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Rebound increase in seizure susceptibility but not isolation-induced calls after single administration of clonazepam and Ro 19-8022 in infant rats

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ABSTRACT

The purpose of our study was to determine whether a single administration of anticonvulsant doses of two ligands of benzodiazepine receptors, clonazepam and Ro 19-8022, leads to development of rebound phenomena in immature 12-day-old rats. Three tests were used: pentylenetetrazole (PTZ)-induced seizures, isolation-induced ultrasonic vocalizations, and motor performance. Susceptibility to the convulsant effects of PTZ decreased 24 hours, but increased 48 hours, after clonazepam administration. Ultrasonic vocalizations were completely suppressed 30 minutes and 3 hours after clonazepam; a moderate inhibitory effect persisted even at 48 hours. Motor abilities were slightly compromised up to 3 hours. Similar effects of Ro 19-8022 on PTZ-induced seizures and ultrasonic vocalizations were observed 24 and 48 hours after administration; motor performance was not affected. Rebound proconvulsant effects followed different time courses after administration of the two benzodiazepine receptor ligands in developing animals. Anxiolytic-like effects of these drugs were still present at the time when animals exhibited rebound proconvulsant effects.

1. Introduction

Benzodiazepines are some of the most frequently prescribed drugs because of their anxiolytic, sedative, hypnotic, and anticonvulsant properties. However, central nervous system adverse reactions, tolerance, and withdrawal syndromes have increasingly been recognized. Available data demonstrate that the development and severity of withdrawal or rebound phenomena, the contiguous or even overlapping syndromes, are highly related to the duration of therapy and also to the elimination half-life and dose (for review, see [1]). Insomnia, agitation, increased muscle tension, anxiety, and autonomic dysfunction are the most frequently reported rebound phenomena in humans. Development of epileptic seizures or status epilepticus is observed, particularly after abrupt discontinuation of long-lasting therapy; patients with epilepsy are at special risk.

Withdrawal and rebound symptoms were observed not only in adult patients but also in critically ill children iatrogenically exposed to benzodiazepines (for review, see [2]). In these patients, benzodiazepines are usually co-administered with opioids or other less commonly used drugs with the potency to induce dependence, tolerance, and withdrawal or abstinence syndromes [3]; thus, the effects of individual substances are difficult to separate. Also, most of our information concerning withdrawal and abstinence syndromes concerns opioids, with less information available for other agents including benzodiazepines (for review, see [2]). Experimental studies of these symptoms focus only on adult animals, and despite its high clinical relevance and importance, this topic is not studied in developing animals.

Therefore there are no adequate tools for detection and quantification of these symptoms in immature rodents. To fill this gap, two models were selected: pentylenetetrazole (PTZ)-induced seizures and isolation-induced ultrasonic vocalizations (USVs). Both these models were extensively studied and pharmacologically analyzed in immature rats. PTZ elicits two types of seizures in rodents: minimal seizures and generalized tonic–clonic seizures (GTCS). They differ not only in semiology but also in pharmacological sensitivity and developmental profile. Minimal clonic seizures can be reliably triggered by PTZ in animals older than 2 weeks, whereas GTCS can be elicited in all age groups, from the very first postnatal days [4,5]. Rat pups emit USVs in response to a transient separation from mothers and siblings. The function of this behavior is to elicit a retrieval response from the mother. The most effective stimulus for eliciting USVs is thermal stress, that is, temperature lower than the nest [6]. Rat vocalizations during a period of maternal separation are considered to be an indicator of the anxiety the animals are experiencing: the greater the rate of USVs, the greater the level of anxiety [7]. Pharmacological analysis has verified the relevance of both the PTZ and USV models to the human condition (for review, see [8–13].

The aim of the present study was to assess the time course of the anticonvulsant and anxiolytic effects of two model partial agonists of benzodiazepine receptors (BZRs) with a focus on the possible occurrence of a rebound increase in anxiety-like behavior and seizure

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susceptibility in immature rats. For this purpose we selected two substances that are well tolerated in rat pups and that have exhibited a tendency toward rebound reaction in previous studies of their anticonvulsant action: the benzodiazepine clonazepam (CZP) and the quinolizinone Ro 19-8022. Both BZR ligands have exhibited strong anticonvulsant properties in both immature and adult rats [10,14], and their anxiolytic properties were confirmed in adult rodents [15,16]. Twelve-day-old rats were chosen because their brain development corresponds to the very early postnatal stage of infants [17]. The present study was designed to answer three questions: (1) Can single drug administration elicit rebound symptoms in immature rats? (2) Are both anticonvulsant and anxiolytic effects followed by a rebound increase in seizure susceptibility and anxiety? (3) Can the selected tests be used to detect and quantify rebound symptoms in immature rats?

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats (Institute of Physiology, Academy of Sciences, Prague) 12 days old were used in the experiments. Response to PTZ-induced seizures was studied in 122 animals; another 48 rats were used for registration of USVs. Animals were housed under controlled temperature (22 ± 1 °C) and humidity (50–60%) with a 12/12 light/dark cycle (lights on at 6:00 AM). On day 1 (day of birth = day 0), the male pups were randomly fostered to control potential differences in maternal care, and each litter was adjusted to 10 animals. On the testing day, the 12-day-old rat pups were transported to the experimental room in their home cage and separated from the mother. The cage was placed on a heating pad maintained at 34 °C, that is, the nest temperature. The pups were marked, weighed, and injected with a single dose of CZP (1 mg/kg, Rivotril, Roche) or Ro 19-8022 [(*R*)-1-[(10-chloro-4-oxo-3-phenyl-4 H-benzo[a]quinolizin-1-y)carbonyl]-2-pyrrolidine-methanol, 0.5 mg/kg dissolved in saline] in a volume of 1 mL/kg. The doses were chosen to be equipotent according to our previous data on their anticonvulsant action in this age group [14,18]. Control animals received the corresponding volume of vehicle (propylene glycol, ethanol, and water in the ratio 5:2:3 was used for CZP and physiological saline for Ro-19-8022). To avoid the possible litter effect in the behavioral part of this study, one control pup and one experimental pup were assigned to a specific treatment group from each litter. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic, and the project was approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic.

2.2. Seizure susceptibility

To assess possible changes in susceptibility to seizures, animals were injected subcutaneously with PTZ (Sigma) freshly dissolved in physiological saline (100 mg/mL) at two different doses [5]. PTZ 100 mg/kg was administered 12, 24, and 48 hours after administration of the test drugs. A low dose of PTZ (50 mg/kg) was used to detect and further analyze possible rebound effects. Based on the time course profile of the anti-PTZ effects of the BZR agonists tested, the low dose of PTZ was administered 48 hours after CZP and 24 and 48 hours after Ro 19-8022 administration. Animals were individually placed in Plexiglas cages and their behavior was monitored for 30 minutes after PTZ injection by an experienced observer. During the entire period of separation from their mothers, the body temperature of pups was maintained at 34 ± 2 °C with an electric heating pad connected to a digital thermometer to compensate for the immature thermoregulation at this age [19]. The incidence and latency of two types of motor convulsions were registered: minimal seizures (mS), that is, predominantly clonic convulsions involving the head and forelimb muscles with preserved righting reflexes, and GTCS, starting with a short running phase and accompanied by a loss of righting reflexes at the beginning of the tonic phase. Other behavioral phenomena (e.g., isolated myoclonic jerks) and behavioral abnormalities were also recorded. To assess the severity of epileptic phenomena, a 5-point scale was used [20], where 0 = no changes; 0.5 = abnormal behavior (e.g., automatisms, increased orienting reaction); 1 = isolated myoclonic jerks; 2 = atypical minimal seizures, characterized mostly by shuffling of forepaws; 3 = minimal seizures; 4 = generalized seizures without the tonic phase; and 5 = complete GTCS.

Each animal was assigned a score for the most severe behavioral characteristics, and an average score was then calculated for each experimental group. Each interval group contained from 8 to 12 rat pups.

2.3. Ultrasonic vocalizations

Ultrasonic vocalizations were recorded 30 minutes and 3, 24, and 48 hours after drug administration, always for 2 minutes. After testing, the pups were returned to their dams. Testing always started between 9 AM and noon. The isolation of pups from their mothers never exceeded 30 minutes. For recording, pups were placed individually into the test cage (21 × 21 × 21 cm) with the controlled temperature (22 °C) reflecting a high stress state. Ultrasounds were recorded using a high-frequency microphone (MK 201), preamplifier (MV 102), filters (01013) and sound level meter (02022) from RFT Messelektronik Otto Schön, Germany, attached to the wall of the cage pointing downward. The distance between the microphone and the rat pup was 15 cm. Filters were set to pass frequencies over 20 kHz. The signal was fed into a custom made detector with variable high and low trigger levels, which transformed the analog signal into digital. The digital pulses were analyzed using customized software developed for our laboratory (Alcor-ESA). Artifacts (i.e., sounds produced by the pup in the frequency below 30 kHz or by scratches on the surface) were rejected by the software.

Latency to the first call, number and duration of calls, intervals between subsequent calls, and frequency (kHz) of the calls were evaluated.

2.4. Motor performance

Immediately after recording of ultrasound calls, the pups were submitted to two sensorimotor tests routinely used in our laboratory: surface righting and negative geotaxis [14,21]. First, pups were individually placed in a supine position on the laboratory desk, and the time to righting was recorded. The pups were tested for a maximum of 60 seconds. Second, pups were individually placed on an inclined (30°) surface with the head facing downward. The ability of pups to turn to 180° was recorded. The pups were tested for a maximum of 90 seconds. Each test was performed three times in close succession. The mean of the three trials was taken as the score.

Each group (CZP, tricomponent solvent, Ro 19-8022, saline) consisted of 12 animals.

2.5. Statistics

Incidence of either type of seizures was evaluated with the Fisher exact test. Latencies to seizure onset and seizure severity in the drug and appropriate control group at different time intervals were compared with an unpaired *t* test.

Motor performance data were subjected to nonparametric tests because of the presence of cutoff values. To compare the differences in motor performances between four subsequent sessions in individual groups the Friedman repeated-measures analysis of variance on ranks followed by Wilcoxon signed rank test was used. Holm's adjustment