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Research report

Gabra5-gene haplotype block associated with behavioral properties of the full agonist benzodiazepine chlordiazepoxide

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HIGHLIGHTS

- The *gabra5* gene is associated with pharmacological properties of benzodiazepines.
- It is located close to the *pink-eyed dilution* (*p*) locus on mouse chromosome 7.
- We demonstrate an haplotype block at the G*abra5* locus.
- An up-regulation of *Gabra5* mRNA in hippocampi increases chlordiazepoxide effects.
- Anxiety correlates with hippocampal *Gabra5* mRNA increase.

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ABSTRACT

The *gabra5* gene is associated with pharmacological properties (myorelaxant, amnesic, anxiolytic) of benzodiazepines. It is tightly located (0.5 cM) close to the *pink-eyed dilution* (*p*) locus which encodes for fur color on mouse chromosome 7. We tested the putative role of the *gabra5* gene in pharmacological properties of the full non specific agonist chlordiazepoxide (CDP), using behavioral and molecular approaches in mutated *p/p* mice and wild type F2 from crosses between two multiple markers inbred strain ABP/Le and C57BL/6By strain. From our results, using rotarod, light–dark box, elevated maze and radial arm maze tests, we demonstrate that *p/p* mice are more sensitive than WT to the sensory motor, anxiolytic and amnesic effect of CDP. This is associated with the presence of a haplotypic block on the murine chromosome 7 and with an up regulation of *gabra5* mRNAs in *hippocampi* of *p/p* F2 mice.
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1. Introduction

Benzodiazepines (BZs) are chemical compounds which are indicated in treatment of anxiety, insomnia, psychomotor impairment seizures, muscular spasms and alcoholic withdrawal. Their pharmacological properties are mediated via the ionotropic $GABA_A$ receptors, through which they potentiate the GABA action on its receptor [64,65,69]. $GABA_A$ receptors are pentameric ligand-gated Cl channels constituted from several distinct subunit classes (α1–6, β1–3, γ1–3, δ1–2, ε, π and ρ1–3) [17,50–52,63]. Human

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genetics studies have associated the 15q11-q13 chromosomal region with psychiatric, neurological diseases or with pharmacological BZs properties. This region is organized in cluster with three of the $GABA_A$ receptor subunit genes (*GABRB3*, *GABRA5* and *GABRG3*) [24,25]. Single nucleotide polymorphisms (SNPs) present in these genes have been associated in epilepsy susceptibility, drug resistance [37,42], susceptibility to autistic [49] or with bipolar schizoaffective disorders [26] Specifically, GABRA5 was associated with bipolar I disorder [14,49,56]. In the mouse model, four lines of $GABA_A$-receptor point mutated knock-in have attributed different diazepam pharmacological properties to different $GABA_A$-receptor isoforms. Mice carrying a knock-in point mutation in the alpha subunits of the $GABA_A$ receptor [α1 (H101R), α2 (H101R), α3 (H126R), α5 (105R)] have shown phenotypes that were associated with distinct pharmacological properties of the $GABA_A$ receptors. The sedative, ataxic and additive properties of diazepam are attributed to $GABA_A$ receptors containing α1 subunit while the anxiolytic, muscle relaxant and anti-hyperalgesic actions of the BZ are attributed to the α2/α3 subunits [15,28,36,65]. Although a recent study has demonstrated that the α5-subunit of the $GABA_A$ receptor affected locomotion and memory for location of objects [59] the implication of the *gabra5* gene in the behavioral phenotype in the mouse is far less well known than the other members the *gabra* gene family (*gabra1*, *gabra2* or *gabra3*). Here, we focused on the implication of the *gabra5* gene in pharmacological properties of chlordiazepoxide (CDP), a full nonspecific benzodiazepine agonist. For this purpose, we used two different approaches. By an inverse genetics approach, we first constructed Mendelian F2 populations of mice, in which *gabra5* gene co-segregates with a fur color marker gene, thus expressing an easily detectable phenotype (*pink-eyed dilution*, *p*). We then compared reactivity of both F2 populations (mutated *p*/*p* F2 and wild type, WT F2) in behavioral procedures which allow evaluating the sensori-motor, anxiolytic and amnesic-like effects of CDP. In the latter approach, we used techniques of molecular genetics. We first sequenced *gabra5*-cDNA, prior to secondly quantifying *gabra5*-mRNA of mutated and wild-type mice to associate data resulting from behavioral comparison with putative modifications in the cDNA and/or in *gabra5*-mRNA expression. Overall, we report that the phenotype of animals bearing the chromosomal fragment containing both *gabra5* and *p* genes, is associated with a differential sensitivity to the chlordiazepoxide BZ, and we further show that *gabra5*-mRNA is up regulated in the *hippocampus* of these mutated F2 mice.

2. Materials and methods

2.1. Animals

We used F2 populations of mice bread from two multiple markers strains, the ABP/Le (ABP) and the C57BL/6By (B6) strain. The mouse inbred strain ABP carries six homozygous recessive mutations, named after the obvious phenotypes they induce that we will call "marker": *non-agouti* (*a*, chromosome 2), *brown coat color* (*b*, chromosome 4), *waved-one* (*wa-1*, chromosome 6), *pink-eyed dilution* (*p*, chromosome 7), *short-ear* (*se*, chromosome 9) and *belted* (*bt*, chromosome 15). The mouse inbred strain B6 carries the corresponding wild-type allelic forms. By crossing the ABP and the B6 strains, the different mutations segregate in a Mendelian F2 population. Thus, we obtain *p*/*p* male mice in the F2 generation (*p*/*p* F2), which expresses solely the *p* marker and not the other ABP parental markers. The *p*/*p* F2 are easily distinguishable from the WT (?/+ F2) because of hypo-pigmentation of the eyes and fur coloring [9,11]. From the age of 4–5 weeks, mice were reared in group-housed cages (8–10 mice per cage). Following their transfer to our laboratory (Tours, France) mice were reared in a reversed light/dark cycle as soon as they arrived in the facility (the level of illumination in the colony rooms is 200 lx). A 2-week acclimation period was properly chosen in order to enable the animals to adapt to this light/dark cycle reversal. Mice were left under this new environment, a reversed 12-h day–night cycle (light on at 7 p.m.), temperature of 22 °C, relative humidity of 60% until the age of testing (i.e. mice were tested at ages between 3.5 and 4.5 months). Food and water were provided ad libitum. Before being tested, each mutated and WT animal was randomly assigned to the independent groups of treatment. Different groups of mice were run in the different behavioral paradigms. Although sex differences in anxiety-like behavior and memory are known, the scope of the study was not to focus on sex difference but on benzodiazepine sensitivity. The number of mice available when selecting the sole *p*/*p* mice from an F2 generation is very low. Consequently, it was not possible to undertake sex comparisons and we only chosen males for experiment. All experiments complied with the ethical guidelines laid down by the French Ministry of Agriculture and with the European Council Directive 86/609/EEC.

2.2. Behavioral procedures

All the behavioral procedures were done during dark phase and previously described [2,20,27]. Doses of chlordiazepoxide used in behavioral procedures were based on our previous studies [2,3,12]. Thirty minutes before testing, 10 ml/kg of chlordiazepoxide solution (CDP, hydrochloride, Sigma, was dissolved in a saline solution (9‰; control solution) and sonicated for 5 min) was injected i.p. in male WT and *p*/*p* F2 mice. Treatment with CDP 2.5, 5 or 10 mg/kg was used in elevated plus maze, radial arm maze and light–dark box. CDP 10, 15 or 20 mg/kg was used in rotarod. These doses were chosen to account for the fact that the anxiolytic and amnesic effects of CDP are known to occur at lower doses than the ones that elicit myorelaxant action. 8–10 mice per genetic groups and per CDP doses or saline were used. Each animal was used only once and were run in a only one paradigm. The behavioral measures were recorded manually using strop watches.

2.3. Chlordiazepoxide-related myorelaxant effect in rotarod

Twenty-four hours before the assessment, mice were selected for their performance on the rod (diameter 3 cm) according to the criterion corresponding to at least 60 s on the rod at 19 rpm. On the second day, 30 min after treatment (10, 15 or 20 mg/kg CDP or vehicle, i.p.), the latency of the selected mice to fall from the rod was measured (maximum 60 s).

2.4. Chlordiazepoxide-related anxiolytic effect

To test the CDP-related anxiolytic effect, mice behavior has been measured in two environments: the light–dark box and the elevated plus maze apparatus. Both measure the natural avoidance of mice either of lit places or of elevated non protected area.

The light–dark box is composed of two PVC cages (20 cm × 20 cm × 14 cm). One is darkened (the black box) and made of opaque PVC bow while the other is lit by a 500 lx illumination. The two boxes are linked with an opaque plastic tunnel (5 cm × 7 cm × 10 cm). Mice are placed individually in the lit box. The test starts when they enter in the dark box. The time spent in the lit box (TLB) and the number of transitions between the two boxes was recorded automatically during a 5-min period. A mouse that has entered the four paws in the new box was considered as having changed boxes.

The *elevated plus maze* consists in a polyvinyl chloride plus-cross shaped ivory plastic arms (27 cm × 5 cm, 38.5 cm above the floor) originating from a central platform (5 cm × 5 cm). Two opposite arms are enclosed by 15 cm high plastic walls and the two others are open. Testing is performed during the dark phase, under a 350 lx-illumination (lights above the end of the open arms). To start the 5-min session, mice were individually introduced at the center, the head facing an open arm. Behaviors were video-monitored and at the end of the 5-min test, mice were returned to their home cages. A mouse was considered to enter a new arm when it introduced its four paws in the arm. We recorded total activity (number of entries into open arms plus number of entries into closed arms of the maze and calculated the percentage of time spent onto open arm entries (open/total entries). Maze was rinsed between sessions with alcohol and dried with paper towel.

2.5. Chlordiazepoxide-related amnesic effect

The *eight-arm radial-maze* was elevated 110 cm above the floor and consisted of a central circular platform measuring 30 cm in diameter. Eight open arms (48 cm long, 5 cm wide) radiated outwards at equal distances and 1 cm high sides surrounding each arm. Each arm formed a corridor leading to an 8-cm square platform. A 1 cm-diameter cup, is embedded in each platform and contains a hidden 10 mg noodle reward.

No confinement was used between each arm choice. This allows the mouse to move freely in the maze. It also enables the use of odor trails as well as spatial cues or algorithmic strategies in order to perform the task. The maze was placed in small air-conditioned room surrounded by visual cues. The maze was always oriented in space in the same way. It was placed on the floor of a spatially rich structured room. Four large black, white, or black and white striped patterns were hung, one on each of the four walls, to provide particularly salient visual spatial cues. Mice were progressively food deprived so that weight loss reached 85% of initial body weight by the start of testing.

2.5.1. Procedure

Pre-training session: Mice were first given two pre-training sessions at 24 h intervals. They were placed in groups of four on the maze for 20 min per session and could freely explore the eight arms, which were provided with abundant food.

Training session: Following pre-training, mice were then given five training sessions, at 24 h intervals, so as to reach a good level of performance, i.e. three errors or

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