific opioid receptor subtype on neuronal differentiation, since all the past reports were usually based on the pharmacological ligand binding profile to differentiate the subtype of opioid receptor. The precise role of the δ_2-DOR or the endogenous opioid peptide present

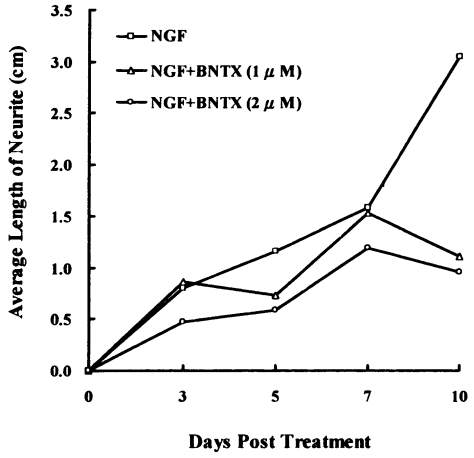

Fig. 4. The influence of BNTX on NGF-induced neurite outgrowth of PC12 cells. Cells were cultured in the presence of NGF alone (□), NGF plus 1μM (△) or 2 μM (○) BNTX for 10 days. The data were quantified from three different independent examiners by directly measuring the cells and neurites on photography taken from two defined areas of each cultured dish. The data from these three independent measurements were averaged.

in the PC12 cells in the NGF-induced differentiation process awaits further investigation.

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[1] Abood, M.E. and Tao, Q., Characterization of a delta opioid receptor in rat pheochromocytoma cells, J. Pharmacol. Exp. Therapeut., 274 (1995) 1566–1573.
[2] Connaughton, J.F., Finnegan, L.P., Schur, J. and Emich, J.P., Current concepts in the management of the pregnant opiate addict, Addict. Dis., 2 (1973) 21–36.
[3] Evans, C.J., Keith Jr, D.E., Morrison, H., Magendzo, K. and Edwards, R.H., Cloning of a delta opioid receptor by functional expression, Science, 258 (1992) 1952–1955.
[4] Froissard, P. and Duval, D., Cytotoxic effects of glutamic acid on PC12 cells, Neurochem. Int., 24 (1994) 485–493.
[5] Gomes, I., Jordan, B.A., Gupta, A., Trapaidze, N., Nagy, V. and Devi, L.A., Heterodimerization of μ and δ opioid receptors: a role in opiate synergy, J. Neurosci., 20 (RC110) (2000) 1–5.
[6] Green, L.A. and Tischler, A.S., Establishment of a noradrenergic clonal cell line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, Proc. Natl. Acad. Sci. USA, 73 (1976) 2424–2428.
[7] Greene, L.A., Aletta, J.M., Rukenstein, A. and Green, S.H., PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation, Methods Enzymol., 147 (1987) 207–216.
[8] Inoue, N. and Hatanaka, H., Nerve growth factor induces specific enkephalin binding sites in a nerve cell line, J. Biol. Chem., 257 (1982) 9238–9241.
[9] Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods, 65 (1983) 55–63.
[10] Portoghese, P.S., Sultana, M., Nagase, H. and Takemori, A.E., A highly selective delta 1-opioid receptor antagonist: 7-benzylidenenaltrexone, Eur. J. Pharmacol., 218 (1992) 195–196.
[11] Sakaguchi, M., Fujimori, T., Satoh, T., Satoh, M., Takeuchi, M. and Matsumura, E., Effects of opioids on neuronal survival in culture of embryonic chick dorsal root ganglion neurons, Neurosci. Lett., 262 (1999) 17–20.
[12] Sofuoglu, M., Portoghese, P.S. and Takemori, A.E., Differential antagonism of delta opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: evidence for delta opioid receptor subtypes, J. Pharmacol. Exp. Therapeut., 257 (1991) 676–680.
[13] Sofuoglu, M., Portoghese, P.S. and Takemori, A.E., 7-Benzylidenenaltrexone (BNTX): a selective delta 1 opioid receptor antagonist in the mouse spinal cord, Life Sci., 52 (1993) 769–775.
[14] Takemori, A.E., Sultana, M., Nagase, H. and Portoghese, P.S., Agonist and antagonist activities of ligands derived from naltrexone and oxymorphone, Life Sci., 50 (1992) 1491–1495.
[15] Yeh, G.C., Hsieh, T.H. and Chang, S.F., The negative influence of endogenous opioid receptor activity on the differentiation of the rat pheochromocytoma PC12 cells induced by nerve growth factor, Neurosci. Lett., 252 (1998) 25–28.
[16] Zagon, I.S. and McLaughlin, P.J., Naltrexone modulates growth in infant rats, Science, 221 (1983) 1179–1180.
[17] Zelson, C., Rubio, E. and Wasserman, E., Neonatal narcotic addiction: 10 year observation, Pediatrics, 48 (1971) 178–189.
[18] Zelson, C., Infant of the addicted mother, N. Engl. J. Med., 288 (1973) 1393–1395.