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The effect of GABA receptor ligands in experimental spina bifida occulta

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Abstract

Background: The pathophysiology behind spina bifida and other neural tube defects (NTDs) is unclear. Folic acid is one variable, but other factors remain. Studies suggest that substances active at the GABA receptor may produce NTDs. To test this hypothesis pregnant rats were exposed to either the GABA a agonist muscimol (1, 2 or 4 mg/kg), the GABA a antagonist bicuculline (.5, 1, or 2 mg/kg), the GABA b agonist baclofen (15, 30, 60 mg/kg), or the GABA b antagonist hydroxysaclofen (1, 3, or 5 mg/kg) during neural tube formation. Normal saline was used as a control and valproic acid (600 mg/kg) as a positive control. The embryos were analyzed for the presence of a spina bifida like NTD.

Results: After drug administration the pregnancies were allowed to proceed to the 21st day of gestation. Then embryos were removed and skeletons staining and cleared. Vertebral arch closure was measured. Results indicate that the GABAa receptor agonist muscimol, the GABAa receptor antagonist bicuculline, and the GABAb agonist baclofen produced NTDs characterized by widening of the vertebral arch. Oppositely the GABAb antagonist hydroxysaclofen produced narrowing of the vertebral arches.

Conclusions: The findings indicate that GABA a or b ligands are capable of altering neural formation. GABA may play a greater than appreciated role in neural tube formation and may be important in NTDs. The narrowing of the vertebral arch produced by the GABA b antagonist hydroxysalcofen suggests that GABA b receptor may play an undefined role in neural tube closure that differs from the GABA a receptor.

Background

Neural tube defects (NTDs) are major malformations of the central nervous system (CNS) due to a defect in the covering of the CNS. They are among the most prevalent of congenital malformations. NTDs are second only to congenital heart defects as a cause of perinatal mortality due to birth defect and range in incidence from 0.5 to 12 per 1000 live births, depending on the country, accounting for 400,000 births world-wide annually.

Factors that predispose individuals to NTDs are numerous. While folic acid deficiency and altered folic acid metabolism have received widespread attention, other contributors are also important. Socio-economic status, genetic factors, maternal illness and maternal drug exposure are important contributors to the risk of NTDs.

Drug models of NTDs are valued because the drug's action provides a possible explanation of the pathophysiol-

ogy of NTDs. Valproic acid (VA) is a well-known teratogen in both animals and humans, with a 5-fold occurrence of spina bifida (SB) in pregnant women exposed to the drug [1]. The mechanism by which VA produces SB is unknown but inhibition of folic acid metabolism is one hypothesis [2,3]. Some investigators have brought the folic acid hypothesis into question by demonstrating that folic acid supplementation has no effect on VA exposed embryos in-vitro [4,5]. These studies suggest that another mechanism may be responsible for the production of SB by VA. Other possible mechanisms include alteration of neuronal membrane conductance, sodium channel blockade or altered neuronal calcium metabolism [6]. VA is also known to allow GABA, the chief inhibitory neurotransmitter of the CNS, to accumulate in tissues.

The role of GABA as a potential site for teratogen activity for VA and other teratogens has been little explored. Preliminary reports have linked ligands active at the GABA receptor with SB or other NTDs, including benzodiazepines, alcohol, and zinc [7–11]. Preliminary work from this laboratory has shown the GABAa receptor agonist muscimol and the GABAb agonist baclofen produce both SB and the Arnold-Chiari malformation, commonly associated with SB [12–14]. VA appears to produce most of its anticonvulsant effects by increasing levels of GABA in the CNS, presumably by inhibiting the enzyme GABA-transaminase (GABA-T). There is other evidence to suggest that substances which alter the function of the GABAergic system may contribute to the formation of NTDs. Alcohol has been associated with NTDs [11] and is known to enhance the functioning of GABA. Benzodiazepines (BDZs), which enhance the activity of the GABA receptor, also enhance the teratogenic effects of VA in humans [10]. Chlordiazepoxide, another BDZ, has been shown to produce NTDs in the hamster [11].

Further support for the contention that VA may produce NTDs by way of GABA activity include the following evidence. One hypothesis of VAs mechanism of action holds that it alters intracelluar pH. This may be the case as GABA can increase intracellular proton levels by intensifying bicarbonate ion conductance through a GABA-gated channel [15] which may act as a "developmental handshake" and regulate neuronal differentiation [16]. The chloride channel, an integral part of the GABA a receptor, has been implicated in embryonic development [17]. GABA receptors are first seen at the time the neural tube formation [18]. Binding sites to GABA agonists and antagonists and the expression of GABA receptor mRNAs are seen starting at 4 days of development and peak at 10–15 days, corresponding to the time of neural tube formation [19]. Many neurotransmitters, including GABA, are growth factor candidates for the CNS [20–22].

Based on the above information we have undertaken this study to systematically examine the effects of GABA agonists and antagonists at both the GABA a and b receptor and the role they may play in SB. We used an established model of SB in the rat to test the hypothesis using VA as a known standard. This model of SB uses the width of the vertebral arch as an indicator of neural tube closure. While this model does not produce a specific SB lesion the widening of the vertebral arch provides a model that resembles human SB in terms of accompanying defects [12–14,23] and response to folate and other drugs [6,11,24,25] and meets the formal definition of a neural tube defect: any defect in the covering of the central nervous system.

We report here the effects of GABA a and b receptor agonists and antagonists administered to rats at 10 days gestation, the period of neural tube formation.

Results and Discussion

A total of 1156 embryos from 123 litters were examined. Measurements from the embryos were averaged for each litter and the litter was used as the unit of analysis. All measurements were made in a blind manner. ANOVA revealed a significant effect of drug treatment of the average vertebral arch distance ($F(13, 109)=7.70$, $p < .0001$). The Bonferroni test was used for follow-up comparisons and the comparisons to the normal saline group are given below and in Table 1 and in Figure 1 where the results are presented graphically. Occasionally other defects were noted in the embryos, chiefly fused ribs, these defects were not appreciable and did not impact the vertebral arch analysis. There is no statistically significant relationship between mean vertebral arch distance and mean littler size.

Valproic Acid produced a significant widening of the vertebral arch in a manner consistent with previous reports ($p < .0005$) [6,24]

The GABAa agonist muscimol produced a significant widening of the vertebral arch at all three doses tested (1, 2, 4 mg/kg) ($p=.06$ for 1 mg/kg, $p < .05$ for 2 & 4 mg/kg). The GABAa antagonist bicuculline also widened vertebral arch distance at the 0.5 and 1 mg/kg doses ($p < .05$), but not at the 2 mg/kg dose.

The GABAb agonist baclofen produced significant widening of the vertebral arch at 30 mg/kg ($p < .05$) but not at 15 or 60 mg/kg. The GABAb antagonist hydroxysaclofen produced a significant narrowing of the vertebral

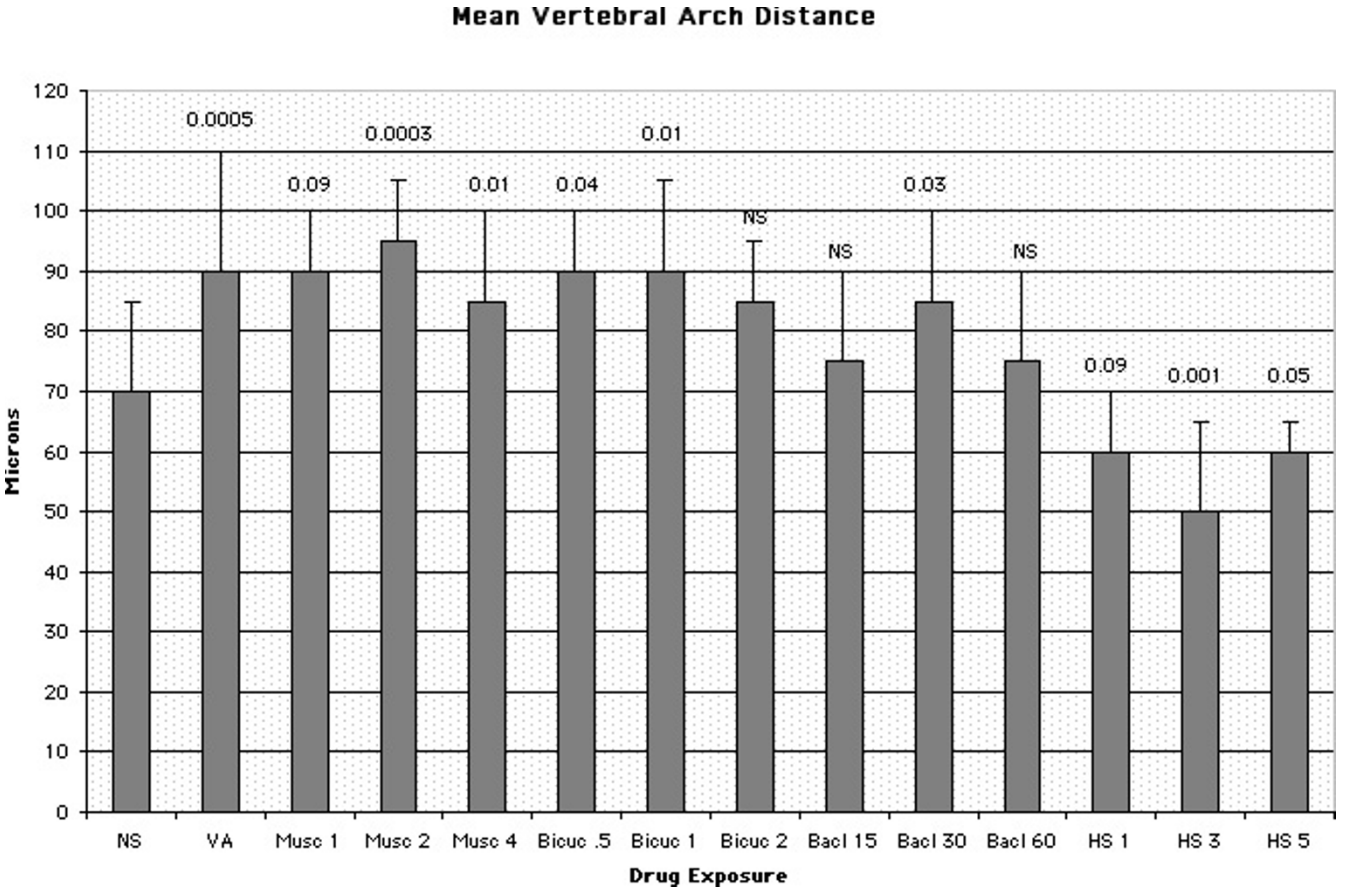

Figure 1
Effect of GABA receptor ligands on vertebral arch distance. Mean vertebral arch distance for drug groups. Error bars represent standard deviations. Exact probability differences from normal saline are given above error bars. See text for comments.

Table 1: Effect of GABA Ligands on Vertebral Arch Width

Drug	Number of Litters	Mean # Embryos per Litter	Mean Arch Width (microns)	S.D.	Sig. (p=)
Normal Saline	15	8.67	71.5	16.4	N/A
Valproic Acid	15	7.20	90.5	19.5	.0005
Muscimol 1	3	10.67	89.3	11.9	.09
Muscimol 2	10	9.9	93.9	8.6	.0003
Muscimol 4	9	11.44	87.25	14.7	.01
Bicuculine 0.5	4	5.25	88.2	9.0	.04
Bicuculine 1	5	5.80	90.5	15.5	.01
Bicuculine 2	3	5.67	82.8	9.0	NS
Baclofen 15	14	8.64	73.6	17.3	NS
Baclofen 30	7	10.43	86.0	13.0	.03
Baclofen 60	10	10.10	75.5	14.6	NS
Hydroxysaclofen 1	10	9.80	61.5	9.6	.09
Hydroxysaclofen 3	9	5.00	50.8	16.2	.001
Hydroxysaclofen 5	9	8.56	59.15	3.15	.05

arch at all three doses tested (p=.09 for 1 mg/kg, p=.001 for 3 mg/kg and p=.05 for 5 mg/kg).

This study indicates that substances active at either the GABA a or b receptors have teratogenic potential. The most striking feature of the drug effects is the differential effect of antagonizing the GABAb receptor with hydroxysaclofen. While the GABAa agonist muscimol and antagonist bicucullin widened the vertebral arch, as did the GABAb agonist baclofen, the GABAb antagonist hydroxysaclofen narrowed the vertebral arch. Narrowing of the vertebral arch was unexpected but has been previously reported. Previous work in this laboratory has demonstrated narrowing of the vertebral arch when zinc is co-administered with baclofen or muscimol [13]. It is unclear what the structural or functional consequence of vertebral arch narrowing is. Work in this laboratory has shown lags in neuromuscular development associated with excessive zinc exposure during neural tube formation and, possibly, accompanying narrowing of the vertebral arches [26]. Another curious finding of this study is that widening of the vertebral arch occurs with either the GABAa agonist muscimol or the antagonist bicuculline. In classic pharmacology it would be expected that the effects would be opposite. However, in this instance it may be that any disruption of the normal function of the GABAa receptor and its integral chloride channel result in widening of the vertebral arch. On the other hand the GABAb receptor is not directly linked to a chloride channel exerting its effect via second messenger systems. This functional difference may allow for a classic agonist-antagonist drug effect during neural tube formation.

One potential confound of this study is the role of developmental delays. Animals exposed to valproate and presumably suffering from widened vertebral arches can show weight differences well into post-natal life. However, behavioral differences persist even when weight differences no longer exist [1]. However, we are unaware of any studies directly examining the spinal columns of animals well into post-natal life, therefore it is unclear if the widening of the vertebral arch seen at 21 days persists. Another limitation to this study is the small sample sizes for some groups and the administration of drugs to only one day. These results need to be replicated using larger sample sizes and administering the drugs at other times during gestation. It is possible that these drugs may disrupt neural tube formation if given outside of the classic time frame for neural tube formation.

GABA is a well-documented neurotrophic agent involved in brain development [27–30]. Most of the work done on the effects of GABA and neural development has been done on embryos and embryonic tissue well past the neural tube stage [27,31,32]. However, there is evidence of glutamic acid decarboxylase (GAD) and GABA receptor expression about the time of neural tube formation [27]. Given that GABA is important to neural development, and the early developmental time frame of the GABA system, it is logical that agents active at the GABA receptor (ethanol, BDZs) can have adverse consequences on CNS development. Nearly all of the substances examined are GABAa receptor ligands. GABAb receptor effects have been little studied and the role of the GABAb receptor in neural development is little known and should be more thoroughly investigated.

Conclusions

GABA may play an important role in neural tube formation and the production of neural tube defects. Substances active at the GABA a or b receptor may be potentially teratogenic. In particular, the findings indicate that GABA a or b ligands are capable of altering neural formation. GABA may play a greater than appreciated role in neural tube formation and may be important in neural tube defects. The narrowing of the vertebral arch produced by the GABA b antagonist hydroxysalcofen suggests that GABA b receptor may play an important, but undefined role in neural tube closure that differs from the GABA a receptor.

Materials and Methods

Female Long-Evans rats 120 days of age were housed with ad-lib access to food and rat chow (Purina, Brentwood, MO) under 12:12 light:dark conditions. The females were mated with males of the same age and strain overnight with the observation of a copulatory plug as evidence of mating and counted as day 0 of pregnancy. The females were then separated and housed singly. At 10 days of gestation the females were treated with one drug as described below. Ten days of gestation corresponds with neural tube formation in the rat. All drugs and chemicals were obtained from Sigma Chemical, St, Louis, MO. Doses were empirically determined with pilot studies. This study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska at Kearney.

Reference groups

Normal saline intraperitoneally (IP) 0.9 cc to establish baseline.

Valproic acid subcutaneously (SC) 1200 mg/kg (600 mg/cc) in two divided doses over 8 hours. Previous studies have shown this dose and route to reliably produce SB in the rat [24]. This group provided a positive control to which the teratogenic activity of other substances could be compared.

GABAa test groups

Muscimol IP at 1, 2, or 4 mg/kg (1 mg/cc). Muscimol is a well-documented specific and potent GABAa agonist [33].

Bicuculline methiodide IP at 0.5, 1 or 2 mg/kg (1 mg/cc). Bicuculline is a well-documented specific and potent GABAa antagonist [33].

GABAb test groups

Baclofen IP at 15, 30, or 60 mg/kg (9 mg/cc as aqueous suspension). Baclofen is a well-documented and specific GABAb agonist [34].

Hydroxysaclofen IP at 1, 3, or 5 mg/kg (1 mg/cc). Hydroxysaclofen is a well-documented specific and potent GABAb antagonist [34].

Embryo studies

After injection gestation was allowed to otherwise progress as normal. At 21 days gestation the females were euthenized with chloroform, the abdomen opened and the uterus and uterine contents removed. Fetuses were removed and had their abdomens opened to allow for the penetration of 10% 0.1 M phosphate buffered formalin in which they were immersed. After three days fixation the fetuses were stained for bone and cartilage as described previously [24]. Briefly, the fetuses were eviscerated and skinned. Cartilage was stained with alcian blue followed by bone staining with alizarin red. The fetuses were cleared in KOH and graded concentrations of glycerol.

After clearing the fetuses were inspected with a dissecting microscope and the width of the vertebral arch gap was measured with an eyepiece micrometer from T-9 to S-4. Previous work has shown that these vertebrae are the most clearly visible and the most frequently effected [6,24]. For the sake of clarity vertebral arch distances from T-9 to S-4 were averaged for the each embryo.

For analysis vertebral arch distance was averaged for each fetus and then for the litter. Data was analyzed with Analysis of Variance with follow-up statistics (Bonferroni test) using Statview 5.0 for the Macintosh.

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