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# Effect of repeated restraint stress and clomipramine on $Na^+/K^+$-ATPase activity and behavior in rats

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ABSTRACT

Activation of the limbic-hypothalamic-pituitary-adrenal axis (LHPA) and the release of glucocorticoids are fundamental for the adaptive response and immediate survival of an organism in reaction to acute stimuli. However, high levels of glucocorticoids in the brain may produce neuronal injury and a decrease of $Na^+/K^+$-ATPase activity, with effects on neurotransmitter signaling, neural activity, as well as the whole animal behavior. Clomipramine is a tricyclic antidepressant that inhibits the reuptake of serotonin and norepinephrine by indirect actions on the dopaminergic system and LHPA axis. Its chronic use increases the body's ability to cope with stress; however, high doses can potentiate its side effects on memory, learning, and sensory motor function. The purpose of the present study was to compare the effect of repeated restraint stress and clomipramine treatment on $Na^+/K^+$-ATPase activity and on the behavior of male rats. Changes in the behavioral response were evaluated by measuring the memory, learning, anxiety, and exploratory responses. Our results showed that exposure to repeated restraint stress reduced levels of $Na^+/K^+$-ATPase in brain structures and changed short and long-term memory, learning, and exploratory response when compared to the control group. Exposure to clomipramine treatment increased anxiety levels and reduced $Na^+/K^+$-ATPase activity in the cerebral cortex as well as short term memory, learning, and exploratory response. In conclusion, the present results provide additional evidence concerning how repeated restraint stress and clomipramine chronically administered at higher dose levels affect the neural activity and behavior of male rats.



## 1. Introduction

$Na^+/K^+$-ATPase is the enzyme responsible for the active transport of sodium and potassium ions in the nervous system, thus maintaining the ionic gradient necessary for neuronal excitability and regulation of neuronal cell volume. Its activity is decreased in patients with bipolar affective disorder and other psychiatric disorders such as depression (Zanatta et al., 2001). As a consequence, a decrease of $Na^+/K^+$-ATPase activity directly affects neurotransmitter signaling and neural activity, as well as the whole animal behavior (Jamme et al., 1995). Chronic stress models alter the functionality of this enzyme (Crema et al., 2010). Three isoforms of the $Na^+/K^+$-ATPase are found in the brain, but vary by cell type and level of expression, and differentially modulate locomotor activity, anxiety-like behavior, spatial learning, and memory (Moseley et al., 2007). The $\alpha 1$ isoform is found in many central nervous system (CNS) cell types; the $\alpha 2$ isoform is predominantly expressed in glia cells, and the $\alpha 3$ isoform is only expressed in neurons (Moseley et al., 2003). Recent studies have shown a significant correlation between chronic treatment with clomipramine and repeated restraint stress on $Na^+/K^+$-ATPase activity in the brain regions of male rats (Balk et al., 2010).

Activation of the LHPA axis and the release of glucocorticoids are fundamental for the adaptive response and immediate survival of an organism in reaction to acute stimuli. However, high levels of glucocorticoids in the brain may produce neuronal injury (Fontella et al., 2005). A substantial amount of literature has focused on anatomical, behavioral, and neuroendocrine changes associated with exposure to chronic stress.

Tricyclic antidepressants (TCAs) have been prescribed for a long time for the treatment of depression. Moreover, indications for their use are associated with anxiety, eating disorders, and chronic

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pain syndromes (Demerdash and Mohamadin, 2004). The mechanism by which TCAs exert their antidepressant effects is not clearly established to date. However, the capacity for blocking the re-uptake of centrally active neurotransmitters is generally accepted as their primary mode of action (Potter et al., 1998).

Clomipramine is a tricyclic antidepressant widely used for the treatment of depression and obsessive compulsive disorder, mainly by inhibiting the re-uptake of serotonin and norepinephrine in the brain (Peters et al., 1990; Trimble, 1990). These neurotransmitters can influence the neuroplasticity in the brain and both are involved in mediating the therapeutic effects of most currently available antidepressants (Delgado, 2004). It has been shown that the chronic administration of clomipramine at relatively low doses reduces depression (Bhagya et al., 2008; Srikumar et al., 2006) as well as the behavioral deficits and cholinergic dysfunction induced by stress in rats (D'Aquila et al., 2000). However, the species and sex of animals, as well as type, duration, and severity of stress can modify the response induced by stress and clomipramine (Consoli et al., 2005). Literature data have also reported that high doses (30 mg/kg) of clomipramine are toxic to experimental animals (Calegari et al., 2007). The adverse effects induced by clomipramine administration due to the blockade of serotonin reuptake include difficulties in learning and memory (Burgos et al., 2005). Recent studies have revealed that endogenous serotonin and norepinephrine can modulate cognitive processes, particularly learning and memory. However, at present, the mechanisms, locations, and durations of serotonergic and noradrenergic system involvement remain unclear (Millan et al., 2000). TCAs inhibit the activity of the enzyme $Na^+/K^+$-ATPase in the rat brain and there is evidence that tricyclic drugs alter the structural organization of the lipid membranes (Pedrazza et al., 2007), causing cognitive deficits.

In this context, the objective of this study was to evaluate the effects of repeated restraint stress in combination with a high concentration of clomipramine to verify whether the association between a stress condition and the use of TCA could affect the $Na^+/K^+$-ATPase activity in different brain structures, and produce changes in the behavior of adult male rats.

## 2. Experimental procedures

### 2.1. Animals

Forty adult male Wistar rats (250–300 g in weight and 60 days old) from our own breeding colony were used. The animals were placed in groups of five animals in cages made of Plexiglas measuring 42 cm × 34 cm × 16 cm, with the floor covered with sawdust. They were kept in a room with light-dark cycle of 12 h with the lights on between 7:00 and 19:00 h and temperature (20–25 °C) controlled receiving water and food *ad libitum*. Rats were first habituated in the room where the behavioral tasks were performed by at least 30 min. When the group was exposed to more than one subsequent behavioral task, there was a period of three days of resting between tasks.

The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources (number of protocol: 23081.007146/2010-17) of the Federal University of Santa Maria, Brazil.

### 2.2. Experimental groups

The animals were divided into two groups: control group, only manipulated for the necessary maintenance of their cages in good health, and stress group, which received a treatment of repeated restraint stress for a forty-day period. On day 14 of this experimental period, each initial group was subdivided into 4 different groups: (1) control, (2) stress, (3) clomipramine, and (4) stress + clomipramine.

### 2.3. Repeated restraint stress model

Stress condition was performed according to the model of repeated restraint stress as described by Ely et al. (1997). The animals were immobilized in plastic tubes measuring 25 cm × 7 cm of diameter adjusted to the size of the animal. The animal behavior was carried out 1 h per day, five days a week, for forty days in the morning between 8:00 and 10:00. The control group was just handled and thus not subject to any repeated stress situation. The repeated restraint stress was applied at least one hour after exposure to behavioral tasks.

### 2.4. Clomipramine treatment

The animals received clomipramine (30 mg/kg) for 27 consecutive days previously described by Calegari et al. (2007) in the drinking water from the fourteenth day of the trial period after the stress-induced behavioral alterations had been established. This dose was chosen because it provides, when administered chronically, possible toxic effects on the body in rodents according to Calegari et al. (2007). The repeated restraint stress continued during the whole treatment period. The rats untreated with the drug received regular water. Clomipramine was placed in dark bottles due to its photosensitivity and the water consumption by animals was analyzed daily in order to adapt the dose to be administered. Oral administration was chosen because it is the most common use of antidepressants in patients with psychiatric disorders according to Lucassen et al. (2004).

### 2.5. Tissue preparation

At the end of the treatment period, rats were killed by decapitation. The brain was removed and the structures like hippocampus, striatum and cerebral cortex were quickly dissected, placed on ice, and immediately homogenized in cold 50 mM Tris–HCl pH 7.4. The homogenates were centrifuged at 4000 × *g* for 10 min to yield the low-speed supernatant fractions that were used for different biochemical assays in all trials.

### 2.6. Sodium potassium ($Na^+/K^+$-ATPase) activity

The $Na^+/K^+$-ATPase activity was estimated by the method of Muszbek (1977). The enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of hippocampal and striatal aliquots. Before, the slices were incubated with Meth (0.05, 0.1, 0.5 and 1 μM) at different times (5 or 15 min). Then, the reaction mixture containing 95 mM NaCl, 15 mM KCl, 1.0 mM ATP (disodium salt), 38 mM Tris–HCl buffer (pH 7.4) was added to aliquot of homogenized slices (50 μg of protein) in a final volume of 0.3 mL. After a 5-min pre-incubation at 37 °C in the presence of 0.1 mM ouabain to specifically inhibit $Na^+/K^+$-ATPase, the reaction was initiated by addition of ATP and terminated after 15 min of incubation by addition of 1 mL of color reagent (Ammonium Molybdate 2%, Triton X 5% solubilized in $H_2SO_4$ 1.8 M). The released inorganic phosphate was measured spectrophotometrically at λ = 405 nm. $Na^+/K^+$-ATPase activity was calculated from the difference between amounts of inorganic phosphate found after incubation in the absence and presence of 1.5 M ouabain.

### 2.7. Behavioral analysis

Aiming to verify whether chronic treatment with clomipramine could or not potentiate the effects caused by repeated restraint stress on cognitive function in animals we performed four behavioral tasks. The Video-Track® system, used for recording the animal behavior, was composed of a camera connected to a computer.

#### 2.7.1. Object recognition task

The object recognition task was performed according to Stangherlin et al. (2008). Novel object recognition is a type of non-aversive and non-spatial memory. Rodents naturally tend to approach and explore novel objects, which are assumed to have no natural significance to the animal and which have never been paired with a reinforcing stimulus. They also show an innate preference for novel over familiar objects. Rodents readily approach objects and investigate them physically by touching and sniffing the objects, rearing upon and trying to manipulate them with their forepaws. This behavior can be easily quantified and utilized to study simple recognition memory as well as more complex spatial-, temporal- and episodic-like memory in rodents. The standard object recognition task measures the spontaneous behavior. The novelty-preference paradigm does not require lengthy training and does not induce high levels of arousal and stress (Stangherlin et al., 2008). The behavioral task was performed in a 45 cm × 45 cm open field surrounded by 30 cm height walls made of brown plywood. The behavioral task was conducted in a moderately lighted room (30 lx). All animals were given a habituation session where they were left to freely explore the open field for 5 min. No object was placed in the box during the habituation trial. Subsequently, four objects were used: A1, A2, B and C. The "A" objects were two identical triangles, the "B" object was a ball and the "C" object was a rectangle. All objects were made of plastic material, with 10 cm × 10 cm (length × height). Each object had the pattern of color, as follows: blue, red and yellow. Twenty-four hours after habituation, training was conducted by placing each individual rat for 5 min into the field, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners, 10 cm from the walls. In a short-term memory (STM) test given 1.5 h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. All objects presented similar textures, colors, and sizes, but distinctive shapes. The percentage of the total exploration time that the animal spent investigating the novel object was