Br. J. Pharmacol. (1990), 99, 11-12 **SHORT COMMUNICATION** 

# Chronic diazepam treatment in rats causes long-lasting changes in central [$^3$H]-5-hydroxytryptamine and [$^{14}$C]-$\gamma$-aminobutyric acid release

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The effects of chronic diazepam administration to rats on the central release of [$^3$H]-5-hydroxytryptamine ([$^3$H]-5-HT) and [$^{14}$C]-$\gamma$-aminobutyric acid ([$^{14}$C]-GABA, *ex vivo*) were examined. Chronic (5 and 21 days) administration of diazepam (4 mg kg$^{-1}$ i.p. daily for 21 days) reduced the K-evoked (20 mM KCl) release of [$^3$H]-5-HT from frontal cortex by approximately 50%. Remarkably, this decrease was still present 1 week after diazepam withdrawal. Chronic diazepam treatment did not significantly affect hippocampal [$^3$H]-5-HT release but after 21 days the K-evoked release of [$^{14}$C]-GABA was more than doubled and remained elevated 30 h after withdrawal; it returned to control levels after 1 week, and decreased below control levels after 2 weeks. This study indicates that chronic diazepam treatment produces striking changes in transmitter release in rats that persist long after treatment has ceased.

**Introduction** The clinical evidence for benzodiazepine-dependence is well established and in a small proportion of patients withdrawal responses persist for a remarkably long time. However, the contributions of the disease state, physical dependence and reaction to media coverage are hard to disentangle. Animal studies have the advantage of allowing an assessment of the direct consequences of chronic drug treatment. As far as we are aware there have been no reports on the effects of chronic benzodiazepine administration on central transmitter release, although from early studies on benzodiazepines changes in release were inferred (Rastogi *et al.*, 1976; 1978). In the present experiments, we have examined the effects of chronic diazepam administration and withdrawal on the release of [$^3$H]-5-hydroxytryptamine ([$^3$H]-5-HT) from the cerebral cortex and both [$^3$H]-5-HT and [$^{14}$C]-$\gamma$-aminobutyric acid ([$^{14}$C]-GABA) from the hippocampus.

**Methods** Male hooded Lister rats (Olac Ltd), weighing 160–200 g at the start of the experiment, were injected (i.p.) daily with either diazepam (4 mg kg$^{-1}$) or vehicle (water/Tween 20) for three weeks. The rats were killed by cervical dislocation following stunning, 30 min after the last injection for drug treatment groups, or at the stated intervals for the withdrawal groups. The brain was rapidly removed and the frontal cortex and hippocampus dissected. Slices (0.2 mm) of both areas were prepared with a McIlwain tissue chopper as described previously (Cunningham & Neal, 1981). Tissue slices were given a preliminary incubation for 10 min at 37°C in Krebs bicarbonate medium. Then [$^3$H]-5-HT (cortex) or [$^3$H]-5-HT and [$^{14}$C]-GABA (hippocampus) were added to the medium to give final concentrations of 0.16 and 0.23 $\mu$M respectively. The medium contained pargyline (50 $\mu$M), ascorbic acid (100 $\mu$M), EDTA (30 $\mu$M) and, for hippocampal slices only, aminooxyacetic acid (AOAA, 50 $\mu$M). After 30 min incubation the slices were inserted between nylon grids in a small chamber (volume 1 ml) where they were superfused at 1 ml min$^{-1}$. Two ml fractions were collected and, following the addition of scintillant (Optiphase HiSafe II), the radioactivity in each sample was measured by liquid scintillation counting. The efflux of radioactivity was expressed as the fractional rate coefficient. An evoked release of [$^3$H]-5-HT and [$^{14}$C]-GABA was achieved by exposing the slices for 2 min to medium containing KCl 20mM.

The mean increases in the FRC were analysed by Analysis of Variance (ANOVA), followed by Duncan's tests. Unless otherwise stated the significance levels quoted are for these tests.

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5-Hydroxy-[G-$^3$H]-tryptamine creatine sulphate (12.6 Ci mmol$^{-1}$) and 4-amino-n-[U-$^{14}$C]-butyric acid (216 mCi mmol$^{-1}$) were obtained from Amersham International. Diazepam was a gift from Roche Products Ltd. Krebs bicarbonate of the following composition was used (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2 and glucose 9.5. The medium was gassed continuously with 5% $CO_2$ in $O_2$.

**Results** *[$^3$H]-5-HT release from cerebral cortex and hippocampus*

*Acute diazepam* A single injection of diazepam had no effect on either the spontaneous resting release (not illustrated) or the K-evoked release of [$^3$H]-5-HT from cortical or hippocampal slices (Figure 1a,b).

*Chronic diazepam* The effects of chronic diazepam on [$^3$H]-5-HT release differed strikingly between the two areas of brain examined. In cortical slices, after 5 days administration of diazepam, the K-evoked release of [$^3$H]-5-HT was reduced by 50% (Figure 1a). This decrease in K-evoked 5-HT release was similar (40% of controls) after 21 days' of drug treatment. Remarkably, when diazepam was withdrawn, the decrease in K-evoked [$^3$H]-5-HT release remained even after 1 week; at this time only there was also a significant ($P < 0.05$) decrease in resting release (not illustrated). In contrast to the effects of diazepam on cortical [$^3$H]-5-HT release, neither chronic treatment of rats with diazepam, nor the withdrawal of the drug, reduced the hippocampal K-evoked release of [$^3$H]-5-HT and if anything, the trend was towards an increased [$^3$H]-5-HT release after drug withdrawal (Figure 1b). However, 30 h after diazepam withdrawal there was a significant ($P < 0.05$) reduction in hippocampal resting release. When hippocampal [$^3$H]-5-HT release was evoked with a higher K concentration (KCl 40 mM), a similar pattern was seen except that after 1 week withdrawal the release was significantly increased compared with controls ($P < 0.05$) (not illustrated).

*[$^{14}$C]-GABA release from hippocampus*

*Acute diazepam* A single injection of diazepam had no effect on either the spontaneous resting release (not illustrated) or the K-evoked release of [$^{14}$C]-GABA from hippocampal slices (Figure 1c).

*Chronic diazepam* The effects of chronic diazepam on the K-evoked release of [$^{14}$C]-GABA from hippocampal slices is illustrated in Figure 1c. Treatment for 5 days had no effect but after 21 days of diazepam administration the K-evoked release of [$^{14}$C]-GABA was more than doubled. This effect was still

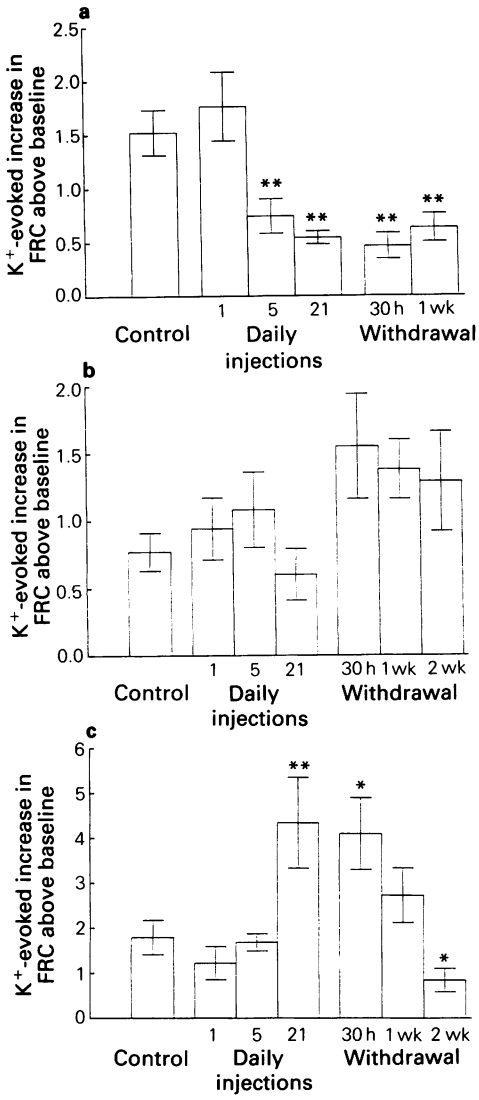


**Figure 1** Effects of diazepam (4 mg kg$^{-1}$, i.p.) and withdrawal on the K-evoked (KCl 20 mM) release (*ex vivo*) of (a) [$^3$H]-5-hydroxytryptamine ([$^3$H]-5-HT) from slices of frontal cortex, (b) [$^3$H]-5-HT from hippocampal slices and (c) [$^{14}$C]-$\gamma$-aminobutyric acid ([$^{14}$C]-GABA) release from hippocampal slices. The columns are mean (s.e.mean shown by vertical bars) of 5 to 9 experiments, ** $P < 0.01$; * $P < 0.05$ significantly different from corresponding controls.

apparent 30 h after diazepam withdrawal, but after 1 week the K-evoked release had returned to control levels. Two weeks after withdrawal the evoked release of [$^{14}$C]-GABA was less than half that of the controls, ($P < 0.05$ Student's $t$ test). The resting release of GABA showed a similar pattern of changes to the evoked release and was significantly increased ($P < 0.05$) 30 h after diazepam withdrawal and significantly decreased ($P < 0.05$) 2 weeks after withdrawal (not illustrated).

**Discussion** There were regional differences in the effects of chronic diazepam on 5-HT release. Whilst this was significantly reduced in the cortex after only 5 days of treatment, there was no decrease in the hippocampus even after 21 days, although longer treatment may have produced a significant drop. Consistent with these regional differences in *ex vivo* release, *in vitro* studies, in which slices were directly exposed to diazepam, showed reduced [$^3$H]-5-HT release from the cortex, and an increased [$^3$H]-5-HT release from slices of substantia nigra (Collinge & Pycock, 1982). Regional differences have also been found in the effects of chronic diazepam on GABA-stimulated chloride influx, with reductions in the cortex but not in the cerebellum (Marley & Gallager, 1989).

The reduction in evoked release of 5-HT is most unlikely to be secondary to a decreased synaptic pool of 5-HT, since benzodiazepines increase both 5-HT and 5-hydroxytryptophan (Johnston & File, 1986). Alternatively, the chronic administration of diazepam may have directly affected the 5-HT release mechanism, for example by decreasing $Ca^{2+}$ influx. Both low and high concentrations of benzodiazepines have been found to decrease voltage-dependent calcium conductance (Polc, 1988).

Whatever the mechanism responsible for the changes in 5-HT release it seems that a different process is involved in the changed GABA release. Diazepam treatment enhanced the GABA release (but this had a very slow onset) and diazepam withdrawal decreased GABA release below the control level, but this also had a very slow onset. A further difference is that the changes in evoked GABA release were paralleled by changes in its resting release, whereas this was not the case for 5-HT. One possible mechanism for these changes in GABA release could be a down regulation of the $GABA_B$ autoreceptors as a result of chronic diazepam treatment, and a subsequent rebound on withdrawal. A change in receptor number may explain the slower time course of the changes in GABA release.

The results of the present study show that chronic diazepam treatment produces significant and long-lasting effects on both 5-HT and GABA mechanisms. Behavioural evidence indicates that different neurochemical mechanisms underlie the development of tolerance to different behavioural effects of the benzodiazepines (File, 1985) and the incidence of different withdrawal responses (Lader & File, 1987). At present we cannot attribute changes in particular behaviours to changes in individual neurotransmitters as we have studied insufficient transmitters and brain regions. However, the long-lasting nature of the changes in release encourage us to believe they may be behaviourally relevant.

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