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Differential Expression of Benzodiazepine Anticonvulsant Cross-Tolerance According to Time Following Flurazepam or Diazepam Treatment

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ROSENBERG, H. C. *Differential expression of benzodiazepine anticonvulsant cross-tolerance according to time following flurazepam or diazepam treatment.* PHARMACOL BIOCHEM BEHAV 51(2/3) 363–368, 1995.—In previous studies in which the anti-pentylenetetrazol (PTZ) effect of benzodiazepines was used to measure tolerance, the results depended on the benzodiazepine used for chronic treatment as well as the benzodiazepine given acutely to test for tolerance. In this study, the time course of tolerance reversal was studied in rats given two treatments known to cause anticonvulsant tolerance, 1-week flurazepam (FZP), and 3-week diazepam (DZP). Neither treatment altered convulsive threshold for IV PTZ, but both treatments decreased the convulsive threshold for bicuculline. Withdrawing DZP, but not FZP, treatment resulted in a loss of body weight. Twelve hours after 1-week FZP treatment, all benzodiazepines were significantly less effective, showing tolerance. Forty-eight hours after the 1-week FZP treatment, tolerance was still observed with DZP, FZP, and zolpidem, but was no longer present with clonazepam or bretazenil. After the 3-week DZP treatment, rats were tolerant to all benzodiazepines tested at 12 h of withdrawal, but had lost tolerance to all the drugs except bretazenil by 48 h. The results suggest differences in the way these benzodiazepines interact with their receptors, allowing differential expression of tolerance, and that chronic DZP and FZP treatments affected interactions of the benzodiazepines with their receptors, but not in the same fashion.

Benzodiazepine Tolerance Convulsion Pentylenetetrazol Bicuculline Convulsive threshold

TOLERANCE to the benzodiazepines appears to be a very complex phenomenon. One indication of this is that the observation of tolerance depends on the benzodiazepine action being evaluated. For example, tolerance readily develops to sedation, but little or none is observed for some behavioral actions of these drugs (2,15,31,33). Though tolerance to the anticonvulsant action of benzodiazepines occurs, it develops more slowly than tolerance to motor impairment (27). Anticonvulsant tolerance has been found to differ according to the benzodiazepine used for chronic treatment. Tolerance to the anticonvulsant action of clobazam appeared more quickly than did tolerance to clonazepam (9,27,40). Several other studies have also compared the development of tolerance during treatment with different benzodiazepines [e.g., (8,27,32)]. Some of the results have led to the suggestion that anticonvulsant tolerance develops more slowly for partial than for full benzodiazepine agonists (3,10,13), although there is only limited information on the relative anticonvulsant efficacies of the benzodiazepines used in these studies. Though the different drugs used in such studies have some pharmacokinetic differences, these differences have not provided an explanation for the variations in abilities of benzodiazepines to evoke anticonvulsant tolerance. Rather, the observation that the rate or degree of tolerance depends on the particular benzodiazepine used for chronic treatment suggests that these drugs do not interact uniformly with their receptors.

The possibility that the various benzodiazepines do not act uniformly with their receptors is also suggested by observations that the measurement of tolerance is partly dependent on the particular drug used to test for tolerance. Some studies have found "differential crosstolerance"; that is, tolerance could be demonstrated with some, but not all, benzodiazepines tested in animals that had all received the same chronic treatment (4,29). In a previous study, we found that anticonvulsant tolerance was present for only some, but not all, benzodiazepines tested 48 h after the end of a 1-week flurazepam (FZP) treatment (28). In rats treated for only a week with midazolam or with diazepam (DZP), there was no anticonvul-

sant tolerance (23). After a 3-week treatment, there was tolerance and cross-tolerance to some benzodiazepines, but the patterns of cross-tolerance differed (23), and also differed from that in FZP-treated rats (28).

In the study of tolerance after FZP treatment (28), rats were tested 48 h after the end of treatment. In subsequent experiments, it was noticed that the pattern of cross-tolerance appeared to differ according to the time after the end of FZP treatment. The present study was undertaken to describe the time course of anticonvulsant tolerance and cross-tolerance after the 1-week FZP treatment. Drug effect was measured by the elevation of the threshold dose of pentylenetetrazol (PTZ) needed to produce convulsions. This was compared to tolerance after the 3-week DZP treatment. The responsiveness of the rats to PTZ was examined at each time point following treatment, as was the effect of bicuculline, to determine if the chronic treatments had affected the threshold dose of these convulsants.

METHOD

Chronic Benzodiazepine Treatment

Male Sprague–Dawley rats, initial weight 200–225 g, were housed in a climate-controlled room with a 12L : 12D cycle, with free access to standard rat food. For FZP treatment, FZP was administered in a 0.02% saccharin solution as drinking water for 1 week, according to the procedure described previously (25,28,34). The concentration of FZP was adjusted daily to provide a daily dose of up to 100 mg/kg for the first 3 days, and 150 mg/kg for the next 4 days (but subject to a maximum concentration of 1.0, then 1.5 mg/ml). Control rats were handled identically, but received undrugged saccharin solution. All the rats used in this study had consumed an average minimum of 100 mg/kg FZP daily. As noted previously (25), this treatment does not cause overt ataxia or sedation, nor are spontaneous withdrawal signs noted following treatment. At the end of the week of FZP treatment, saccharin solution was given as drinking water. Rats were tested 12 or 48 h, or a week later. Each rat was tested only once, and groups of FZP treated and control rats were tested in parallel. Residual drug, which might interfere with the experiment by having an anticonvulsant effect, was not expected to play a role because the amount of active benzodiazepine (FZP plus active metabolites) in brain 12 h after the end of chronic FZP treatment (26) is more than an order of magnitude less than the drug concentration expected in brain after the acute IP benzodiazepine injections used for tolerance testing (28). This is in keeping with the very rapid biotransformation of FZP and its metabolites in rats, and the corresponding plasma half-lives of less than 2 h (14).

For DZP treatment, the drug was administered by implanting DZP-filled Silastic reservoirs, as described previously (6,17). In a previous study from this lab, this treatment was found to produce brain DZP levels of 250–275 ng/g, and resulted in anticonvulsant tolerance (23). On the first day of treatment, rats (initial weight 125–150 g) were anesthetized with methoxyflurane, and two Silastic reservoirs, each containing 90 mg of DZP, were inserted as previously described (6,23). Control animals were handled identically, but received empty, sealed Silastic tubes. An additional tube was implanted, while the rat was anesthetized with methoxyflurane, on day 10 of treatment. All tubes were removed, during methoxyflurane anesthesia, and rats were tested 12 or 48 hr, or a week later. In a previous study using the 3-week DZP treatment, residual DZP measured 12 h after removing the tubes was less than 15 ng/g brain (23), which was far less than the drug concentration expected in brain after the acute IP injection of benzodiazepine doses used in this study (28). As noted above, benzodiazepines, including DZP, are very rapidly biotransformed in rats. After IP injection of 5 mg/kg DZP, DZP and desmethyldiazepam were reported to disappear from brain with half-lives of approximately 1 h (5).

Measurement of Convulsant Threshold

To measure the threshold for PTZ-induced convulsive activity, a 20-mg/ml solution of PTZ (prepared fresh daily), in physiological saline, was infused at a constant speed of 0.5 ml/min using a 25 ga "Butterfly" infusion set inserted in a lateral tail vein. The time to onset of clonus of the front legs was recorded and the PTZ threshold dose calculated. A similar technique was used for bicuculline. A 10-mg/ml bicuculline stock solution (in 0.1 N HCl, pH adjusted to approximately 3 with NaOH) was made fresh daily and diluted 100-fold with normal saline to a final concentration of 0.1 mg/ml. This was infused into a lateral tail vein at a rate of 0.5 ml/min, and the time to onset of clonus recorded. In all experiments, each rat was tested only once.

Anticonvulsant Activity of Benzodiazepines

FZP dihydrochloride was prepared in distilled water as a 100-mg/ml stock solution and the pH adjusted as previously described (25), then diluted to 20 mg/ml with physiological saline for injection of the 20-mg/kg dose. The other benzodiazepines used for tolerance testing were DZP (5 mg/kg), clonazepam (1 mg/kg), bretazenil (5 mg/kg), and zolpidem (5 mg/kg). Each of these benzodiazepines was injected IP, using a volume of 1 ml/kg, in a standard vehicle (40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, 0.2 M Na benzoate, and 0.02 M benzoic acid in distilled water). This vehicle had no effect on the PTZ or bicuculline threshold, as determined by comparing the effects of physiological saline with those of this vehicle in the control rats. A benzodiazepine or vehicle was injected 30 min before PTZ infusion, and the drug action was measured as the increase in PTZ threshold (mg/kg PTZ), compared to the threshold after vehicle injection. The dose of each benzodiazepine was chosen in preliminary experiments to cause an approximately threefold increase in PTZ convulsive threshold in control rats. Tolerance was indicated by a significant difference in the PTZ threshold between chronically treated and control rats determined 30 min after the acute IP injection of a benzodiazepine. The results observed after acute vehicle pretreatment provided a measure of any effect that chronic treatment may have had on sensitivity to PTZ. On each test day, groups of treated rats and their corresponding controls were tested in parallel. Comparisons were made by Student's t-test, with $p < 0.05$ required for significance. Changes in body weight were evaluated by ANOVA, using time after withdrawing treatment and treatment group as grouping variables.

RESULTS

The effect of chronic FZP treatment on PTZ threshold was measured at each of the three time points, 12 h, 48 h, and 1 week following the end of the 1-week FZP treatment. There was no significant effect of the FZP treatment on PTZ convulsive threshold measured after saline pretreatment, or after pretreatment with the propylene glycol-based vehicle (Figs. 1–3). Each of the benzodiazepines chosen for testing produced

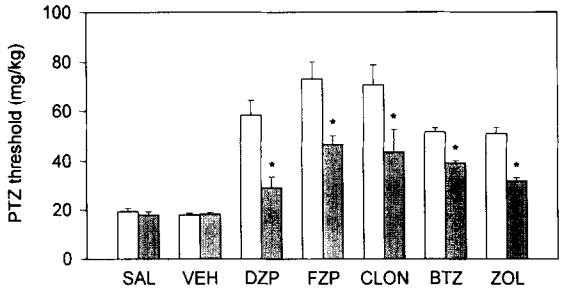

FIG. 1. Threshold PTZ dose for clonus in rats tested 12 h after the end of 1-week FZP treatment (shaded bars) and in controls (open bars). PTZ was infused as a 20-mg/ml solution at 0.5 ml/min beginning 30 min after IP injection of 1 ml/kg physiological saline (SAL), the propylene glycol-based vehicle (VEH), 5 mg/kg DZP, 20 mg/kg FZP, 1 mg/kg clonazepam (CLON), 5 mg/kg bretazenil (BTZ), or 5 mg/kg zolpidem (ZOL). Bars indicate mean + SEM, n = 8-10. *$p < 0.05$.

a clear anticonvulsant effect, noted by the increase in PTZ threshold in control rats tested after acute benzodiazepine pretreatment as compared to the vehicle-pretreated controls. In rats tested 12 h after the end of the 1-week FZP treatment, there was significant tolerance to FZP, as well as significant cross-tolerance to each of the other benzodiazepines tested (Fig. 1). However, in those rats tested 48 h after the end of the 1-week FZP treatment, differential tolerance was found. Rats were still tolerant to FZP, DZP, and zolpidem, but there was no longer a difference between treated and control rats in the anticonvulsant effects of clonazepam or bretazenil (Fig. 2). Other rats were tested with FZP, DZP, or zolpidem a week after the chronic FZP treatment, and it was found that tolerance could no longer be demonstrated (Fig. 3). There was no indication of rebound hyperresponsiveness to these drugs, or to clonazepam, a week after the end of the FZP treatment (Fig. 3).

The effects of the chronic DZP treatment were also measured at three time points after treatment had been withdrawn by removal of the SC reservoirs. There was no significant effect of the DZP treatment on PTZ threshold (Figs. 4 and 5). In rats tested 12 h after the end of the 3-week DZP treatment, there was tolerance to DZP as well as tolerance to all the other benzodiazepines used in this study (Fig. 4). However, at 48 h after the end of DZP treatment, there was no tolerance to DZP or any of the other drugs, except for bretazenil, which still had a significantly smaller anticonvulsant effect than in

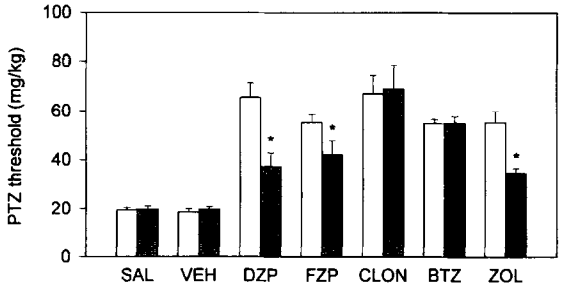

FIG. 2. Threshold PTZ dose for clonus in rats tested 48 h after the end of 1-week FZP treatment (shaded bars) and in controls (open bars). See Fig. 1 for methods and abbreviations. n = 8.

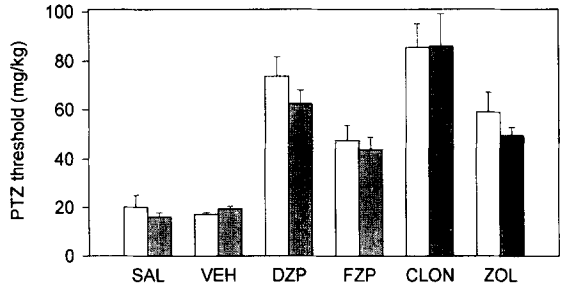

FIG. 3. Threshold PTZ dose for clonus in rats tested 1 week after the end of 1-week FZP treatment (shaded bars) and in controls (open bars). See Fig. 1 for methods and abbreviations. n = 8.

the corresponding control rats (Fig. 5). In rats tested for tolerance to bretazenil a week after chronic DZP, there was no longer a significant difference in PTZ threshold measured 30 min after bretazenil pretreatment (controls, 49.8 ± 2.7 mg/kg; DZP treated, 47.5 ± 3.9 mg/kg, mean ± SEM, n = 7 in each group).

Though neither chronic treatment had affected PTZ threshold, both FZP and DZP treatments resulted in a significant decrease in bicuculline threshold in rats tested 12 h after the end of treatment (Fig. 6). No significant difference in bicuculline threshold was noted in either FZP- or DZP-treated rats tested 48 h after treatment had been withdrawn.

It was also noted that rats that had been treated for 3 weeks with DZP had an obvious, transient weight loss 12 and 48 h after treatment was withdrawn (Fig. 7). There was a much smaller and very brief decrease in the controls, which then continued to gain weight. Thus, the weight loss in DZP-treated rats was not solely a consequence of the surgery needed to remove the DZP reservoirs at the end of treatment. In contrast, both the FZP-treated rats and their controls continued to gain weight following the end of FZP treatment (Fig. 7).

DISCUSSION

In previous work, we had noted that anticonvulsant tolerance was present for some but not for other benzodiazepines, when rats were tested 48 h after the end of FZP treatment

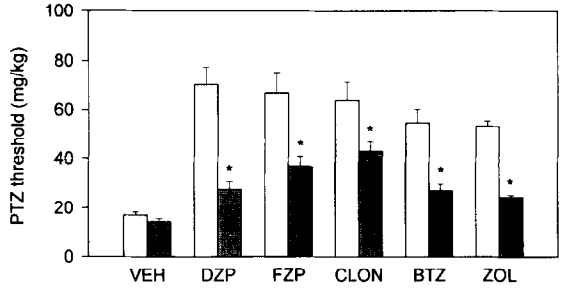

FIG. 4. Threshold PTZ dose for clonus in rats tested 12 h after the end of 3-week DZP treatment (shaded bars) and in controls (open bars). PTZ was infused as a 20-mg/ml solution at 0.5 ml/min beginning 30 min after IP injection of 1 ml/kg propylene glycol-based vehicle (VEH), 5 mg/kg DZP, 20 mg/kg FZP, 1 mg/kg clonazepam (CLON), 5 mg/kg bretazenil (BTZ), or 5 mg/kg zolpidem (ZOL). Bars indicate mean + SEM, n = 6-8. *$p < 0.05$.

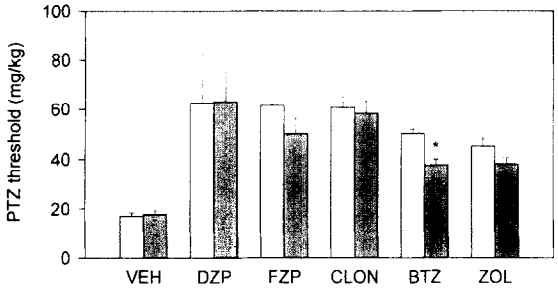

FIG. 5. Threshold PTZ dose for clonus in rats tested 48 h after the end of 3-week DZP treatment (shaded bars) and in controls (open bars). See Fig. 4 for methods and abbreviations. n = 7–8.

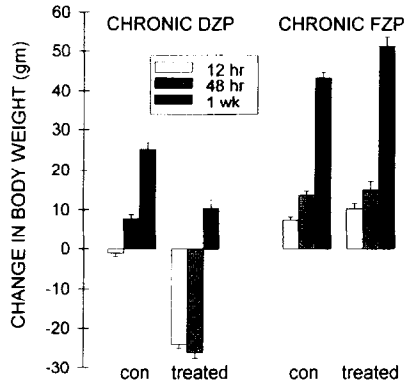

FIG. 7. Weight change following withdrawal of 3-week DZP treatment or 1-week FZP treatment, and the corresponding control (con) groups. The control groups showed the weight gain expected for male Sprague-Dawley rats. DZP treatment had a significant effect, shown by the interaction between time after withdrawing treatment and treatment group (F = 22.4, $p < 0.00001$). There was no significant interaction between time after FZP treatment and treatment group.

(28). In a subsequent study, such differential cross-tolerance was also noted in rats studied 12 h after a 3-week treatment with midazolam, whereas rats were tolerant to each of four different benzodiazepines tested 12 h after a 3-week DZP treatment (23). The present study further defines these results, and shows that, though differential cross-tolerance was present after both FZP and DZP treatments, it is a time-dependent phenomenon. After either FZP or DZP treatments, rats were tolerant to each benzodiazepine used in this study. However, by 48 h after the end of the chronic treatment, tolerance could be demonstrated for some, but not all, benzodiazepines. It is worth noting that the treatments differed not only in the drugs used but also in the route of administration. Moreover, DZP but not FZP treatment entailed brief periods of anesthesia to implant or remove the tubes. Though the controls were also subjected to the same anesthesia, it was still possible that the anesthesia and/or different handling of the rats may have contributed to the results. However, the pattern of cross-tolerance suggested that this was not a simple result of DZP and FZP treatments causing differing degrees of tolerance, or of differing time courses for tolerance reversal following the end of treatment. A clear example of this was provided comparing the effects of DZP and FZP treatments on the anticonvulsant actions of zolpidem and bretazenil. In rats tested 48 h after FZP treatment, tolerance to three of the five drugs tested, including zolpidem, was still clearly evident, whereas

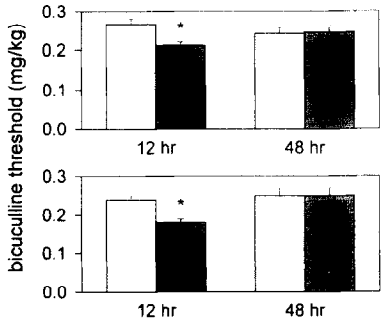

FIG. 6. Threshold bicuculline dose for clonus. Top panel: rats tested 12 or 48 h after the end of 1-week FZP treatment (shaded bars) and in controls (open bars). Lower panel: rats tested 12 or 48 h after the end of 3-week DZP treatment. Bicuculline, prepared as described in the Method section, was infused IV as a 0.1-mg/ml solution at 0.5 ml/min. Bars indicate mean + SEM, n = 4–8. *$p < 0.05$.

tolerance to bretazenil had been lost. In contrast, DZP treatment produced the opposite result; of all the benzodiazepines tested, including zolpidem, bretazenil was the only benzodiazepine to which significant tolerance could be demonstrated 48 h after treatment had been withdrawn. These data show that the ability to detect tolerance after a chronic benzodiazepine treatment is dependent on which drug is used to test for tolerance, and the time course for any loss of effect of that particular drug. Furthermore, the opposite patterns of cross-tolerance suggests that DZP and FZP treatments had caused different effects on neural function.

Several previous studies have compared the development of anticonvulsant tolerance during treatment with various benzodiazepines. Bretazenil, which is a partial agonist, appeared to be less able to produce tolerance than other benzodiazepines examined (10,13). Observations such as these led to the hypothesis that anticonvulsant tolerance develops less to partial than to full benzodiazepine agonists (1,3,8). The present results show that, even though bretazenil may not readily evoke tolerance, rats that have been made tolerant to another benzodiazepine will be cross-tolerant to the anticonvulsant effect of bretazenil. Other studies have suggested that little tolerance develops during zolpidem treatment. In a study of tolerance to the ability of benzodiazepines to reduce reinforced behavior, zolpidem was found to not produce tolerance (30). In another study, the effects of chronic midazolam and zolpidem were compared against PTZ, maximal electroshock, and isoniazid convulsions (21). Tolerance to the anticonvulsant effect of midazolam, but not zolpidem, was seen for all three types of convulsions. These findings show that zolpidem also causes less tolerance than other benzodiazepines, though the present findings showed that rats made tolerant to other benzodiazepines readily show cross-tolerance to zolpidem. In the earlier study (21), cross-tolerance was suggested by the fact that midazolam-treated mice showed less of an anticonvulsant effect when tested with zolpidem against isoniazid convulsions, though this could not be separated from the observation of reduced latency to isoniazid convulsions in midazolam-treated mice. This demonstrated the importance of recognizing any increased susceptibility to convulsions following the

end of chronic treatment, which would confound the measurement of tolerance.

Neither the 1-week FZP treatment nor the 3-week DZP treatment caused a significant change in PTZ threshold. This was important because it showed that the measurement of tolerance was not hindered by a decrease in convulsive threshold, which might have given the appearance of tolerance. However, both treatments did cause a transient decrease in the threshold for IV bicuculline convulsions. A similar finding was reported by Nutt et al. (19), who found that mice treated with FZP had a greater sensitivity to bicuculline, but not to PTZ. If reduced convulsive threshold was simply a measure of withdrawal hyperexcitability, it might be expected that there would be increased sensitivity to all convulsants. The specificity shown by these results suggests that the decreased threshold for bicuculline, but not for PTZ, is related to differing mechanisms of action of the convulsants, and that using benzodiazepine effects on bicuculline threshold as a means to measure tolerance may not provide accurate results.

Though the lack of any change in PTZ threshold argues against withdrawal hyperexcitability, it should be noted that flumazenil-precipitated abstinence, signifying the presence of physical dependence, has been described in rats given a similar DZP treatment (17,36). In the present study, the weight loss seen after withdrawing DZP, but not FZP, treatment may also be an indicator of withdrawal. In fact, weight loss has been noted to be one sign of DZP withdrawal in rats (16).

The results of this and previous studies, showing differential cross-tolerance, support the contention that benzodiazepines do not all interact with their receptors uniformly. If these drugs do interact in different ways or with different receptors, their chronic effects might also differ, as suggested by the difference in patterns of cross-tolerance after FZP as compared to DZP treatment. The molecular basis of different actions may involve the $GABA_A$ receptor, which includes the benzodiazepine receptor as a modulatory site. Binding experiments have shown that benzodiazepine receptor number can be decreased by a 4-week FZP treatment (24,25,39), and some localized downregulation can be detected after the 1-week FZP treatment used in the present study (35). Using the "type-1" benzodiazepine receptor-selective ligand [^{3}H]zolpidem, even greater downregulation was found (39), and decreased binding could be seen in brain homogenates of rats treated with FZP for only a week (37). However, 3-week DZP treatment did not reduce the binding of [^{3}H]flunitrazepam, [^{3}H]zolpidem, or other benzodiazepine receptor ligands in brain homogenates (11,23,39). These findings suggested that FZP and DZP treatments have differing effects on the $GABA_A$ receptor. The idea that chronic exposure to different benzodiazepines might produce qualitatively different effects is also supported by the comparison of chronic alprazolam and lorazepam treatments on benzodiazepine binding and GABA-dependent chloride uptake in mice (7), and by the comparison of withdrawal phenomena in physically dependent animals (18).

The differing effects of FZP and DZP treatments on the $GABA_A$ receptor have also been shown by measurements of mRNAs for various $GABA_A$ receptor subunits. Though both FZP and DZP treatments can decrease mRNA levels, they differ in the particular subunits and brain regions affected (12,20,22,38,41,42). The relationship between these apparent changes in gene expression and tolerance or receptor regulation is not known. However, the data do serve to demonstrate that DZP and FZP treatments have differing effects on $GABA_A$ receptor subunit mRNA expression. It is hypothesized that these ultimately result in changes in the receptor subunit composition, which determine, at least in part, the presence and pattern of tolerance.

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