INFLUENCE OF SOME GABAERGIC AGENTS ON NITRAZEPAM-INDUCED SLEEP IN THE DOMESTIC FOWL (GALLUS DOMESTICUS)

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Abstract—The effects of gamma amino butyric acid (GABA), bicuculline and amino-oxyacetic acid (AOAA) on nitrazepam-induced sleep were studied in young chicks. GABA (200–3200 mg/kg) induced a marked sedation in young chicks. It also potentiated nitrazepam (1.6 mg/kg)-induced sleep. Bicuculline (1.25–5.00 mg/kg) effectively antagonized nitrazepam-induced sleep. In addition, it effectively antagonized GABA (1600 mg/kg)-induced potentiation of nitrazepam sleep. AOAA (2.5–7.5 mg/kg) delayed the onset of nitrazepam sleep, but significantly prolonged its duration. Nitrazepam (1.6 mg/kg) synchronized the electroencephalogram (EEG) of the hyperstriatum, optic tectum and reticular formation. Similarly, the electromyograph (EMG) activity was markedly reduced. GABA (1600 mg/kg) synchronized the EEG of the hyperstriatum, optic tectum and reticular formation while the EMG activity was reduced. Administration of GABA (1600 mg/kg) into nitrazepam (1.6 mg/kg)-pretreated chicks induced desynchronization of the EEG of the hyperstriatum, while the EEG of the reticular formation was synchronized. In addition, the EMG activity was reduced. Bicuculline (5 mg/kg) activated the EEG of the hyperstriatum; this effect was antagonized by GABA (1600 mg/kg). Similarly, GABA (1600 mg/kg)-induced decrease in EMG activity, synchronization of the EEG of the optic tectum and reticular formation was antagonized by bicuculline. The present data suggest that GABA potentiated nitrazepam-induced behavioral and electroencephalographical sleep.

The sedative-hypnotic effect of benzodiazepines in various mammalian species is well established (1–3). Braestrup and his co-worker (4) reported that highly specific recognition sites exist in the brain for the benzodiazepines. Although the classical monoamine transmitters have been implicated in the mechanism of action of the benzodiazepines, the primary site of action within the synaptic processes is still uncertain (5–7). GABA has also been postulated to play a primary role in the mechanism of the behavioral effects of the benzodiazepines (8, 9). However, the precise sites of action are still unknown (7).

There has been no documented evidence in the literature to our knowledge on the influence of GABAergic agents on nitrazepam-induced sleep using chicks. Although GABA does not cross the blood-brain barrier, it has been possible to study the direct effects of GABA in this project because the young chick (1–28 day old) lacks a functional blood-brain barrier (10). It is therefore the objective of this project to investigate the influence of GABA, AOAA and bicuculline on nitrazepam-induced sleep in young chicks. In addition, the influence of these GABAergic agents on the EEG and EMG of young chicks was studied.
Materials and Methods

White ranger cockerels (obtained from Arewa Agricultural Enterprises Nigeria Ltd., Zaria), 4–6 day old and weighing between 35 and 40 g, were used in all the experiments. The chicks were regarded as sedated when they were quiet and immobile with or without closure of their eyes over 5 min. The criteria for behavioral sleep in chicks were those used by Fugner and Hoefke (11). The procedures of Osuide and Wamba (12, 13) for behavioral sleep experiments, preparation and implantation of EEG (unipolar and bipolar) and EMG electrodes into young chicks were followed in this project. The electrodes were prepared in our laboratories using stainless steel insect pins (about 0.12 mm diameter, while the polar distance of the bipolar electrodes was about 0.11 mm.) Four chicks were used per behavioral experiment and each experiment was repeated three times. Control chicks (injected with the appropriate solvent vehicle omitting the drug) were studied concurrently with chicks injected with GABAergic agents. Each chick was used only once.

Two specially-designed behavioral cages containing 15 compartments each were used in this project. After the implantation process was completed, the chicks were kept warm using an angle poise lamp and allowed 24 hr for full recovery from halothane before EEG and EMG recording using a Grass polygraph (model 79 D). The implanted, freely moving and conscious chick was placed inside a screened cage and allowed sometime to settle before recording the EEG and EMG. Control recording was taken for about 30 min prior to injection of the drugs, after which recording was continued for 90 min. In view of the circadian fluctuation in brain monoamine levels which would consequently influence the gross behavior of the chicks, all the experiments were performed during the same period of the day (i.e., between 1300 and 1700).

Nitrazepam (mogadon, Roche Products), GABA (ICN Pharmaceuticals), AOAA (Sigma Chemical Company) and bicuculline (Sigma Chemical Company) were injected intraperitoneally. Nitrazepam and bicuculline were each suspended in 3% v/v Tween 80. GABA and AOAA solutions were prepared by dissolving them in normal saline. The solutions of AOAA and bicuculline were freshly prepared on the days of the experiments.

The duration of drug pretreatment before administration of nitrazepam for all the drugs used in this project was 10 min. The pretreatment period was established from preliminary studies in our laboratory.

Results

Effect of GABA on 4–6 day old chicks: GABA (800–3200 mg/kg) sedated the animals. The onset of sedation was reduced, while the duration was increased as the dose of GABA was progressively increased (Table 1).

| Doses of GABA (mg/kg) | Number sedated/number used | Onset of sedation (min) Mean ± S.E.M. | Duration of sedation (min) Mean ± S.E.M. |
|-----------------------|-----------------------------|---------------------------------------|------------------------------------------|
| 200                   | 0/12                        | -                                     | -                                        |
| 400                   | 0/12                        | -                                     | -                                        |
| 800                   | 3/12                        | 15.6 ± 2.8                            | 25.8 ± 4.6                               |
| 1600                  | 12/12                       | 9.4 ± 3.7                             | 14.2 ± 2.2                              |
| 3200                  | 12/12                       | 8.4 ± 1.6                             | 15.6 ± 2.7                              |
Influence of GABA on nitrazepam-induced sleep using 4–6 day old chicks. GABA (12.5–1600 mg/kg) prolonged the onset of sleep, increased the number of animals that slept, while the sleeping time was considerably prolonged with an increase in dose. Three per cent v/v Tween 80 diluted appropriately with normal saline (i.e., the solvent vehicle for nitrazepam) had no observable behavioral effect on the animals (Table 2).

Influence of bicuculline on nitrazepam-induced sleep using 4–6 day old chicks. Bicuculline (1.25–5.0 mg/kg) delayed the onset of sleep and reduced the proportion of chicks which slept following the administration of nitrazepam (1.6 mg/kg). Nitrazepam sleeping time was also decreased with elevated doses of bicuculline. Apparently,

| Doses (mg/kg) Nitrazepam | GABA | Number asleep/ number used | Onset of asleep (min) | Duration of sleep (min) |
|--------------------------|------|---------------------------|-----------------------|-------------------------|
| 1.6                      | 12.5 | 6/12                      | 5.9 ± 1.5             | 10.3 ± 2.9              |
| 1.6                      | 25.0 | 9/12                      | 8.4* ± 0.8            | 16.6* ± 2.4             |
| 1.6                      | 50.0 | 9/12                      | 8.8 ± 1.3             | 12.7 ± 2.8              |
| 1.6                      | 100.0| 10/12                     | 7.0 ± 1.1             | 18.4** ± 3.6            |
| 1.6                      | 200.0| 11* /12                   | 11.7*** ± 2.7         | 12.7 ± 3.2              |
| 1.6                      | 400.0| 11* /12                   | 11.3*** ± 2.1         | 16.2 ± 3.5              |
| 1.6                      | 800.0| 12** /12                  | 10.5** ± 3.7          | 17.3* ± 3.7             |
| 1.6                      | 1600.0| 12** /12                  | 11.0* ± 3.2           | 39.6*** ± 5.0           |

* and ** are significantly different from the controls (same dose of nitrazepam) and represent P<0.05 and P<0.005, respectively. Chi-squared test with Yate's correction for continuity. ** and *** are significantly different from the controls (same dose of nitrazepam) and represent P<0.01, P<0.025, and P<0.005, respectively, Student's t-test.

| Doses (mg/kg) Nitrazepam | Bicuculline | GABA | Number asleep/ number used | Onset of sleep (min) | Duration of sleep (min) |
|--------------------------|-------------|------|---------------------------|-----------------------|-------------------------|
| 1.6                      | 1.25        |      | 12/26                     | 5.9 ± 1.6             | 10.3 ± 2.9              |
| 1.6                      | 2.50        | 6/12 | 8.6** ± 0.9               | 13.3 ± 3.1            |
| 1.6                      | 5.00        | 7/12 | 11.0** ± 2.6              | 11.6 ± 2.9            |
| 1.6                      | 5.00        | 4/12 | 12.4** ± 2.9              | 5.5 ± 0.9             |
| 1.6                      | 5.00        | 1600| 0/4                       | 5/4                   | 5/4                     |
| 1.6                      | 2.50        | 1600| 10/12                     | 10.4*** ± 3.1         | 17.8** ± 3.1            |
| 1.6                      | 5.00        | 1600| 2/12                      | 21.8** ± 3.2          | 8.2 ± 1.3               |

++ and *** are significantly different from the controls (same dose of nitrazepam) and represent P<0.025 and P<0.005, respectively. Student's t-test.
bicuculline (5.0 mg/kg) antagonised GABA-induced potentiation of nitrazepam sleep (Table 3).

Influence of amino oxyacetic acid (2.5–7.5 mg/kg) on nitrazepam-induced sleep: Low doses of amino oxyacetic acid (2.5–5.0 mg/kg) reduced the proportion of chicks that slept and delayed the onset of nitrazepam sleep. However, a higher dose of amino oxyacetic acid (7.5 mg/kg) increased the proportion of chicks that slept but delayed the onset of nitrazepam sleep (Table 4).

Effect of nitrazepam on chick EEG and EMG using 4 day old chicks: Nitrazepam synchronized the EEG of the hyperstriatum and the pontine reticular formation. In addition, the amplitude of the EMG was increased while the frequency was reduced (Fig. 1).

Effects of GABA on the EEG and EMG: GABA (1.6 g/kg) synchronized the EEG of the hyperstriatum, optic tectum and pontine reticular formation, while the EMG activity was reduced (Fig. 2).

Table 4. Effect of AOAA on nitrazepam-induced sleep using 4–6 day old chicks

| Dose (mg/kg) Nitrazepam | AOAA | GABA | Number asleep/number used | Onset of sleep (min) Mean ± S.E.M. | Duration of sleep (min) Mean ± S.E.M. |
|-------------------------|------|------|---------------------------|------------------------------------|--------------------------------------|
| 1.6                     |      |      | 6/12                      | 5.9 ± 1.5                           | 10.3 ± 2.1                           |
| 1.6                     | 2.5  |      | 4/12                      | 8.7 ± 2.0                           | 13.0 ± 2.5                           |
| 1.6                     | 5.0  |      | 5/12                      | 10.9* ± 2.8                         | 8.7 ± 1.4                            |
| 1.6                     | 7.5  |      | 9/12                      | 10.00* ± 1.0                        | 17.9* ± 3.3                          |
| 1.6                     | 7.5  | 1600 | 8/12                      | 14.4*** ± 2.4                       | 14.8 ± 3.2                           |

* and *** are significantly different from the controls (same dose of nitrazepam) and represent P<0.01 and P<0.005, respectively. Student's t-test.

Fig. 1. Effect of nitrazepam on chick EEG and EMG using 4 day old chicks. A: Control (solvent vehicle 0.4 ml). B: 5 min after injection of 1.6 mg/kg nitrazepam. C: 20 min after. D: 60 min after. Abbreviations in this and subsequent tracing HS=hyperstriatum, OT=optic tectum, RF=pontine reticular formation.
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Fig. 2. Effect of GABA on chick EEG and EMG using 5 day old chicks. A: Control (solvent vehicle 0.4 ml). B: 5 min after injection of 1.6 g/kg GABA. C: 20 min after. D: 30 min after.

Fig. 3. Effect of GABA 10 min later plus nitrazepam on chick EEG and EMG using 4 day old chicks. A: Control (solvent vehicle 0.4 ml). B: 10 min after injection of GABA (1.6 g/kg). C: 10 min after injection of GABA (1.6 g/kg) and nitrazepam (1.6 mg/kg). D: 20 min after.

Effects of GABA and nitrazepam on the EEG and EMG: Administration of both GABA (1.6 g/kg) and nitrazepam (1.6 mg/kg) desynchronized the EEG of the hyperstriatum and synchronized the EEG of the pontine reticular formation. The EMG activity was slightly reduced (Fig. 3).

Effects of GABA and bicuculline on the EEG and EMG: Bicuculline (5 mg/kg) activated the EEG of the hyperstriatum; this effect was antagonised by 1.6 g/kg of GABA (Fig. 4).

Discussion

The sedative effect of GABA was dose-dependent. Thus, 200–400 mg/kg of GABA had no observable effect on gross behavior, while 800–3200 mg/kg produced obvious
sedation in a dose-dependent manner (Table 1). The proportion of chicks that were sedated increased while the onset of sedation decreased with an increase in dose. These results indicate an increase in the intensity of the sedative effect of GABA with increase in dose. It is therefore possible that the sedative effect of GABA in chicks might be directly related to the amount of the drug that reached the brain. The present data agrees with the results of some workers who observed that intravenous or intracisternal injection of GABA in mice produced a tendency to sleep (14–17). Thus, the mechanisms underlying GABA-induced sedation in mice and young chicks might be similar.

GABA profoundly potentiated nitrazepam-induced sleep in young chicks. For example, 200–1600 mg/kg of GABA, dose-dependently increased the proportion of animals that slept upon injection of nitrazepam. The potentiation of nitrazepam-induced sleep by GABA was statistically significant vis-a-vis the proportion of animals that slept (P<0.005). In addition, GABA delayed the onset and prolonged the duration of nitrazepam sleep. The delay in onset might be due to a direct effect of GABA on its receptors or an effect mediated via the interaction of GABA with other putative transmitters (e.g., release of dopamine). On the other hand, the result might be related to a non-specific effect of GABA on some cortical neurones. However, GABA significantly prolonged the duration of nitrazepam-induced sleep (P<0.05). From the present data, it can be suggested that nitrazepam may induce sleep via GABAergic mechanisms. Diazepam is known to enhance presynaptic inhibition in the spinal cord (8). Since GABA has also been postulated to mediate presynaptic inhibition, the hypothesis that diazepam may act via GABA neurons was proposed by Polc et al. (18). This hypothesis agrees with our observation that GABA potentiated nitrazepam-induced sleep.

Bicuculline (a GABA receptor antagonist) antagonized nitrazepam-induced sleep. Thus, the proportion of animals that slept reduced and the onset of nitrazepam sleep was delayed by bicuculline. The onset of nitrazepam-induced sleep was profoundly delayed by prior administration of bicuculline (P<0.005). Such data support our finding that nitrazepam

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Fig. 4. Effect of GABA 20 min later plus bicuculline on chick EEG and EMG using 5 day old chicks. A: Control (solvent vehicle 0.4 ml). B: 20 min after injection of 5 mg/kg bicuculline. C: 10 min after injection of GABA (1.6 g/kg) and bicuculline (5 mg/kg), D: 30 min after.
sleep was potentiated by GABA. It is therefore possible that endogenous GABA might be involved in nitrazepam sleep in chicks. Since diazepam and possibly other benzodiazepines have been postulated to enhance presynaptic inhibition (8), the above data agrees with the possible mechanism of action of the antagonism produced by bicuculline against nitrazepam-induced sleep.

Just like GABA, AOAA delayed the onset of nitrazepam-induced sleep, but significantly prolonged the duration of sleep (P<0.01). This result indicates a marked potentiation of nitrazepam-induced sleep and agrees with the previous suggestion that nitrazepam may act via GABA mechanisms. This proposal is supported by the report that AOAA is a powerful inhibitor of GABA transaminase (GABA-T)—an enzyme which metabolizes GABA (19), thereby leading to an increase in brain GABA levels. The increased brain GABA levels might be responsible for the potentiation of nitrazepam sleep observed with AOAA in this study.

Nitrazepam synchronized the EEG of the hyperstriatum, optic tectum and the pontine reticular formation. In addition, sleep spindles were observed on both the hyperstriatum and the reticular formation with an associated EMG activation. These electrocortical observations indicate behavioral sleep. Thus, the EEG results agree with the behavioral data obtained in this project.

GABA synchronized the EEG of the hyperstriatum, optic tectum and reticular formation. In addition, the EMG activity was reduced. This result conforms with the sedative effect of GABA (17).

Administration of both GABA and nitrazepam apparently desynchronized the EEG of the hyperstriatum but synchronized the EEG of the reticular formation, while the muscle activity (EMG) was slightly reduced. Such data indicates electrocortical sleep. Thus, the EEG results agree with the behavioral observation vis-a-vis potentiation of nitrazepam sleep by GABA.

Bicuculline activated the EEG of the hyperstriatum, which might be due to antagonism of endogenous GABA. Bicuculline-induced activation of the EEG of the hyperstriatum was antagonized by GABA. Similarly, GABA-induced synchronization of the EEG of the optic tectum and reticular formation as well as reduced EMG activity were apparently antagonized by bicuculline. Thus, the EEG and EMG results agree with the behavioral observations that GABA potentiated nitrazepam sleep.

These results indicate that nitrazepam-induced sleep in young chicks may involve enhancement of GABA neurotransmission.

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