Full-length article

Changes of brain neuropeptide Y and its receptors in rats with flurazepam tolerance and dependence[1]

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Key words

Abstract

Aim: Anticonvulsant tolerance and dependence are two obstacles that restrict the clinical use of benzodiazepines (BDZ). In order to explore the mechanism of these two adverse reactions, changes of neuropeptide Y (NPY) and its receptors in the hippocampus of rat models, in relation to flurazepam (FZP, a member of BDZ) tolerance and dependence, were investigated. **Methods:** The mRNA of preproNPY and its receptors (Y_1, Y_2, and Y_5) in the hippocampus were determined by competitive RT-PCR, and the distribution of NPY in the hippocampus was examined by immunohistochemistry. **Results:** A decrease of preproNPY mRNA in the hippocampus was found in tolerant and dependent rats. The level of preproNPY mRNA in the hippocampus was reversely correlated with the degree of tolerance and dependence, measured by the threshold of pentylenetetrazol-induced seizures. Immunohistochemistry indicated a decrease of NPY-immunoreactive material in neurons of the CA1, CA3, and dentate gyrus regions of both tolerant and dependent rats. The mRNA of NPY receptors Y_1 and Y_5 decreased in tolerant rats but did not change in dependent rats. The mRNA of NPY receptor Y_2 increased in tolerant rats but decreased in dependent rats. **Conclusion:** A decrease of NPY in the hippocampus might be involved in anticonvulsant tolerance and dependence following long-term treatment with FZP. Y_1, Y_2, and Y_5 mRNA were also altered in FZP tolerance and dependence.

Introduction

Benzodiazepines (BDZ) have been widely used as anxiolytics, sedatives, hypnotics and anticonvulsants. BDZ produce therapeutic effects through the major fast inhibitory neurotransmitter receptors, $GABA_A$ receptors, in the central nervous system (CNS). BDZ positively modulate $GABA_A$ receptors by increasing their affinity for GABA and thus reduce the excitability of the post-synaptic neurons.

However, long-term treatment with BDZ often leads to drug tolerance, which means the therapeutic efficacy of the drug decreases after a period of continuous administration. Another consequence of chronic administration of BDZ is the dependence manifested by the aggravation of primary symptoms and/or appearance of abstinence syndrome, which includes anxiety, discomfort, diarrhea, loss of bodyweight and salivating when the drug is withdrawn abruptly. Tolerance and dependence are frequently encountered in the clinical use of BDZ as an antiepileptic medicine. The usefulness of BDZ is therefore limited, although most have primarily satisfactory antiepileptic efficacies.

Many studies have focused on the mechanisms of tolerance and dependence. Changes of binding capacity, gene expression and metabolism in neural receptors, including $GABA_A$ receptors[1], glutamatergic receptors[2] and peripheral BDZ receptors[3], were studied in the presence of tolerance and/or dependence. Other factors, such as nitric oxide[4], calcium-channel blockers[5], protein kinases[6] and bicarbonate radicals[7] were also studied. However, the exact mechanism underlying the tolerance and dependence is still unknown.

Neuropeptide Y (NPY) is a polypeptide of 36 amino acid residues widely distributed in the nervous system. This neuropeptide regulates various functions such as food

uptake, blood pressure, circadian rhythm and anxiety. It induces the central and peripheral activities through at least six receptor subtypes called Y_1–Y_6 that belong to the G-protein coupled receptor superfamily[8].

After a period of BDZ treatment, the glutamatergic system was modified, which was thought to be one of the reasons underlying tolerance to BDZ. For example, an increase of 206% in *in vitro* glutamate release was found in the hippocampus of animals chronically treated with lorazepam[2]. Based on the facts that the NPYergic system inhibited the release of glutamate from presynaptic membranes[9] and that NPY was reduced in the CNS in alcohol dependence[10], the changes of NPYergic system in rat models with BDZ tolerance and dependence attracted our attention.

In this study, rat models of anticonvulsant tolerance and dependence to flurazepam (FZP, a BDZ) were established and the changes of NPY and its receptors (Y_1, Y_2, and Y_5) in the hippocampus of these rat models were investigated.

Materials and methods

The establishment of rat models and the experiments on rats in this study were approved by the Ethics Committee of Animal Experiments of Peking University First Hospital.

Rat model of FZP tolerance The rat model of FZP tolerance used was produced by following the method developed by Rosenberg[11]. Male Sprague-Dawley rats (initial weight 180–200 g) were housed in a climate-controlled room with free access to standard rat food. FZP dissolved in a 0.02% saccharin solution was given as drinking water for 1 week (FZP-tolerant group, *n*=10). The concentration of FZP was adjusted to provide a daily dose of up to 100 mg/kg for the first 3 d and 150 mg/kg for the next 4 d. All rats consumed an average concentration of more than 100 mg/kg FZP daily. Control rats (*n*=10) were handled identically, but received 0.02% saccharin solution without the drug. After one week of treatment, rats were given saccharin solution as drinking water. Withdrawal signs such as overt ataxia, anxiety and hyperactivity were not found with this treatment[12].

Tolerance to FZP was evaluated 12 h after discontinuation of FZP intake. Because of the very rapid biotransformation of FZP and its metabolites in rats, and the corresponding plasma half-life ($T_{1/2}$) of less than 2 h, the residual drug should not interfere with the anticonvulsant test at this time[13]. FZP dissolved in distilled water (100 mg/mL) was diluted to 20 mg/mL with normal saline for injection. This solution was injected intraperitoneally to deliver a dose of 20 mg/kg. After 30 min, 20 mg/mL pentylenetetrazol (PTZ, freshly prepared in normal saline) was infused at a con-

stant rate of 0.5 mL/min into a tail vein, and clonus of the front legs was monitored to determine the threshold of PTZ-induced seizures. The onset time of clonus was recorded, and the PTZ threshold (mg/kg) was then derived. Rats in the control group were tested along with those in the FZP-tolerant group using the same procedure. FZP tolerance was determined by the decrease of PTZ threshold as compared with that of the control group. After the PTZ threshold test, the brain was removed and stored at -70 ºC immediately.

Rat model of FZP dependence We integrated the methods reported by Rosenberg[11] and Izzo *et al*[14] to establish a rat model of FZP dependence. The animals used, drug dosage, method of drug intake and PTZ threshold test were from the method reported by Rosenberg[11], but the days of drug administration and the dependence test were from the method reported by Izzo *et al*[14]. To obtain a more stable blood concentration, male Sprague-Dawley rats were given FZP dissolved in 0.02% saccharin solution as drinking water instead of an oral gavage of diazepam three times daily[14]. For rats in the FZP-dependent group (*n*=10), FZP was given at increasing doses for 14 d: d 1–3, 100 mg·kg^{-1}·d^{-1}; d 4–7, 150 mg·kg^{-1}·d^{-1}; d 8–10, 200 mg·kg^{-1}·d^{-1}; and d 11–14, 250 mg·kg^{-1}·d^{-1}. Control rats (*n*=10) were handled identically, but received 0.02% saccharin solution without the drug as drinking water. The emergence of dependence was determined by the susceptibility to PTZ-induced seizures 96 h after termination of the 14-day FZP administration. PTZ (20 mg/mL) was infused at a constant rate of 0.5 mL/min into a tail vein and the infusion was discontinued at the first sign of tonic-clonic seizures. The PTZ threshold (mg/kg) was derived from the amount of PTZ used to induce tonic-clonic seizures. Rats in the control group were treated identically. FZP dependence was determined by the decrease of the PTZ threshold as compared with that of the control group. After the PTZ threshold test, the brain was removed and stored at -70 ºC immediately.

PTZ-induced seizures themselves may also cause changes in the brain. To minimize these interferences, we killed rats immediately after the PTZ threshold test and compared the changes in the hippocampus with those from the respective control rats.

Reagents FZP was purchased from DaZhong Pharmaceutical (Shanghai, China).

Rats were from the Animal Department of Peking University Health Sciences Center. PTZ and anti-NPY antibody were from Sigma-Aldrich (Saint Louis, MO, USA). RNase-free DNase I was obtained from Promega (Madison, WI, USA), Trizol reagent and M-MuLV reverse transcriptase were from Gibco (Rockville, MD, USA). The sequences of oligonucle-

otide primers for the amplification of preproNPY (386 bp), Y_1 (426 bp), Y_2 (393 bp), and Y_5 (391 bp) cDNA are: NPY-F, 5'-TATCCCTGCTCGTGTGTTTG-3'; NPY-R, 5'-AACGACAA-CAAGGGAAATGG-3'; Y_1-F, 5'- ACTCTCACAGGCTGTC-TTAC-3'; Y_1-R, 5'-ATAGTCTCGTAGTCGTCGTC-3'; Y_2-F, 5'-AGCCTTTCCACCCTGCTAAT-3'; Y_2-R, 5'-GTGAATGGCA-TCCAACCTCT-3'; Y_5-F, 5'-CACCTAGCCGTTCCAGAA-AA-3'; Y_5-R, 5'-GGGCTCTCAAGTCTGCTTTG-3'.

Measurement of preproNPY and NPY receptor cDNA in hippocampus by competitive RT-PCR Animals were decapitated immediately after the PTZ threshold test. The hippocampus was quickly removed and stored at -70 ºC until use. Brain tissue was homogenized in Trizol reagent in a precooled mortar following manufacturer's protocol[15]. Total RNA samples were treated with RNase-free DNase I to eliminate genomic DNA contamination before reverse transcription. Total RNA of 3 µg was reversely transcribed into cDNA using oligo d $(T)_{15}$ as the primer and M-MuLV reverse transcriptase.

The internal competitive standards for the measurement of preproNPY, Y_1, Y_2, and Y_5 cDNA were made using PCR. These DNA fragments had the same sequences as their respective PCR products, except that approximately 80 bp at the downstream site of the forward primers were deleted. Quantitative PCR was performed in a 0.2 mL tube containing brain cDNA 1 µL, internal competitive standard 1 µL, 5 µmol/L each primer mixture 1µL, 2.5 mmol/L each dNTP mixture 1 µL, 1.5 mmol/L $MgCl_2$, 10×PCR buffer 2.5 µL, and 1.25 unit *Taq* DNA polymerase, in a total volume of 25 µL. PCR was run at 94 ºC 30 s, 62 ºC 30 s, and 72 ºC 40 s (93 ºC 1 min, 65 ºC 2 min and 72 ºC 2 min for the amplification of Y_1 cDNA) for 35 cycles. PCR products were separated in a 1.5% agarose gel and stained using ethidium bromide. The ratio of the optical density of the two DNA bands under UV light was measured by a gel image analysis system. A standard curve of the ratio was drawn using the same conditions as described above, except that brain cDNA was changed to a serial dilution of the purified PCR product. The relative amount of the cDNA in a sample was then obtained from the standard curve.

Immunohistochemistry of NPY in hippocampus After anesthesia, rats were perfused through the left ventricle with 0.9% sodium chloride followed by 4% paraformaldehyde in phosphate buffer solution for approximately 30 min. The removed brain was sequentially soaked in 4% paraformaldehyde in phosphate buffer solution for 24 h and 30% sucrose at 4 ºC for 72 h. Frozen sections of 8-mm thickness in a coronal plane were used for immunohistochemistry. One brain section from the treated rat and one from the respective control rat were placed on one slide and processed together. After treatment with 0.3% H_2O_2 to block the endogenous peroxidase, sections were incubated with 1:50 polyclonal anti-NPY antibody overnight at 4 ºC. The secondary antibody was biotinylated anti-rabbit IgG antibody (Vector, Burlingame, CA, USA). Signals were visualized by horseradish peroxidase conjugated avidin and diaminobenzidine. The relative density of NPY-immunoreactive material in neurons in the pyramidal cell layer of CA1, CA3 region and granular cell layer of the dentate gyrus region was measured in a defined area by a densitometer.

Statistical analysis Data were expressed as mean±SD. Paired *t*-tests were used to evaluate the significance of intergroup differences. $P<0.05$ was considered as significant.

Results

Rat models of FZP tolerance and dependence

FZP-tolerant model The rat model of FZP tolerance was evaluated by studying the FZP anticonvulsant efficacy after discontinuing the 7-d FZP treatment for 12 h when the brain FZP should have been metabolized[13]. Anticonvulsive efficacy of a single dose of 20 mg/kg FZP was measured by studying the threshold of PTZ-induced seizures after 30 min. In comparison with the control group, the average PTZ threshold decreased by more than 3 times in the FZP-tolerant group, indicating the successful establishment of an FZP-tolerant model in rats (Table 1).

FZP-dependent model In contrast to the evaluation of FZP tolerance, FZP dependence was estimated by studying the PTZ threshold after discontinuing the 14-d FZP treatment for 96 h when maximum withdrawal signs were expected to occur[14]. As shown in Table 1, the average PTZ threshold in the FZP-dependent model was lower than that in its control group by more than 2 times, indicating that the FZP-dependent model is acceptable for further study (Table 1).

Changes of preproNPY cDNA in hippocampus of FZP-tolerant and -dependent rats In the hippocampus, preproNPY

Table 1. Extent of FZP tolerance and dependence evaluated by the threshold of PTZ-induced seizures. $n=10$. $^{b}P<0.05$ *vs* the respective control groups.

Group	Threshold of PTZ-induced seizures/mg·kg^{-1}
FZP-tolerant	22.47±10.24^{b}
Control	71.74±23.68
FZP-dependent	5.69±1.77^{b}
Control	15.91±2.45

cDNA was dramatically reduced both in FZP-tolerant and -dependent groups, as compared with that of the respective control groups (Table 2, Figure 1).

Table 2. Relative amount of preproNPY, Y_1, Y_2, and Y_5 cDNA in hippocampus of rats with anticonvulsant tolerance and dependence to FZP. n=8. Mean±SD. [b]$P<0.05$ *vs* those of the respective control groups.

Group	PreproNPY	Y_1	Y_2	Y_5
FZP-tolerant	0.09±0.01[b]	0.99±0.13[b]	16.32±6.26[b]	0.64±0.16[b]
Control	5.37±1.06	3.67±1.10	3.11±1.13	3.75±1.33
FZP-dependent	0.78±1.56[b]	2.89±1.31	0.54±1.59[b]	3.41±1.66
Control	5.16±1.70	3.61±1.79	3.33±1.37	3.51±0.86

NPY-immunoreactive cells were found in many brain regions, with the hippocampus showing the most remarkable changes in FZP tolerant and dependent rats. In the hippocampus of control animals, NPY-immunoreactive cells were mostly located in CA1, CA3, and dentate gyrus regions. NPY-immunoreactive cells were large multipolar or fusiform neurons, and NPY positive material was found in their cytoplasm and processes (Figure 2A, 2B). In the hippocampus from FZP-tolerant and FZP-dependent rats, NPY-immunoreactive material in the cytoplasm and processes was significantly reduced as compared with that in the same regions of the respective control rats (Figure 2C, 2D, Table 3).
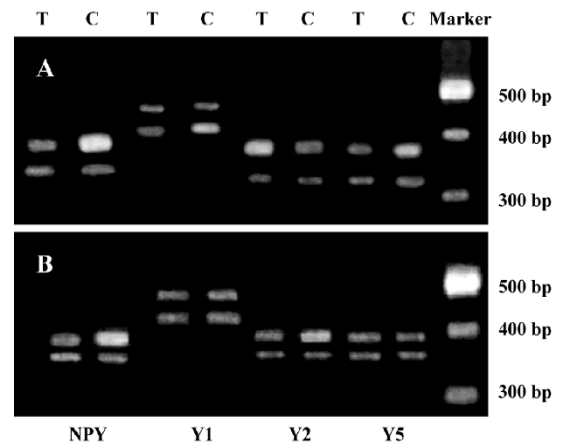

Figure 1. Representative electrophoresis profiles of competitive PCR for the quantification of preproNPY, Y_1, Y_2, and Y_5 cDNA in the hippocampus of FZP-tolerant model (A) and FZP-dependent model (B). The density ratio of the upper/lower bands in a lane is positively proportional to the amount of the cDNA in the hippocampus. Lane T, samples from FZP-treated rats; lane C, samples from control rats.

Changes of NPY receptor cDNA in hippocampus of FZP-tolerant and -dependent rats Y_1 and Y_5 cDNA were decreased in the tolerant group as compared with those of the respective control group. However, no quantitative changes of these two cDNA were found in the FZP-dependent group. In contrast, Y_2 cDNA was increased significantly in the FZP-tolerant group, but was decreased in the FZP-dependent
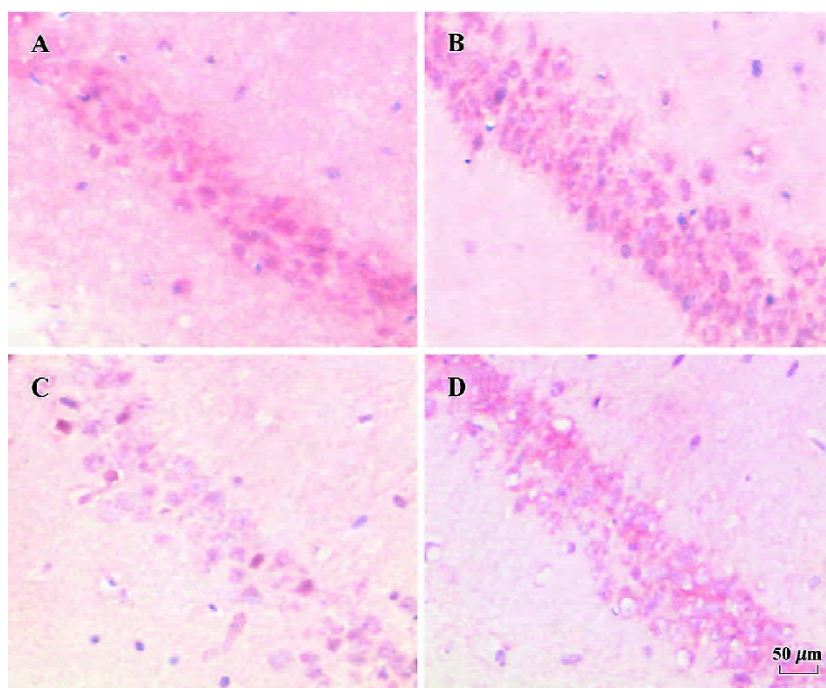

Figure 2. NPY-immunoreactive cells in pyramidal cell layers of CA1 region detected by immunohistochemistry (counterstained by hematoxylin ×400). (A), A sample from a control rat for FZP-tolerant group and (B), a sample from a control rat for FZP-dependent group show plenty of NPY-immunoreactive material (brown-red colour) in the cytoplasm of most neurons as well as neurofibers in white matter. (C), A sample from an FZP-tolerant rat and (D), a sample from an FZP-dependent rat show reduced NPY-immunoreactive material in the neuronal cytoplasm and neurofibers.

Table 3. Relative density of NPY-immunoreactive material in neurons of the hippocampus (area of 0.05 mm^2). $n=6$. [b]$P<0.05$ *vs* control groups.

Group	CA1	CA3	Dentate gyrus
FZP-tolerant	31.12±5.43[b]	33.04±7.13[b]	30.58±5.99[b]
Control	209.47±11.18	221.84±14.05	218.58±13.94
FZP-dependent	75.33±9.86[b]	71.65±7.25[b]	72.97±6.34[b]
Control	202.51±10.09	214.59±13.28	227.76±11.62

group, as compared with that of the respective controls (Table 2, Figure 1).

Correlations between the threshold of PTZ-induced seizures and the levels of preproNPY, Y_1, Y_2, and Y_5 cDNA in hippocampus of FZP-tolerant and -dependent rats The correlations were examined to show whether changes of NPY and its receptors in the hippocampus were related to the pathogenesis of FZP tolerance and dependence. The PTZ threshold was positively correlated with the level of preproNPY cDNA in the hippocampus both in FZP-tolerant and -dependent groups (Figure 3A, 3B). In other words, the

lower the expression of NPY in the hippocampus, the higher degree of tolerance and dependence the rat showed. The PTZ threshold was negatively correlated with the level of hippocampal Y_2 cDNA in the FZP-tolerant group and was positively correlated with the level of hippocampal Y_2 cDNA in the FZP-dependent group (Figure 3C, 3D). No correlation between the PTZ threshold and the changes of Y_1 and Y_5 cDNA were found (data not shown).

Discussion

Pharmacologically, BDZ produce their sedative, anxiolytic and antiepileptic activities through a modulation action on GABA$_A$ receptors. However, tolerance to BDZ and dependence on BDZ are the two major adverse reactions in clinical practice. Neuronal hyperexcitability may be the common basis for the tolerance and dependence, although they may have different manifestations.

Hippocampus is implicated in the generation and modulation of seizure activities, and plays a central role in controlling excitability of the CNS[16]. In the present study, the hippocampus was separated to investigate the changes of NPY and its receptors (Y_1, Y_2, and Y_5) in rat models with
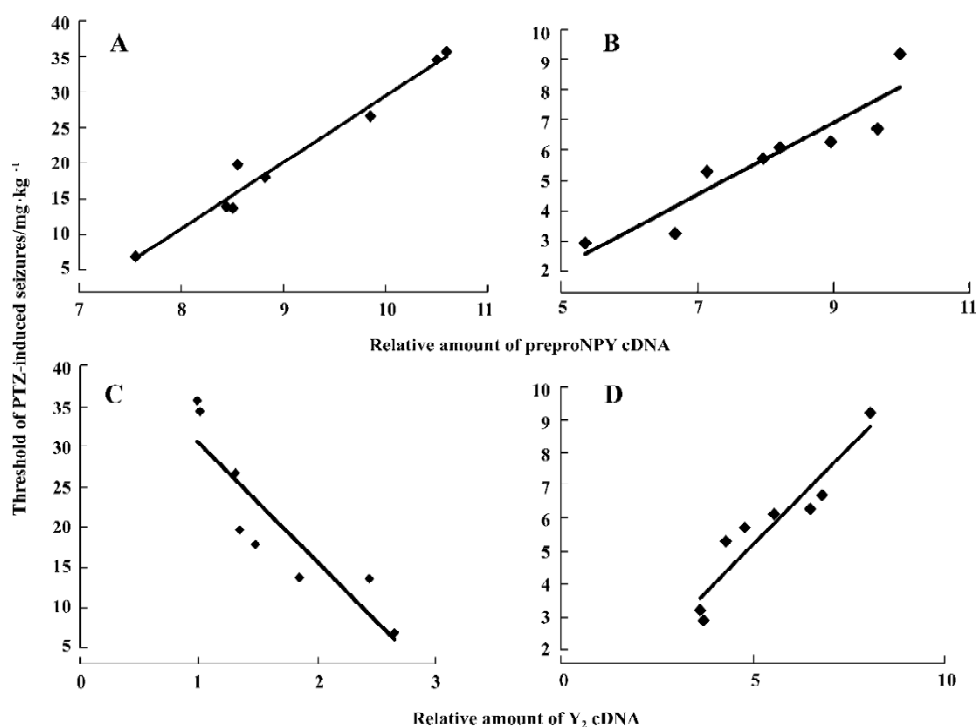

Figure 3. Correlations between the PTZ threshold and the level of hippocampal preproNPY cDNA: (A), in the FZP-tolerant group ($r=0.9855$, $P<0.01$) and (B), in the FZP-dependent group ($r=0.9295$, $P<0.01$). Correlation between the PTZ threshold and the level of hippocampal Y_2 cDNA: (C), in FZP-tolerant group ($r=-0.8986$, $P<0.01$) and (D), in FZP-dependent group ($r=0.9414$, $P<0.01$).

anticonvulsant tolerance and dependence to FZP.

We found that preproNPY cDNA in the hippocampus was significantly decreased in both the FZP-tolerant and FZP-dependent groups. Immunohistochemistry also showed the reduction of NPY-immunoreactive material in the neuronal cytoplasm and neurofibers of CA1, CA3, and dentate gyrus regions. Y_1 and Y_5 receptor cDNA were decreased in the tolerant group but were not changed in the dependent group. Y_2 receptor cDNA was increased in the tolerant group but was decreased in the dependent group.

To date, only one paper has been published on the relationship between the NPYergic system and drug tolerance and/or dependence[17]: contingent tolerance to carbamazepine after repeatedly giving the drug may be associated with the down-regulation of NPY. However, no data about the changes of the NPYergic system following long-term treatment of BDZ have been found in the published literature.

The level of preproNPY cDNA in the hippocampus reversely relating to the degree of tolerance and dependence may suggest that the changes of preproNPY cDNA are involved in the pathogenesis of FZP tolerance and dependence. NPY, one of the most abundant and widely distributed neuropeptides in the central and peripheral nervous systems, acts as an endogenous modulator to reduce seizure activities. In the hippocampus, the inhibition of epileptiform discharges after NPY treatment was attributed to the decrease of glutamate released from the presynaptic nerve terminals through blocking of the G-protein dependent calcium channel[18]. Therefore, the decrease of cellular NPY we found may result in neuronal hyperexcitability that is the fundamental pathological state of BDZ tolerance and dependence.

Six subtypes of NPY receptors, Y_1–Y_6, have been identified. Their pharmacological characteristics are very similar, although the identity of amino acid sequences is as low as 30% among these receptor subtypes[19]. Of the six receptor subtypes, Y_1, Y_2, and Y_5 are the major functional receptors and are expressed abundantly. In rodents, changes in the Y_1 receptor and its mRNA in the CNS can be induced by various stimuli, suggesting the multiple functions of the Y_1 receptor[20]. The Y_2 receptor demonstrates a predominant role in modulation of seizure activity[21]. The Y_5 receptor may also be involved in the modulation of anticonvulsant activity in rodents[22,23]. Theoretically, drug tolerance and dependence are caused by the imbalance of excitation over inhibition in the brain, and NPY receptors would decrease in association with the decrease of NPY. The different changes to NPY receptors between the FZP-tolerant and FZP-dependent group and the increase of Y_2 in FZP-tolerant rats in this study can not be satisfactorily explained because of the in-sufficient and conflicting data regarding Y_1, Y_2, and Y_5 receptors in the regulation of neuronal excitability at the present time. More experiments should be performed on the distribution and ligand binding capacity of the three subtype receptors in FZP tolerance and dependence.

Our preliminary results suggest that the decrease of NPY in the hippocampus plays an important role in the generation of anticonvulsant tolerance and dependence following long-term FZP treatment. The NPYergic system may be a new target for the comprehension of these adverse effects of BDZ.

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