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The Effects of Benzodiazepines in Newborn Rats Suggest a Function for Type 2 Receptors

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FILE, S. E. AND L. J. WILKS. *The effects of benzodiazepines in newborn rats suggest a function for type 2 receptors.* PHARMACOL BIOCHEM BEHAV **25**(6) 1145–1148, 1986.—In day 4 female rats lorazepam, diazepam and clonazepam produced dose-related increases in forward walking and loss of righting and diazepam produced a dose-related increase in paddling. Lorazepam and diazepam increased jerks of the fore- and hind-limbs and the whole body, and clonazepam increased the latter two; these increases were not dose-related. Some doses of lorazepam and the lowest dose of diazepam increased tonic-clonic movements. Thus the benzodiazepines were observed to have two kinds of stimulant effect in day 4 rats. One is to cause hyperactivity and this effect is dose-related. The other is to cause a type of seizure-like behavior, although this action is not dose-related and the responses can be distinguished from those caused by convulsant compounds. The effects of the benzodiazepine antagonist Ro 15-1788 resembled those of the benzodiazepines. It increased hind-limb and whole body jerks, forward walking, paddling, loss of righting and tonic-clonic movements. CL 218,872, which is selective for type 1 benzodiazepine receptors, was devoid of significant effects. This suggests that the behavioral changes observed with the other compounds were mediated by the type 2 receptors.

Benzodiazepines Type 2 receptors Neonate Convulsions Hyperactivity

WHILST it has been known for some time that there are two sub-types of benzodiazepine receptors, for which benzodiazepines have equal affinity [7], the functional relevance of these two receptor sub-types has remained obscure. A recent report [1] that benzodiazepines cause convulsions in newborn rats may have provided the first evidence as to the functional relevance of the two types of benzodiazepine receptors. The finding that benzodiazepines cause convulsions may seem strange since they are used clinically as anticonvulsants. However, Barr and Lithgow suggested that benzodiazepines have two distinct actions: one, to cause convulsions, through actions at type 2 receptors, and the other to counteract convulsions, through actions at type 1 receptors. In the adult the latter action predominates, whereas in the newborn rat, which only has type 2 receptors [2] convulsions are seen.

A later paper by Pappas and Walsh [10] queried the conclusion that the newborn rats were convulsing and suggested that benzodiazepines were simply causing hyperactivity. They compared the effects of two benzodiazepines with those of clonidine (which produces a hyperactivity syndrome in the young rat) and of the convulsant pentylenetrazole. They found that only this last drug reliably produced signs of seizures (head-shaking, limb or body clonus). Loss of righting reflex was markedly induced by clonidine and diazepam, less so by chlordiazepoxide and not at all by pentylenetetrazole. Loss of righting was one of the signs used by Barr and Lithgow to indicate "convulsions." The most striking effects of clonidine were on forward walking, swimming and wall climbing. Since Barr and Lithgow had also included swimming movements in their convulsion-related behavior, Pappas and Walsh suggested that the behavior they had seen might have been hyperactivity rather than seizures.

The problem over what consitutes a seizure in a neonatal rat is by no means trivial. It arises because the rat is born at such an early developmental stage; synapses are not fully developed, myelination is incomplete, and motor patterns are primitive. It is not possible to have recourse to EEG measures during the first week, since the cortex has not developed. Thus one cannot be certain whether the baby rat is awake, asleep or convulsing. This raises the possibility that benzodiazepines were promoting sleep and that the incidence of sleep-jerks was mistaken for seizures. These are strong clonic jerks of the fore- or hind-limbs, often accompanied by a whole body spasm.

In order to try to clarify the behaviors induced by benzodiazepines we conducted a pilot study in which we compared the effects of chlordiazepoxide, diazepam, clonidine and the convulsant picrotoxin on 4 day old pups (see Table 1). Our observations convinced us of the difficulty of interpreting neonatal rat behavior. The seizures caused by picrotoxin (2 and 4 mg/kg) were reliable and unmistakeable and

TABLE 1

BEHAVIORAL RESPONSES OF DAY 4 RATS TO PICROTOXIN (PICRO), CHLORDIAZEPOXIDE (CDP), DIAZEPAM (DZ) AND CLONIDINE (CLON)

		Picro		CDP		DZ		Clon	
	Control	2	4	5	20	0.5	2	0.2	1
Frequent Jerks	2/16	6/8	8/8	8/9	6/9	4/8	5/9	3/9	4/10
Tonic-Clonic Movements	0/16	3/8	6/8	0/9	0/9	0/8	0/9	2/9	0/10
Tremor	1/16	5/8	8/8	2/9	0/9	1/8	2/9	0/9	0/10
Head-Shake	0/16	2/8	4/8	1/9	0/9	1/8	0/9	0/9	0/10
Paddling	0/16	0/8	0/8	1/9	7/9	2/8	6/9	1/9	0/10
Forward Walk	0/16	0/8	0/8	0/9	3/9	1/8	7/9	5/8	10/10
Wall Climbing	0/16	0/8	0/8	0/9	0/9	0/8	0/9	4/8	8/10
Squeaking	0/16	0/8	0/8	0/9	0/9	0/8	0/9	4/9	7/10

All doses in mg/kg. Scores are numbers of rats responding/number tested.

consisted of head-shaking, frequent clonus, violent body tremor, tonic-clonic movements and the adoption of a body posture with all four limbs extended. The hyperactivity and vocalisations caused by clonidine (0.2 and 1 mg/kg) were equally unmistakeable. The effects of the benzodiazepines were different from either of these two drugs and from each other, see Table 1. In order to minimise interpretations of the behaviors in the main study we scored each type of jerk and motor response separately. These behavioral categories yeilded inter-observer reliability of >90%.

The doses used in the studies so far have corresponded with the doses that would be used in adult rats. Garattini *et al.* [6] showed that in the newborn rat (age not specified) the anticonvulsant ED_{50} of diazepam was 15 times less than in adults. It is therefore possible that the seizures and/or hyperactivity simply reflect a high dose, toxic effect. In very high doses benzodiazepines can cause seizures in adult rats [11]. The purpose of the present study was to compare over a wide dose range the effects in the neonate of three different benzodiazepines, diazepam, lorazepam and clonazepam; of the benzodiazepine antagonist Ro 15-1788 (flumazepil); and of CL 218,872 (3-methyl-6[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-6]pyridazine) which acts at the benzodiazepine receptors, but is selective for type 1 [7].

METHOD

Drugs

Lorazepam (Ativan injection, Wyeth) was further diluted with distilled water to give injection volumes of 4 μl/g. The lorazepam vehicle was similarly diluted. CL 218,872 (Lederle) was dissolved in distilled water, and diazepam, clonazepam and Ro 15-1788 (Hoffmann-La Roche) were suspended in distilled water with a drop of Tween-20, in various concentrations to give injection volumes of 4 μl/g.

Animals

Female offspring of hooded Lister rats (Olac Ltd. Bicester) were left with their mothers and male siblings until day 4 (day of birth=day 0). The pups weighed 6–8 g on the day of test.

Procedure

A total of 211 female pups were removed from their nests and allocated at random among the various drug groups: controls (half received diluted lorazepam vehicle and half received water-Tween; lorazepam (0.01, 0.025, 0.5, 1 or 2 mg/kg); diazepam (0.2, 0.5, 1 or 2 mg/kg); clonazepam (0.1, 0.25, 0.5, 2.5 or 5 mg/kg); Ro 15-1788 (5, 10 or 20 mg/kg); CL 218,872 (5, 10 or 20 mg/kg). The pups were injected IP and observed immediately afterwards for the next 30 min, by an observer blind to the drug treatment. Each pup was placed singly on a plastic tray lined with paper in a room maintained at 33°C. Single clonic jerks were scored separately according to the body region concerned: head, tail, fore-limb, hind-limb and whole body. Head-shaking (side to side waving of the head), forward walking, paddling (rapidly alternating movements of the fore-limbs without forward progression), writhing, fine body tremor, loss of righting, sqeaks, and tonic-clonic movements were also scored. The behaviors that occurred as discrete events were counted (e.g., jerks); others that occurred in bouts were rated on a three-point scale of 1 for a brief occurrence (1 sec), 2 for a duration of 2–10 sec, and 3 for a bout >10 sec. For these behaviors the final score for each rat was the sum of the incidence × rating.

RESULTS

None of the drugs significantly altered the incidence of head-jerks, tail-jerks, head-shakes, writhing, fine body tremor or squeaks.

Lorazepam singificantly increased fore-limb jerks, hind-limb jerks, whole body jerks, forward walking, loss of righting and tonic-clonic movements (see Table 2). Diazepam had a very similar effect, but also increased paddling; however, only the lowest dose increased tonic-clonic movements (see

TABLE 2

MEDIAN SCORES (FREQUENCY × INTENSITY) FOR EACH BEHAVIOR DURING A 30 MIN OBSERVATION PERIOD FOLLOWING IP INJECTION

		Lorazepam (mg/kg)					Diazepam (mg/kg)				Clonazepam (mg/kg)					Ro 15-1788 (mg/kg)			CL218872 (mg/kg)		
	Controls	0.01	.025	0.5	1	2	0.2	0.5	1	2	0.1	0.25	0.5	2.5	5	5	10	20	5	10	20
Forelimb Jerk	0	0	1*	0.5	1.5*	0	0	0	0	1†	0	0	0	0	0	0	0	0	0	1	0
Hindlimb Jerk	0	1*	1	3*	3*	4†	4.5*	1.5*	6‡	2*	2.5*	1.5*	5*	2.5*	0	2*	1.5*	3‡	0	1	0
Whole Body Jerk	5	5	10*	5	5	9	23‡	15*	21‡	21.5†	15.5*	10	10.5	12*	3	22‡	16†	16†	5	5	5
Forward Walk	0	2*	1	8‡	14.5‡	10‡	0	6*	4.5*	10.5†	6.5‡	6‡	6.5†	5†	13‡	2	3*	0	0	0	1
Paddling	0	0	0	1	1	0	2.5	8.5*	13*	19.5‡	1.5	1	1	1	0	12*	19‡	22‡	0	0	2
Loss of Righting	0	4*	4*	7‡	7.5‡	10‡	1.5	9.5‡	17‡	23‡	10.5†	4.5*	6*	11†	20‡	7*	10†	11†	0	0	0
Clonic-Tonic Movements	0	0	4*	2†	0	1*	2*	0	0	0.5	0	0	0	0	0	1*	0	2*	0	0	0
n=	25	9	9	9	10	9	10	10	11	10	10	10	10	11	10	9	9	9	7	7	7

‡$p<0.001$; †$p<0.01$; *$p<0.05$. Mann-Whitney U-tests compared with controls.

Table 2). Clonazepam increased jerks of the hind-limbs and whole body only, but like the other benzodiazepines increased forward walking and loss of righting. It was without effect on tonic-clonic movements (see Table 2).

All three doses of Ro 15-1788 increased hind-limb and whole body jerks, and 5 and 20 mg/kg increased tonic-clonic movements. All three doses increased paddling, but only 10 mg/kg increased forward walking. All doses increased loss of righting. CL 218,872 had no significant effect on any of the behaviors scored (see Table 2).

DISCUSSION

In the adult rat there are two main types of seizure: full tonic-clonic convulsions and myoclonic jerks. In addition in the neonate rat head-shaking, writhing and fine body tremor have been observed following administration of convulsants (Table 1, [8,10]). These three behaviors were not changed by any of the drug treatments used in this study, nor was there an increased incidence of head and tail jerks. However, hind-limb and whole body jerks might reflect myoclonus and these were increased by all three of the benzodiazepines and Ro 15-1788. Since the benzodiazepines caused hyperactivity it is unlikely that the increased incidence of jerks reflected sleep-jerks. Tonic-clonic movements were seen with Ro 15-1788 and lorazepam (not dose-related) and with the lowest dose of diazepam. The absence of tonic-clonic movements with higher doses of diazepam or with clonazepam suggests that this is not just a toxic response to high doses of potent compounds.

The tonic-clonic movements seen with the benzodiazepines differed from those observed in the pilot study after the administration of picrotoxin, and nor did the benzodiazepines produce violent body tremor or the characteristic body posture with all four limbs extended. However, even in adult rats different convulsant compounds can cause seizures with conspicuously different characteristics, e.g., the seizures caused by the benzodiazepine Ro 5-4864 differ markedly from those caused by picrotoxin [3]. We therefore feel that it is possible that the hind-limb and body jerks and the tonic-clonic movements caused by benzodiazepine administration to newborn rats may reflect a type of seizure-like activity.

The loss of righting did seem to be dose-related and was caused by all three benzodiazepines and by Ro 15-1788. Hyperactivity, as indicated by forward walking, was also caused by all the benzodiazepines and Ro 15-1788 and was dose-related. Paddling was markedly increased by diazepam and Ro 15-1788, but not by the other benzodiazepines. Although there are some differences in the detailed effects of the different benzodiazepines it is clear that all changes produced were in the direction of stimulant, rather than sedative, effects.

The similarity between the effects of the benzodiazepine antagonist Ro 15-1788 and the benzodiazepines is interesting. Little and Nutt [8] have reported increases in locomotor activity in day 3 rat pups injected IP with the convulsant β-carbolines β-CCM and DMCM (both at 5 mg/kg) or with pentylenetetrazole (100 mg/kg). They did not find an increase with flurazepam (10 mg/kg), but only this single dose was studied. Since we found this effect to be dose-related, it seems likely that both agonists and inverse agonists at the benzodiazepine receptor and convulsants acting at other sites on the receptor complex can cause hyperactivity. It therefore seems that at this early stage of development the distinction between agonist and inverse agonist sites is not apparent. Little and Nutt also found that pentylenetetrazole,

but not the other compounds, caused a loss of righting. However, the use of single doses precludes generalisations from their data.

Our results suggest the possibility that benzodiazepine administration to neonatal rats might have two distinct stimulant effects. One is to cause hyperactivity, indicated by forward walking and paddling and possibly by loss of righting (this usually occurred during periods of vigorous activity). The second effect is possibly related to some sort of seizure activity, indicated by jerks of the hind-limbs and whole body and sometimes by tonic-clonic movements. However, the overall pattern of responses was not the same as that seen after picrotoxin and although jerks were seen they were not as prolonged or as vigorous. The clonic-tonic movements seen after benzodiazepines were not dose-related and were much less evident with clonazepam. In both types of behavior the effects of Ro 15-1788 resembled those of the benzodiazepines and thus it could be said to be having agonist-like activity in the day 4 rat. However, since β-carbolines with inverse agonist actions cause similar changes [8] it is difficult to categorise the behavior.

CL 218,872 (5–20 mg/kg) caused none of these behavioral changes. In the adult it is about half as potent as chlordiazepoxide and about one tenth as potent as diazepam [5,9] and it is therefore unlikely that we were looking at an inappropriate dose-range. The incidence of jerks and tonic-clonic movements were not dose-related, so we feel it is unlikely that lower or higher doses of CL 218,872 would have had significantly different effects. Since CL 218,872 has selectivity for type 1 benzodiazepine receptors [7] that are not present at day 4, our results suggest that all the behavioral changes we observed were mediated through actions at type 2 receptors. This provides some confirmation for the fascinating possibility that the type 2 receptors mediate the stimulant effects of benzodiazepines seen in adult animals, whereas the type 1 receptors mediate the sedative effects. This would explain why sedative effects predominate in the adult, since the type 1 receptors predominate. If different receptors are involved in the stimulant and sedative effects of the benzodiazepines this would also explain why tolerance does not develop to the former, but rapidly develops to the latter [4,12].

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