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Differential behavioral profile induced by the injection of dipotassium chlorazepate within brain areas that project to the nucleus accumbens septi

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Abstract:
Background: The effect of the agonism on γ-aminobutyric acid (GABA) receptors was studied within medial prefrontal cortex (mPFC), amygdala (AMY) and ventral hipocampus (VH) in the plus-maze test in male rats bilaterally cannulated. These structures send glutamatergic projections to the nucleus accumbens septi (NAS), in which interaction and integration between these afferent pathways has been described. In a previous study of our group, blockade of glutamatergic transmission within NAS induced an anxiolytic like effect.
Methods: Three rat groups received either saline or dipotassium chlorazepate (1 or 2 μg/1 μl solution) 15 min before testing. Time spent in the open arms (TSOA), time per entry (TPE), extreme arrivals (EA), open and closed arms entries (OAE, CAE) and relationship between open- and closed-arms quotient (OCAQ) were recorded.
Results: In the AMY injected group TSOA, OAE and EA were increased by the higher doses of dipotassium chlorazepate ($p < 0.01$). In the mPFC, TPE was decreased by both doses ($p < 0.05$). Injection within ventral hippocampus (VH) decreased TSOA, OAE and OCAQ with lower doses ($p < 0.05$). When the three studied saline groups were compared, TSOA, OAE, EA and OCAQ were enhanced in the VH group when compared to mPFC and AMY ($p < 0.001$). Insertion of inner canula ($p < 0.001$, $p < 0.01$, $p < 0.01$) and saline injection showed an increasing significant difference ($p < 0.001$ in all cases) with the action of guide cannula alone within VH in TSOA, OAE and EA.
Conclusion: We conclude that the injection of dipotassium chlorazepate has a differential effect depending of the brain area, leading to facilitatory and inhibitory effects on anxiety processing.

Key words:
GABA, amygdala, medial prefrontal cortex, ventral hippocampus, nucleus accumbens septi, anxiety, plus-maze, schizophrenia

* Equal contribution

Introduction

Amygdala (AMY) has been classically related to anxiety [26, 42, 58]. Its role has been highlighted in the response to acute [56] as well as chronic [59] stress. However, some additional structures and systems appear to modulate or participate in anxiety processes [10, 55, 58]. It is the case of nucleus accumbens septi (NAS) that has been involved in anxiety processes by us and another group [38, 44, 45]. Glutamatergic transmission within NAS appears to be a key to understand this modulation [44, 45]. It has been postulated that hippocampus (Hip) exerts a facilitatory and modulatory effect on NAS afferents from AMY and prefrontal cortex (PFC) that converges in NAS sending glutamatergic projections [28, 29]. Ventral hippocampus (VH) has been pointed as the involved substructure [5–7]. It is relevant to explain the action of VH gating prefrontal impulses on NAS, leading or not to goal directed behaviors, but also to explain emotional driving, disrupted in schizophrenia [28]. A similar role could be supposed in the case of PFC since recently, it has been observed that under special conditions PFC could also drive sustained up states in NAS [33].

In humans, an important number of recent studies have focused on several structures involved in a wide range of anxiety disorders [9, 60, 63], even in structures classically linked to cognitive functions, like PFC [1]. In this way, recent neuroimage techniques have implicated some NAS projecting structures, like AMY, medial prefrontal cortex (mPFC), and Hip in post traumatic stress disorder (PTSD) as related brain substrates [60]. Interestingly, these structures exert interactions within the NAS, and they have been studied in schizophrenia models [28, 29]. Recent findings appear to show that these interactions have an important role in various pathologies, and may be they could explain some phenomenon in normal anxiety processing.

A correlation has been proposed between severity in PTSD and AMY responsiveness, and during symptomatic states it appears to be very clear. The processing of trauma-unrelated affective information appears to produce also relevant modifications. The mPFC influence appears to be less important, and its responsiveness lowered during symptomatic states [60]. These evidences could be suggesting an interactive balance between both structures that has been reported in previous electrophysiological studies [28, 29, 49–52]. Furthermore, mPFC hypofunction has been proposed as underlying difficulties in emotional cognitive tasks performance, and it has been correlated to anatomic and functional Hip disorders in PTSD patients [60]. Other disorders in emotional cues management are present in schizophrenia, and a Hip dysfunction has been proposed as its cause [28, 29, 51].

Acquisition and extinction have been widely studied in animal models leading to some interesting findings in anxiety therapy starting from conditioned fear. Neuroimaging studies during exposition to emotional ambiguous stimuli have claimed the attention about the relevance of an interaction between AMY and PFC functioning as a whole circuitry, leading to new possible treatment schedules [9]. The perception and the encoding of arousing material appear to activate some parts of the PFC that could be exerting a modulating function, suggesting its possible role under stress conditions and mainly during retrieval [63]. Furthermore, an increased activity of both AMY and Hip has been reported in situations of encoding and consolidation of highly emotive, dangerous or stressor situations, suggesting the relevance of these memory related structures in these kinds of events [63]. In the same way, stress mediators, like corticotrophin releasing hormone (CRH), that have a significant anxiogenic effect when were injected to rats [22], exert their anxiogenic action within AMY [20]. Anxiety could disrupt attention and perception, mainly registering potential indicators of risk or danger, leading to misunderstandings in the interpretation of expressions, suggesting its influence in cognitive processes [9].

Present study aims to a comprehensive approach of brain dynamics in anxiety situations. Additionally, it is oriented to understand how the benzodiazepines, acting in some brain areas, exert different effects that are overlapped in the systemic use of these drugs. It has been proposed that philogenetically higher structures appear to have different sensitivity and response to depressor drugs. The purpose of the present study is to delimitate the specific behavioral patterns induced by the action of benzodiazepines injection in different brain nuclei related to anxiety.

Materials and Methods

Animals

Male rats from a Holtzman-derived colony aged 90 days and weighing 240–290 g were used (n = 90).

They were maintained under controlled temperature conditions (22–24°C) and lighting (lights on from 05.00 to 19.00 h). All tests were conducted under the light cycle. Standard rat chow and water were freely available.

Bioethical considerations

The animal's housing and experimental procedures were carried on following project approval criteria of the National University of Cuyo, accordingly to the guidelines set by European Community Council (Directive 86/609/EEC), to the bioethical rules established by the Faculty of Medicine of the National University of Cuyo, and to Argentine law.

Surgery

Animals were anesthetized with ether (Andes Laboratories, Mendoza, Argentina) and stereotaxically implanted with bilateral stainless steel cannulae into the Amy, the mPFC and the VH. The cannulae were double barreled and the set was composed of an outer guiding cannula stainless steel tubing (23 gauge, 15 mm in length), provided with a removable stylet (30 gauge, 15 mm in length) to avoid its obstruction. Cannulae were fixed to the skull using a screw and dental acrylic (Subiton, Argentina), as in previous studies [4, 42, 44, 45]. Coordinates for Amy in respect to bregma were: A = –1 mm; L = ± 4.5 mm; V = –6.7 mm; for mPFC were: A = + 4 mm; L = ± 1 mm; V = –2.5 mm; for VH were: A = –3.6 mm; L = ± 4.5 mm; V = –4 mm. After surgery, rats were housed individually and maintained undisturbed for a week recovery period.

Apparatus

The plus-maze was made of wood and consisted of two open arms, 50 × 10 cm (length per wide), and two enclosed arms 50 × 10 × 50 cm (length per wide per height), arranged such that the two arms of each type were opposite to each other. The maze was elevated to a height of 50 cm. The room was illuminated by a 60 W bulb 1.5 m above the apparatus.

Drug

Dipotassium chlorazepate for use in humans was used (kindly provided by El Puente Pharmacy, Mendoza, Argentina). This benzodiazepine, a dipotassium salt, was elected due to its water and saline solution solubility.

Experimental procedure and treatment

Animals were injected under gentle manual restraint 15 min before testing. A 30 gauge, 17 mm long stainless steel injection cannula (dimensioned to precisely reach the goal area) attached to a 10 µl microsyringe (Hamilton) was introduced into the guide cannula. Volumes of 1 µl solution were gradually injected over 1 min periods into both the left and right brain structure. Volume was designed aiming to reach the whole studied structure. The injection cannulae were left in place for an additional 1 min to allow for diffusion. The rats were placed individually in the center of the plus-maze apparatus, facing to the open arm, and allowed 5 min for free exploration. All the experiments were carried out between 08.00 and 12.00 h (light cycle).

According to previous works, we measured the time spent in the open arms (TSOA), time per entry (TPE, quotient between time spent in the open arms divided by the number of entries to open arms) [13, 38, 42, 44, 45], open and closed arms entries (OAE and CAE, respectively [19]; arm entry defined as all four paws into an arm), relationship between open and closed arms entries (OCAQ, open/closed arms quotient), and extreme arrivals (EA, defined as number of times the rat reaches the end of an open arms).

Experiment 1

Rats were bilaterally injected under gentle manual restraint bilaterally in AMY with either saline (1 µl) or dipotassium chlorazepate (1 and 2 µg/1 µl) 15 min before testing. According to previous studies [13, 24, 42, 44], we measured TSOA, TPE, OAE, CAE, OCAQ and EA.

Experiment 2

Rats were injected bilaterally in mPFC with either saline (1 µl) or dipotassium chlorazepate (1 and 2 µg/1 µl) 15 min before test, and the experiment followed the same routine that experiment 1.

Experiment 3

Rats were injected bilaterally in Hip with either saline (1 µl) or dipotassium chlorazepate (1 and 2 µg/1 µl) 15 min before the test, with the same procedure as in previous experiments.

Experiment 4

The saline groups of previous experiments were statistically compared, aiming to verify or exclude possible differences between controls, and search for its corresponding meaning.

Experiment 5

Rats bilaterally implanted in VH were systematically studied, in three different conditions. In the first case, a condition of guide cannula chronic placement with injection-like handling; in the second case, a guide cannula chronic placement was performed, with acute insertion of the injection cannula, and in the third case, the guide cannula chronic placement with acute placement of injection cannula condition was followed by saline injection (1 µl). All procedures were carried on 15 min before test.

Histological analysis

After the experiments, rats were euthanized with overdose of ether and injected with saturated methylene blue solution (1 µl) through the cannula. Brains were removed from the skull and fixed in 20% formalin solution. The fixed brains were sectioned and examined with a 10× magnifying lens and the sections containing the injection sites were saved. Microscopic inspection of these sections served to ascertain the location of the cannula that was transferred to standard sections taken from a brain atlas [53]. We only report statistics data for those rats with correct placements of cannulae. The compromise of AMY (experiment 1, Fig. 1), PFC (experiment 2, Fig. 3), and VH (experiments 3 and 5, Fig. 5) in the diffusion area of the methylene blue solution diffusion was checked in all cases.

Statistical data analysis

The Kolmogorov Smirnov test was used to ascertain parametric distribution of data. One way ANOVA followed by Dunnett's *post-hoc* test was used. In all cases, a $p < 0.05$ (two tailed) was considered significant. The results are reported as the means ± standard error of the mean (SEM, n = 14–20 for each group).

Results

Experiment 1

When saline or dipotassium chlorazepate were injected within AMY (Fig. 1), TSOA was modified by treatment [$F(2, 47) = 5.085$, $p = 0.0100$], showing a significant increase with the higher dose of dipotassium chlorazepate (2 µg/1 µl, $p < 0.01$, Fig. 2, top left). OAE were also modified by treatment [$F(2, 47) = 5.508$, $p = 0.0071$], and increased by the higher dose of dipotassium chlorazepate (2 µg/1 µl, $p < 0.01$, Fig. 2, top right). A modification by treatment was also observed in the case of EA [$F(2, 47) = 4.862$, $p = 0.0120$], and a significant increase was observed also with the higher dipotassium chlorazepate dose (2 µg/1 µl, $p < 0.01$, Fig. 2, bottom).

Experiment 2

When saline or dipotassium chlorazepate were injected within mPFC (Fig. 3), only TPE was modified by the treatment [$F(2, 42) = 4.179$; $p = 0.0221$], with a significant decrease induced by both doses of the drug (1 and 2 µg/1 µl, $p < 0.05$, Fig. 4).

Experiment 3

When saline or dipotassium chlorazepate were injected within VH (Fig. 5), TSOA was modified by treatment [$F(2, 43) = 3.777$; $p = 0.0308$], and decreased by the lower dipotassium chlorazepate dose (1 µg/1 µl, $p < 0.05$, Fig. 6, top left). OAE were modified by treatment [$F(2, 43) = 3.580$; $p = 0.0365$] and also decreased by the lower dose (1 µg/1 µl, $p < 0.05$, Fig. 6, top right). OCAQ was modified by treatment [$F(2, 43) = 4.252$; $p = 0.0206$] and, again, decreased only by the lower dose (1 µg/1 µl, $p < 0.05$, Fig. 6, bottom).

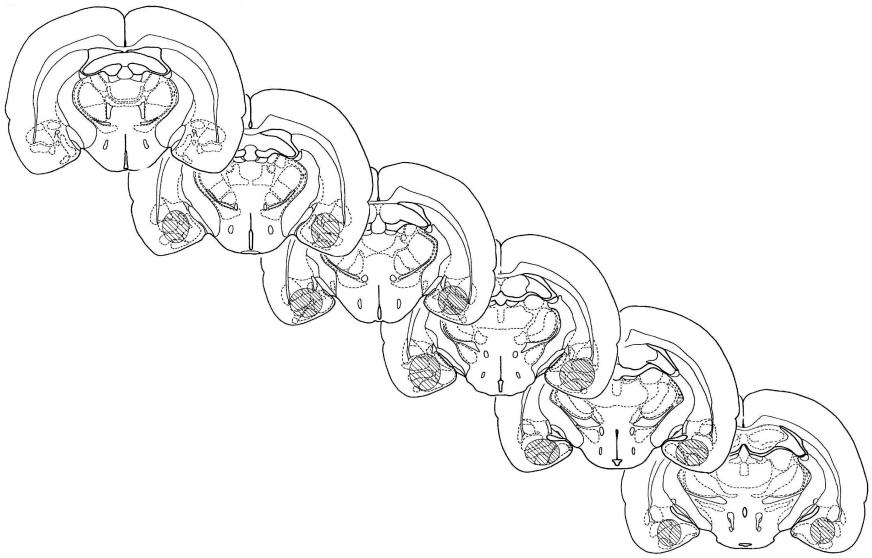

Fig. 1. Schematic representation of histology of rats used in the plus maze test injected within AMY. Frontal brain sections are showing the location of the injection site in a schematic representation of AMY diffusion area [51]

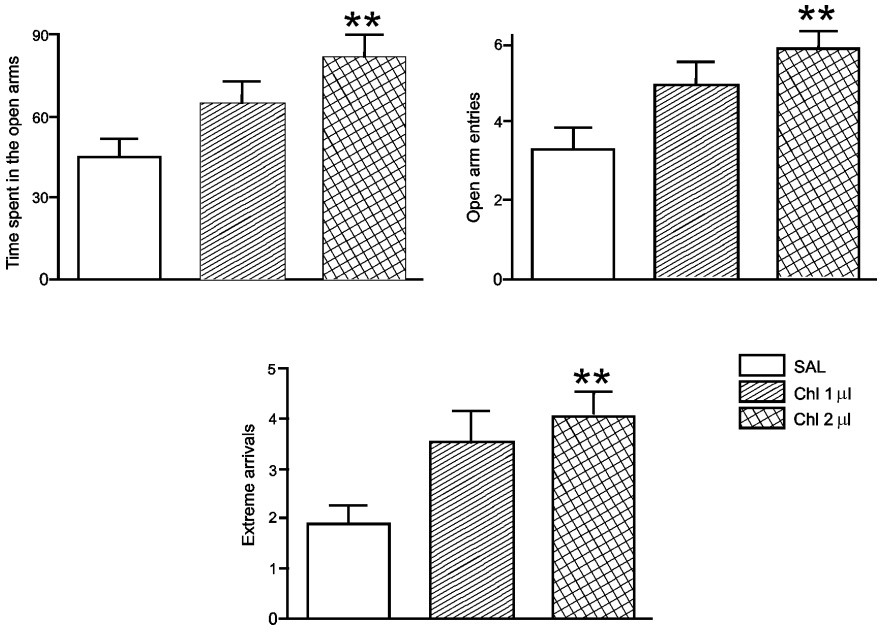

Fig. 2. Behavioral profile displayed by rats injected into the AMY with saline (1 µl), or dipotassium chlorazepate (Chl, 1.0 and 2.0 µg/1 µl). On the top left, time spent in the open arms (TSOA); top right, open arms entries (OAE); bottom left, extreme arrivals (EA). Results are reported as the mean ± standard error of the mean (SEM) (n = 15–20 rats, ** $p < 0.01$)

Experiment 4

When a comparison was made between saline groups of all the areas here studied (mPFC, AMY, HV), TSOA [$F(2, 41) = 25.01$; $p < 0.0001$, Fig. 7, top left], OAE [$F(2, 41) = 19.26$; $p < 0.0001$, Fig. 7, top right], EA [$F(2, 41 = 16.28$; $p < 0.0001$, Fig. 7, bottom left], and OCAQ [$F(2, 41) = 11.82$; $p < 0.0001$, Fig. 7, bottom right] were modified by treatment, with highly significant increases for VH injections ($p < 0.001$ for all cases).

Experiment 5

When a comparison was made within the VH between implanted cannula, inner injection cannula placement and saline injection, TSOA was modified by the treatment [$F(2, 35) = 11.14$; $p = 0.0002$], showing an increase induced by the mere acute introduction of this inner cannula but also by acute saline injection through the inner cannula ($p < 0.001$ for both groups, Fig. 8, top left). OAE were modified by the treatment

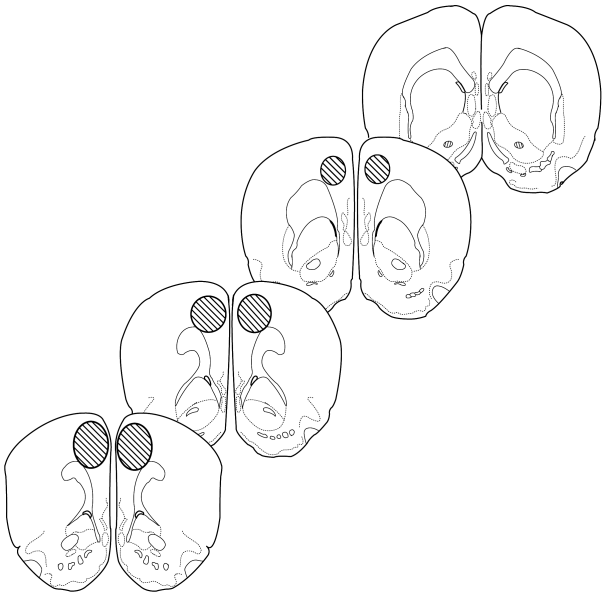

Fig. 3. Schematic representation of histology of rats used in the plus maze test injected within medial prefrontal cortex (mPFC). Frontal brain sections are showing the location of the injection site in a schematic representation of diffusion area [51]

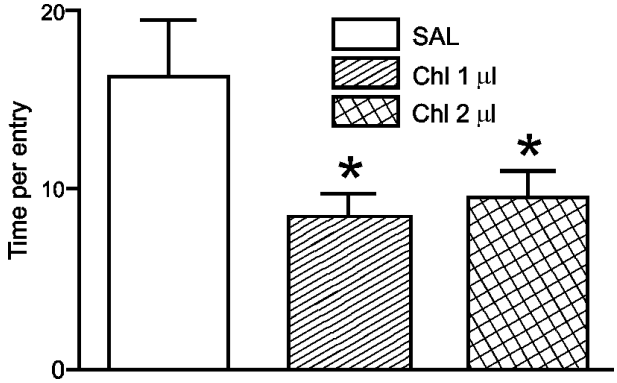

Fig. 4. Time per entry (TPE) of rats injected into the medial prefrontal cortex (mPFC) with saline (1 µl), or dipotassium chlorazepate (Chl, 1.0 and 2.0 µg/1 µl). Results are reported as the mean ± standard error of the mean (SEM) (n = 14–16 rats, * $p < 0.05$)

[F (2, 35) = 10.32; p = 0.0003], and increased by the location of the inner cannula and the saline injection ($p < 0.01$ and $p < 0.001$, respectively, Fig. 8, top right). EA were modified by the treatment [F (2, 35) = 9.538; p = 0.0005] and increased again by the acute location of the inner cannula and acute saline injection ($p < 0.01$ and $p < 0.001$, respectively, Fig. 8, bottom).

Discussion

In the present study, we observed that in the first experiment an increase in several parameters was observed when dipotassium chlorazepate was injected within AMY (Fig. 2). In this way, TSOA, OAE and EA reached clear levels of statistical significance ($p < 0.01$ for all). The effect was mainly observed with the higher dose, but not with the lower one, even when a dose response relation was here observed. The fact that TSOA was increased, but not the TPE could be considered related to motor variables, since the increase in the time could be more related to an increase in open arm visits than an increase in the time employed in each visit. The fact that also OAE and EA were increased with the higher dose could allow us to think that AMY benzodiazepine effect could be more related to a disinhibitory action than typical and specific anxiolytic effect. This precise effect has been postulated to be related to an increase in TPE [13, 38, 42, 44]. However, an increase in locomotor activity as an anxiolytic collateral parameter could not be ruled out. The fact that a dose response curve was not present could allow to suppose that here AMY is acting in an "all or nothing" manner. It may be that the whole AMY could be acting through its action on NAS, in which we have observed actually an increase in TPE [42, 44], as we have also observed injecting intra ventricular GABAergic agonist compounds [38]. These NAS afferences appear to integrate the affective facilitation, given by AMY, with the goal directed motor plans, given by the PFC, and the contextual constraints, integrated by Hip [28, 50]. A disturbance in these circuitries has been postulated as the neural substrate of schizophrenic disorders by previous studies [28] and recent findings of our group [4].

In the second experiment, the benzodiazepine injection within mPFC decreases only TPE (Fig. 4, $p < 0.05$ for both doses). The fact that both doses clearly modified this parameter could be an argument about the higher sensibility of this zone to the depressive benzodiazepines action. Following the same previously mentioned schedule [28], the multiple motor plans given by the prefrontal cortex, and here interfered by benzodiazepines, could explain the decrease of the TPE, as a stereotyped behavior without permanence guided for search finality. However, an additional explanation regarding anxiety increase cannot be ruled out. Recently, a mechanism of prefrontal

down regulation of the AMY has been proposed. In this way, GABAergic intercalated cells (ITC) are activated through glutamatergic projections from mPFC, and these ITC inhibit the central medial nucleus of the AMY [9, 54]. An alternative proposed pathway involves glutamatergic projections from mPFC to basolateral AMY exciting GABAergic interneurons [9, 30]. In present results, since a stimulation of mPFC has a decremental effect on central AMY, a decrease of mPFC influence induced by GABAergic depression could be reflected by an increase of AMY excitatory influences. By this way, intra-accumbens and extra-accumbens pathways would be involved in this interaction.

In the case of the injection within VH (Fig. 6), some unexpected results were found. Firstly, it was shown the high level of TSOA of the saline group. This fact led us to additional experiments here described, i.e., the saline groups comparison and the cannulae and saline injection *per se*, without any drug. Curiously, starting from these elevated saline basal levels, the effect was the inverse than it could be expected in other nuclei, such as AMY. A significant decrease was observed with the lower dose ($p < 0.05$), with an important tendency with the higher dose, that was not, actually, different from the other. This effect appears to be symmetrically inversed when compared to the finding obtained with AMY injections, and par-

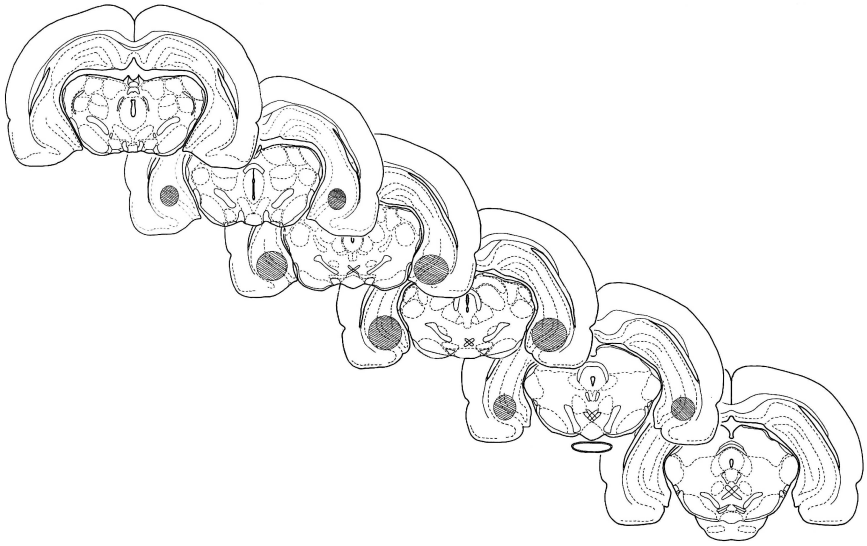

Fig. 5. Schematic representation of histology of rats used in the plus maze test injected within ventral hippocampus (VH). Frontal brain sections are showing the location of the injection site in a schematic representation of diffusion area [51]

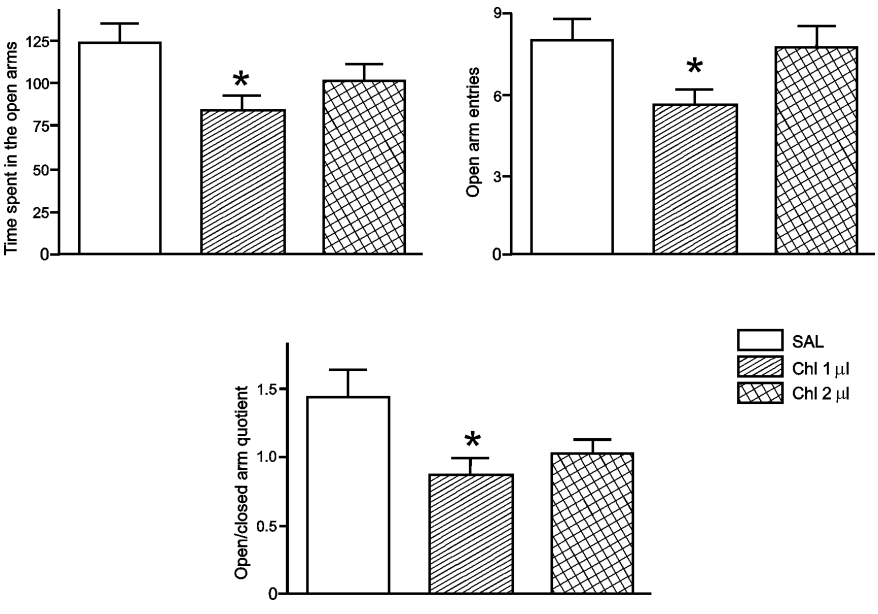

Fig. 6. Time spent in the open arms (TSOA), open arms entries (OAE) and open/closed arms quotient (OCAQ) of rats injected into the ventral hippocampus (VH) with saline (1 µl), or dipotassium chlorazepate (Chl, 1.0 and 2.0 µg/1 µl). Results are reported as the mean ± standard error of the mean (SEM) (n = 14–16 rats, * $p < 0.05$)

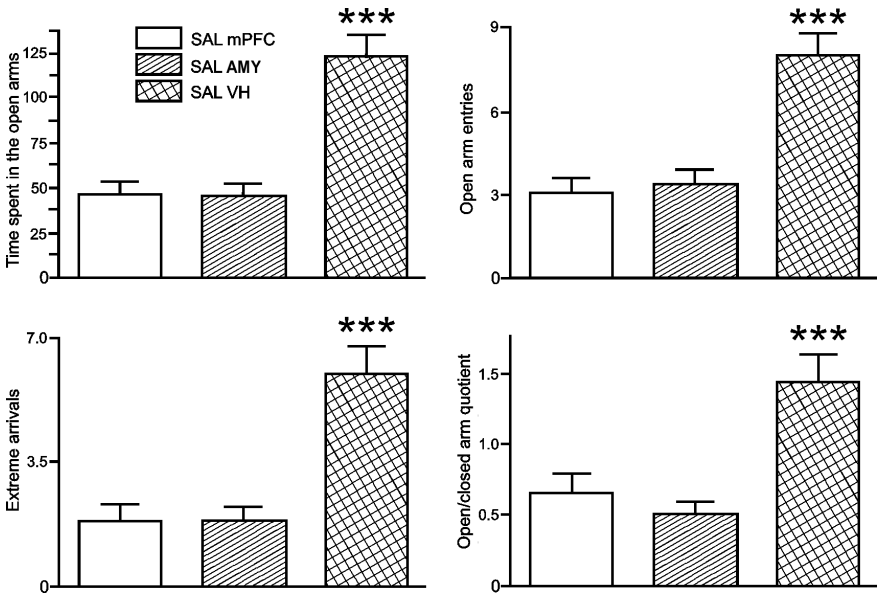

Fig. 7. Comparison of the effects induced by saline injections within mPFC, AMY and VH. Time spent in the open arms (TSOA), open arms entries (OAE), extreme arrivals (EA) and open/closed arms quotient (OCAQ) of rats injected with saline (saline groups, 1 µl). Results are reported as the mean ± standard error of the mean (SEM) (n = 14–16 rats, *** $p < 0.001$)

allel to those observed in the case of mPFC. The synergic activity of Hip and PFC has been well described in some conditions [28, 29]. The decrease in OAE and OCAQ appears to run in the same way in the case of both structures. The first parameter could be indicating inhibitory effects and the second an aversive effect of open arms. This effects could be interfering AMY projections to NAS [28, 29], that lead to a decrease in these parameters.

As a fourth experiment (Fig. 7), saline groups differences of the previous three experiments were compared aiming to verify if they were or were not significant, taking into account high values observed in saline group in experiment 3 (TSOA, OAE and OCAQ). Interestingly, saline injection into the VH led us to observe that TSOA, OAE, EA and OCAQ were clearly increased by VH saline injections when compared to AMY and mPFC ($p < 0.001$ for all cases, Fig. 7). This very clear effect appears to indicate a high susceptibility of VH to the effectuated manipulations. Curiously, in the previous experiment, three of the four parameters here studied showed a lowering response to benzodiazepines within VH, starting from high saline injection values when compared to other

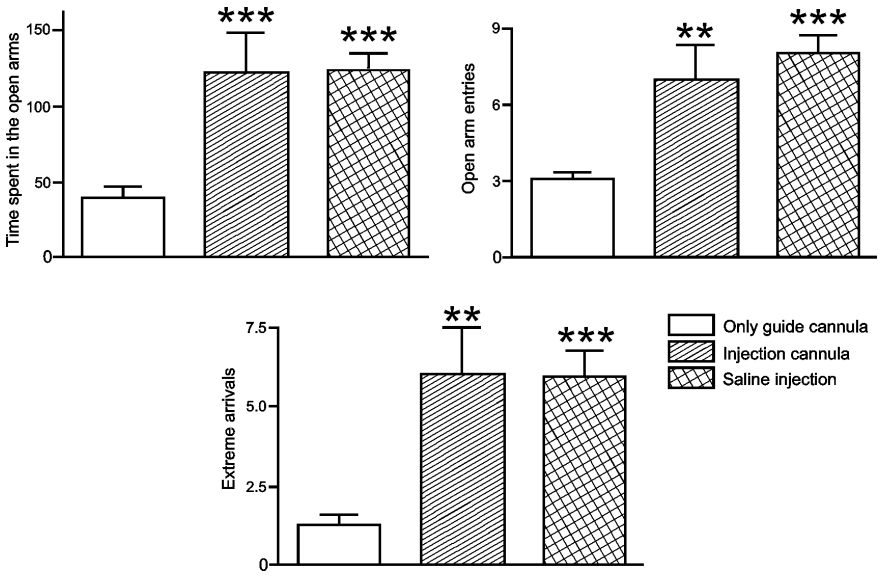

Fig. 8. Time spent in the open arms, open arms entries and extreme arrivals of rats with cannula, no injection, with cannulae implanted within ventral hippocampus (Hip, only guide cannulae) but not injected, and rats injected within Hip with saline injection (1 µl). Results are reported as the mean ± standard error of the mean (SEM) (n = 14–16 rats, ** $p < 0.01$, *** $p < 0.001$)

areas. It is the case of TSOA, OAE and the OCAQ (Fig. 6). These parameters appear to be selectively influenced by VH treatments. In this fourth experiment, the high values obtained in TSOA, OAE, EA and OCAQ could be interpreted as the behavioral effect of an increase in VH inputs into NAS, with an anxiolytic-like manifestation. In this case, VH afferent projections to NAS could be blocking anxiogenic inputs, such as those coming from AMY. Aiming to see if this anxiolytic-like effect could be attributed to acute VH tissue irritation, an additional experiment was designed, comparing the chronic cannulae placement with two potential irritation treatments: acute injection cannulae placement and acute injection cannulae placement followed by saline injection. For this reason, the fifth experiment was designed.

In the fifth experiment, a comparison was made between an implanted cannula group, an implanted cannula group with acute inner cannula placement, and an implanted cannula with acute saline injection within the VH through an inner cannula placement (Fig. 8), aiming to see if acute tissue irritation has an effect on anxiety. By this way, TSOA, OAE, EA and OCAQ were increased in a highly significant manner by the introduction of this inner cannula but also by saline injection through the inner cannula (Fig. 8). By this manner, results are congruent with an anxiolytic-like effect due to acute VH tissue irritation. The decrease in TSOA, OAE, and OCAQ in the third experiment could be explained, facing to these last evidences, as a decrease of anxiolytic like effect due to VH irritation, because of the inhibitory effects that dipotassium chlorazepate exerts within VH tissue.

Finally, it is interesting to remark that closed arms entries, classically linked to an increase in locomotor activity [19], were not modified by any treatment in all these experiments, suggesting a specific effect not strictly related to motor variables. The difference between anxiolytic effect and unspecific actions has been signaled. In some animal models this property has been designed as "behavioral disinhibition" [41]. Anxiolytic treatments appear to facilitate this effect in some models, and it could be considered as a collateral effect of them. In the mice, chlordiazepoxide and diazepam have an effect in exploration in different tests, like hole-board, and in two-chambered apparatus; furthermore, they exert a disinhibitory action on social interaction in aversive conditions (high light) and another anxiety tests [14]. In our present conditions, these effects have not masked our results, since the main locomotor variable, the closed arm entries, was not here influenced. When both variables (entries to the open and closed arms) were considered together there was no significant difference between groups injected with saline or benzodiazepine (data not shown).

Globally considered (see Tab. 1), all these findings could be pointing that irritation of VH could be producing a discharge increase that leads to a decrease in AMY influences on NAS. In the same way, VH inhibition by benzodiazepine leads to a decrease in most of the parameters studied, and it may be mediated by a prevalence of anxiogenic-like AMY inputs on NAS, like in mPFC inhibition, considering that facilitator effect of NAS-Hip pathways on frontocortical inputs has been described [28, 29, 49, 50, 52]. In the inverse way, the AMY blockade led to increases like those observed when the VH was stimulated by inner cannula and saline injection, suggesting the same interactions previously described [28, 29, 49, 50, 52].

As it was previously said, NAS has been early involved in anxiety processes by us and another group [37, 44, 45], and after it, an important number of laboratories are showing additional evidences on the role of NAS in anxiety, citing our findings [12, 15, 16, 43, 65]. All these studies give support to a previous study using immunohistochemical staining for fos-like activity, mapping functional activation of discrete brain areas induced by anxiogenic situations. In this previous study in rats, more stressing situations (plus maze and footshocks) activated PFC, AMY and NAS, and lower stressors (air puff) did not activate NAS fos-like activity, suggesting a necessary degree of stress to activate it [18]. The interactions between limbic and striatal structures has been described and mainly related to pathways from AMY to NAS [11]. However, as it was previously suggested [9], other pathways connecting the involved areas could be acting in these processes [8, 35, 36] and cannot be excluded. An anxiolytic effect mediated by glutamatergic projections from PFC to AMY stimulating accumbal GABAergic neurons has also been described [30, 34, 35]. The effect of PFC inhibition induced here by benzodiazepine injection could explain the anxiogenic-like effect observed, with a decrease in time per entry. Furthermore, short circuitries could be involved within the studied structures, like those described within AMY for CRH pathways [20].

Another relevant point to be considered is the fact that in present study an important volume (1 μl) was

Tab. 1. Schematic results presentation: Time spent in the open arms (TSOA), time per entry (TPE), open arm entries (OAE), closed arm entries (CAE), open/closed arm entries (OCAQ) and extreme arrivals (EA). All parameters studied are here considered, and the arrow number is indicating significance levels obtained in the experiments compared to control (correspondence of one arrow by one asterisk; ↑ $p < 0.05$, ↑↑ $p < 0.01$, ↑↑↑ $p < 0.001$). Direction of the arrow is indicating if the values were increasing (↑) or decreasing (↓) when compared to respective controls. The negative sign is indicating absence of significance

Studied parameters	Experiment 1 ChI AMY		Experiment 2 ChI mPFC		Experiment 3 ChI VH		Experiment 4 Saline groups		Experiment 5 Inj. cann. and saline	
TSOA	–	↑↑	–	–	↓	–	–	↑↑↑	↑↑↑	↑↑↑
TPE	–	–	↓	↓	–	–	–	–	–	–
OAE	–	↑↑	–	–	↓	–	–	↑↑↑	↑↑	↑↑↑
CAE	–	–	–	–	–	–	–	–	–	–
OCAQ	–	–	–	–	↓	–	–	↑↑↑	–	–
EA	–	↑↑	–	–	–	–	–	↑↑↑	↑↑	↑↑↑

used, aiming to reach the whole structures. This volume allows us to have clear and predictable results, mainly in AMY and PFC. Several previous studies have signaled the role of discrete nucleus within the brain zones here involved. It is the case of AMY [46, 47, 57, 61, 62] and PFC [35, 39]. In future studies, the role of these nuclei would be studied. In the present paper our intention is to delimitate the effects exerted by BDZ in the whole structures.

All these present evidences allow us to think that present findings could be correlated with previously described circuitries interactions, like those described for AMY and PFC circuitries [9], AMY and NAS [11], and AMY, PFC, Hip and NAS [28, 29, 49, 50, 52]. Like it was previously said, an activation of AMY, Hip and some zones of PFC has been correlated with stress arousal and its correlative anxiety [63]. Recent conceptualizations consider previously postulated hierarchical structure in a different way that conceived by Jackson following levels of evolution of the nervous system, in which each level has complete somatic representation. New higher levels do not keep down lower ones. Today, it is considered that new levels are integrated in a schedule in which inputs are processed in distinct processing channels [17, 64]. Furthermore, outputs of different brain levels, such as FC, AMY and Hip are integrated within NAS as different and integrating inputs, and coordinating complex functions [28], such as those involved in anxiety. These findings give additional support to the idea that NAS is not only related to reward, but also to responses to processing of environmental stimulus, such as pleasant and unpleasant stimuli, as it has been reported in humans using functional magnetic resonance images (fMRI) [40]. Animal and human studies appear to support the idea that this nucleus is related to complex and sophisticated functions, explaining its importance and role in human illness. However, the involvement of additional circuitries cannot be ruled out.

Clinically, present basic findings could lead to a better understanding of benzodiazepines therapeutic and paradoxical effects, such as behavioral disinhibition and hostility rage reactions. These collateral effects have been previously reported in some studies a long time ago [3, 27, 48], and some authors attributed them to several factors such as dosage, time administration, psychopathological and medical condition and individual variables [3, 48]. The inhibition prevalence on different structures here studied could lead to anxiogenic or anxiolytic effects induced by benzodiazepines, as it was observed in present results.

Additionally, present findings could give an interpretation about some phenomena present in schizophrenia, in which these circuitries are dysfunctioned [25, 28, 29]. It has been described an emotional driving disturbance in schizophrenia [28], and it has been attributed to a Hip failure to gate PFC inputs to NAS, leading to an AMY inputs prevalence [28]. We have previously observed that a glutamatergic blockade decrease anxiety. We have also suggested that this effect in anxiety could be interpreted as a homologous sign of schizophrenic affective flattening, since a decrease in anxiety could be considered as a certain level of indifference coping by an anxiogenic stimulus [44]. We have induced some schizophrenic homologous signs

in animals, suggesting that a glutamatergic dysfunction within NAS is related to positive [2, 21, 23–25, 45] and negative [23, 24, 44] symptoms, and also, to working memory schizophrenic dysfunction [4]. These later findings are in accordance with the fact that NAS is considered today a switchboard for goal-directed behaviors [32]. Pharmacological glutamatergic NAS blockade induced an increase in PFC activity [4], in accordance to other evidences showing that a disinhibited prefrontal cortex impairs cognitive flexibility [31]. Present results give a wider view about these facts, studying separately the effects of projecting structures to NAS in anxiety levels. In this task in the elevated plus maze, anxiety levels and exploratory strategies are present and playing reciprocally in the same instance.

We conclude that the injection of dipotassium chlorazepate has a differential profile depending of the brain area in which it is acting, leading to anxiolytic effects (mediated by its injection within AMY) or anxiogenic effects (mediated by its administration within PFC or Hip). Anxiogenic effects could mediate paradoxical effects induced by benzodiazepines. In this way, facilitative and inhibitory effect on anxiety processing appears to depend on benzodiazepines action in specific brain areas.

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