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Research Report

Role of 5-HT$_3$ and 5-HT$_{2C}$ receptors located within the medial amygdala in the control of salt intake in sodium-depleted rats

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ABSTRACT

In the present study, we investigated the role of 5-HT$_3$ and 5-HT$_{2C}$ receptors located within the medial amygdala (MeA) in the control of water and salt intake in sodium-depleted rats. Pharmacological activation of 5-HT$_3$ receptors located in the medial amygdala by the selective 5-HT$_3$ receptor agonist *m*-CPBG significantly reduced salt intake in sodium-depleted rats, an effect that is reverted by pretreatment with the selective 5-HT$_3$ receptor antagonist ondansetron. In addition, the injection of ondansetron alone into the medial amygdala had no effect on salt intake in sodium-depleted and in sodium-repleted rats. Pharmacological stimulation of 5-HT$_{2C}$ receptors located in the medial amygdala by the selective 5-HT$_{2C}$ receptor agonist *m*-CPP failed to modify salt intake in sodium-depleted rats, whereas the blockade of these receptors by the selective 5-HT$_{2C}$ receptor antagonist SDZ SER 082 significantly reduced salt intake in this same group of animals. These results lead to the conclusion that the pharmacological activation of 5-HT$_3$ receptors located within the MeA inhibits salt intake in sodium-depleted rats and that, in this same brain region, the functional integrity of 5-HT$_{2C}$ receptors is required to achieve the full expression of sodium appetite in sodium-depleted rats.

1. Introduction

Food and water intake are behavioral processes simultaneously regulated by a wide array of anatomically distinct brain areas operating a myriad of neurotransmitters, including serotonin. The massive amount of research on the central serotonergic control of food intake has definitely confirmed a major role of this indolamine in the regulation of the hunger/satiety balance (Halford et al., 2005; Meguid et al., 2000; Simanski, 1996). Less attention has been given to the role of central serotonin in the control of water and salt intake, leading us to concentrate our efforts on elucidating the mechanisms of action of this important serotonergic function.

Previous studies carried out by our group, in which we have used the laboratory rat in several experimental protocols, led us to conclude that different subtypes of central serotonin receptors seem to participate in the intricate control of water and salt intake. Indeed, in a previous paper, our results showed that the pharmacological activation of central 5-HT$_{1D}$ receptors leads to a significant inhibition of water intake induced by central cholinergic, angiotensinergic and adrenergic stimulation (De Castro e Silva et al., 1997). Moreover, we

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have shown that central 5-HT_4 receptors exert a dualist role on the control of water intake, potentiating angiotensin-II-induced drinking and inhibiting thirst induced by central cholinergic activation (Castro et al., 2000). We have also shown that the 5-HT_2 receptor family appears to participate in thirst and sodium appetite regulation. In fact, the pharmacological activation of central 5-HT_{2C} receptors inhibits water intake elicited by different thirst-inducing physiological stimuli (Castro et al., 2002a) and decreases sodium appetite in sodium-depleted rats (Castro et al., 2003). Additionally, we have also established that the central activation of 5-HT_3 receptors significantly diminishes water intake in experimental protocols in which different physiological stimuli are used to promote thirst (dehydration, hypovolemia and hyperosmolarity) and following central angiotensinergic and cholinergic activation (Castro et al., 2002b). Finally, we have shown that the activation of central 5-HT_3 receptors inhibits salt intake in sodium-depleted rats (Castro et al., 2003).

In all of these previous studies, we have used pharmacological approaches in which selective serotonergic drugs were injected directly into the third ventricle and we analyzed their effects on water or salt intake. This allowed us to study the effects promoted by serotonergic stimulation or inhibition when the various pharmacological agents interact with many of the brain areas located in the vicinities of the ventricular system. It was not feasible to study the roles played by serotonin in any particular central site with this initial protocol. However, with the methodology used in the present study, we were able to investigate the putative roles played by specific serotonergic circuitries in the control of water and salt intake.

A complex interactive network of inhibitory and stimulatory inputs, involving different brain neurotransmitters and areas, controls water and salt intake (Johnson and Thunhorst, 1997). The amygdaloid complex, a region in which several serotonin receptor subtypes coexist (Hoyer et al., 2002), plays a crucial role in the control of water and salt intake. In fact, this structure, which is connected with both the prosencephalic and rhomboencephalic areas involved in the control of hydrosaline balance, sends inputs to the higher integrative areas that induce the motor patterns related to the acquisition of water and sodium (Johnson et al., 1999).

As far as we know, the role of 5-HT_3 and 5-HT_{2C} receptors located within the MeA in the mechanisms controlling salt intake has yet clarified. Therefore, in the present study, we investigated the role of these receptors in water and salt intake in rats submitted to sodium depletion.

2. Results

The characteristic site of bilateral injections into the medial amygdala is shown in Fig. 1. Table 1 shows the results obtained with animals that received misplaced injections of *m*-CPBG and ondansetron. Table 2 presents the results obtained with animals that received misplaced injections of *m*-CPP and SDZ SER 082. The analysis of the data summarized in both Tables 1 and 2 indicates that no effect was observable when the drugs were injected into sites located outside the medial amygdala.

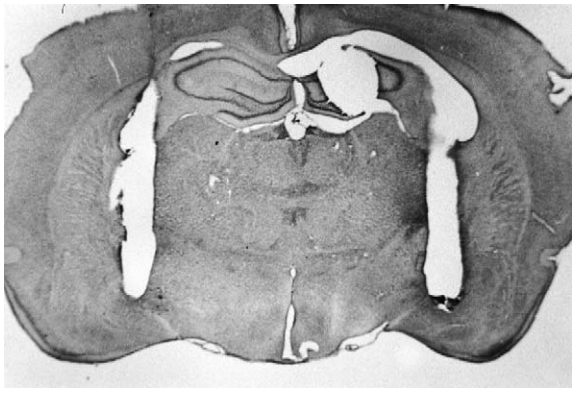

Fig. 1 – Photomicrograph of a coronal section showing the typical position of the cannula within the MeA.

Fig. 2A shows the effect of bilateral MeA injections of the selective 5-HT_3 receptor agonist *m*-CPBG, at different doses (40, 80 and 160 nmol), on salt intake in sodium-depleted rats. Analysis of variance indicated significant treatment and time main effects and significant treatment × time interaction [$F(4,31) = 34.31$; $P < 0.0001$; $F(5,20) = 43.70$, $P < 0.0001$; $F(20,155) = 5.34$, $P < 0.0001$, respectively]. As expected, salt intake increased significantly in control, sodium-depleted animals receiving MeA injections of isotonic saline solution when compared with animals not submitted to sodium depletion also receiving injections of saline into the MeA. At the lowest dose used (40 nmol), the central administration of *m*-CPBG failed to modify the high salt intake observed in sodium-depleted rats. At the intermediate dose of 80 nmol, the central administration of *m*-CPBG significantly reduced salt intake in sodium-depleted animals in the first 45 min of the experiment. At the highest dose used, *m*-CPBG inhibited salt intake in sodium-depleted rats for the duration of the experiment.

Fig. 2B shows the effect of bilateral MeA injections of the selective 5-HT_3 receptor agonist *m*-CPBG, at different doses (40, 80 and 160 nmol), on water intake in sodium-depleted rats. Analysis of variance indicated no significant treatment and time main effects and no significant treatment × time interaction [$F(4,31) = 1.63$; $P = 0.1914$; $F(5,20) = 1.74$, $P = 0.1290$; $F(20,155) = 1.09$, $P = 0.3667$, respectively]. As expected, water intake was negligible in sodium-depleted rats and remained unaltered by any of the treatments.

Fig. 3A displays the effect of the pretreatment with the selective 5-HT_3 receptor antagonist ondansetron (160 nmol) on the antinatriorexic response induced by MeA injections of *m*-CPBG (160 nmol) in sodium-depleted rats. Analysis of variance indicated significant treatment and time main effects and significant treatment × time interaction [$F(3,27) = 45.49$; $P < 0.0001$; $F(5,15) = 32.67$, $P < 0.0001$; $F(15,135) = 5.34$, $P < 0.0001$, respectively]. After two consecutive MeA injections of saline solution, sodium-depleted rats (sodium-depleted saline + saline) drank significantly more hypertonic saline than normonatremic controls also receiving central injections of saline (saline + saline). A significant decrease in salt intake similar to that observed in the previous experimental set was seen in sodium-depleted animals receiving injections of *m*-CPBG (160 nmol) but pretreated with saline (sodium-depleted saline + *m*-CPBG) into the amygdala. On

Table 1 – Cumulative water and salt intakes (ml/100 g body weight) in sodium-depleted animals receiving injections of *m*-CPBG and ondansetron, at various doses, in rats bearing cannulas in sites surrounding the MeA

Time	Intakes	15	30	45	60	90	120	ANOVA	
Treatment								Water	Salt
Saline (9)	Water	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	Factor A — drug	Factor A — drug
	Salt	3.1 ± 0.3	4.3 ± 0.4	4.8 ± 0.5	5.1 ± 0.5	5.6 ± 0.3	5.8 ± 0.3	$F(3,30) = 1.57$;	$F(3,30) = 1.13$;
m-CPBG	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	$P = 0.2182$	$P = 0.3520$
160 nmol (8)	Salt	1.8 ± 0.6	3.3 ± 0.6	4.4 ± 0.4	4.7 ± 0.4	5.1 ± 0.5	5.3 ± 0.6	Factor B — time	Factor B — time
m-CPBG	Water	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	$F(5,15) = 8.45$;	$F(5,15) = 82.57$;
80 nmol (8)	Salt	1.8 ± 0.4	4.2 ± 0.4	5.1 ± 0.3	5.5 ± 0.3	5.8 ± 0.4	6.1 ± 0.3	$P < 0.0001$	$P < 0.0001$
m-CPBG	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	Factor A × Factor B	Factor A × Factor B
40 nmol (9)	Salt	2.4 ± 0.5	3.9 ± 0.5	4.4 ± 0.4	4.6 ± 0.3	4.8 ± 0.3	4.8 ± 0.3	$F(15,150) = 2.31$;	$F(15,150) = 1.65$;
								$P = 0.0056$	$P = 0.0683$
Saline (6)	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	Factor A — drug	Factor A — drug
	Salt	1.3 ± 0.4	3.8 ± 0.6	4.5 ± 0.5	4.8 ± 0.5	5.2 ± 0.4	5.9 ± 0.1	$F(3,19) = 0.490$;	$F(3,19) = 1.27$;
Ond	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	$P = 0.6937$	$P = 0.3126$
160 nmol (4)	Salt	3.0 ± 0.3	4.7 ± 0.5	4.9 ± 0.5	5.0 ± 0.5	5.2 ± 0.4	5.2 ± 0.4	Factor B — time	Factor B — time
Ond	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	$F(5,15) = 2.315$;	$F(5,15) = 59.39$;
80 nmol (9)	Salt	1.6 ± 0.4	2.9 ± 0.5	3.6 ± 0.4	4.3 ± 0.5	4.4 ± 0.4	4.8 ± 0.4	$P = 0.0495$	$P < 0.0001$
Ond	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	Factor A × Factor B	Factor A × Factor B
40 nmol (4)	Salt	1.8 ± 0.8	3.5 ± 0.6	3.9 ± 0.5	3.9 ± 0.5	4.2 ± 0.3	4.2 ± 0.3	$F(15,95) = 0.393$;	$F(15,95) = 1.96$;
								$P = 0.9780$	$P = 0.0262$

Results are shown as mean ± SEM. There were no statistically significant differences among the groups. The number of animals used in each experimental set is indicated in the parenthesis.

the other hand, there was a significant blockade in the antinatriorexic effect of *m*-CPBG in animals receiving *m*-CPBG (160 nmol) but pretreated with 160 nmol of ondansetron (sodium-depleted ondansetron + *m*-CPBG).

Fig. 3B shows the effect of pretreatment with ondansetron (160 nmol) on water intake after injections of *m*-CPBG (160 nmol) into the amygdala in sodium-depleted rats. Analysis of variance indicated no significant treatment main effects and no significant treatment × time interaction [$F(3,27) = 2.03$; $P = 0.1327$; $F(5,15) = 3.54$, $P = 0.0048$; $F(15,135) = 1.74$, $P = 0.0502$, respectively]. Here, as in the previous experiment, water intake was negligible in sodium-depleted rats and remained unchanged by any of the treatments.

Table 2 – Cumulative salt and water intakes (ml/100 g body weight) of sodium-depleted animals receiving injections of *m*-CPP and SDZ SER 082, at various doses, in rats bearing cannulas in sites surrounding the MeA

Time	Intakes	15	30	45	60	90	120	ANOVA	
Treatment								Water	Salt
Saline (7)	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	Factor A — drug	Factor A — drug
	Salt	2.2 ± 0.5	3.4 ± 0.2	4.4 ± 0.5	4.9 ± 0.6	5.5 ± 0.4	5.6 ± 0.4	$F(3,27) = 0.775$;	$F(3,27) = 0.575$;
m-CPP	Water	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	$P = 0.5179$	$P = 0.3662$
160 nmol (8)	Salt	2.3 ± 0.5	3.6 ± 0.4	4.2 ± 0.4	5.0 ± 0.3	5.0 ± 0.3	5.1 ± 0.3	Factor B — time	Factor B — time
m-CPP	Water	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	$F(5,15) = 2.221$;	$F(5,15) = 71.976$;
80 nmol (8)	Salt	2.6 ± 0.7	4.0 ± 0.7	4.2 ± 0.7	4.9 ± 0.4	5.3 ± 0.4	5.5 ± 0.5	$P = 0.0557$	$P < 0.0001$
m-CPP	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	Factor A × Factor B	Factor A × Factor B
40 nmol (8)	Salt	1.6 ± 0.4	3.1 ± 0.3	3.9 ± 0.4	4.4 ± 0.3	4.8 ± 0.3	4.8 ± 0.3	$F(15,135) = 1.005$;	$F(15,135) = 0.0446$;
								$P = 0.4534$	$P = 0.9621$
Saline (7)	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	Factor A — drug	Factor A — drug
	Salt	1.5 ± 0.7	3.1 ± 0.8	3.8 ± 0.6	4.1 ± 0.5	4.1 ± 0.5	4.5 ± 0.4	$F(3,16) = 2.35$;	$F(3,16) = 0.819$;
SDZ SER	Water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	$P = 0.1113$	$P = 0.5020$
160 nmol (8)	Salt	0.8 ± 0.4	2.6 ± 0.5	3.8 ± 0.3	4.3 ± 0.3	4.7 ± 0.4	4.9 ± 0.3	Factor B — time	Factor B — time
SDZ SER	Water	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	$F(5,15) = 1.28$;	$F(5,15) = 55.925$;
80 nmol (8)	Salt	0.8 ± 0.8	2.2 ± 1.0	3.1 ± 0.9	3.3 ± 0.9	3.9 ± 0.6	4.2 ± 0.4	$P = 0.2808$	$P < 0.0001$
SDZ SER	Water	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	Factor A × Factor B	Factor A × Factor B
40 nmol (8)	Salt	1.9 ± 1.4	4.6 ± 0.1	5.0 ± 0.5	5.3 ± 0.3	5.3 ± 0.3	5.3 ± 0.3	$F(15,80) = 1.42$;	$F(15,80) = 0.958$;
								$P = 0.1574$	$P = 0.5066$

Results are shown as mean ± SEM. There were no statistically significant differences among the groups. The number of animals used in each experimental set is indicated in the parenthesis.

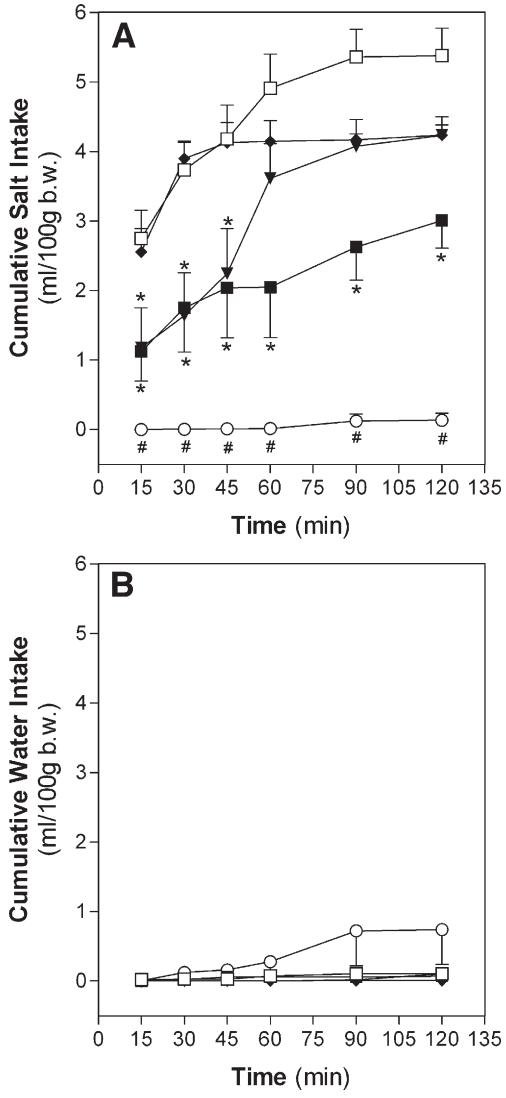

Fig. 2 – Cumulative salt (A) and water (B) intakes (ml/100 g body weight) of sodium-depleted animals treated with bilateral injections of *m*-CPBG, at various doses, into the MeA. The following groups are presented: saline (□; *n* = 07); *m*-CPBG 40 nmol/rat (♦; *n* = 07); *m*-CPBG 80 nmol/rat (▼; *n* = 07); *m*-CPBG 160 nmol/rat (■; *n* = 06). An additional control group of animals not submitted to sodium depletion and receiving injections of saline into the MeA is also shown (○; *n* = 09). Data are presented as mean ± SEM. Asterisks indicate a statistically significant difference (two-way ANOVA followed by Newman–Keul's test; *P* < 0.05) when the distinct groups of animals are compared to control sodium-depleted animals receiving injections of saline into the MeA. # indicates a statistically significant difference when the group of rats not submitted to sodium depletion is compared to sodium depletion animals receiving saline. Each curve in the graph has been obtained from a naive group of animals.

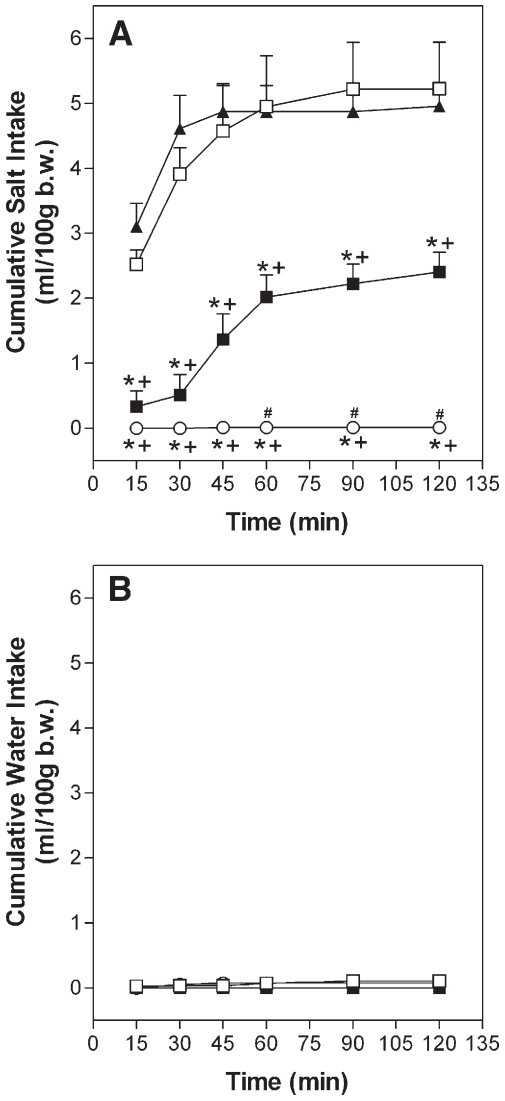

Fig. 3 – Cumulative salt (A) and water (B) intakes (ml/100 g body weight) of sodium-depleted animals treated with bilateral injections of *m*-CPBG (160 nmol) or saline into the MeA but pretreated with injections of ondansetron (160 nmol) or saline into the MeA. The following groups are presented: saline + saline (□; *n* = 07); saline + *m*-CPBG (■; *n* = 09); ondansetron + *m*-CPBG (▲; *n* = 7). An additional control group of animals not submitted to sodium depletion and receiving MeA injections of saline is also shown (○; *n* = 08). Data are presented as mean ± SEM. Asterisks indicate a statistically significant difference (two-way ANOVA followed by Newman–Keul's test; *P* < 0.05) when the distinct groups of animals are compared to control sodium-depleted animals (saline). # indicates a statistically significant difference when the group of rats not submitted to sodium depletion is compared to sodium-depleted rats receiving saline + *m*-CPBG. + indicates a statistically significant difference when the groups of rats not submitted to sodium depletion receiving saline as treatment and as pretreatment and submitted to sodium depletion receiving *m*-CPBG but pretreated with saline are compared to the other groups. Each curve in the graph has been obtained from a naive group of animals.

Fig. 4A shows the effect of bilateral MeA injections of ondansetron alone (40, 80 and 160 nmol) on salt intake in sodium-depleted rats. Analysis of variance indicated significant treatment main effects and significant treatment × time interaction [$F(4,29) = 53.07$; $P < 0.0001$; $F(5,20) = 47.98$, $P < 0.0001$;

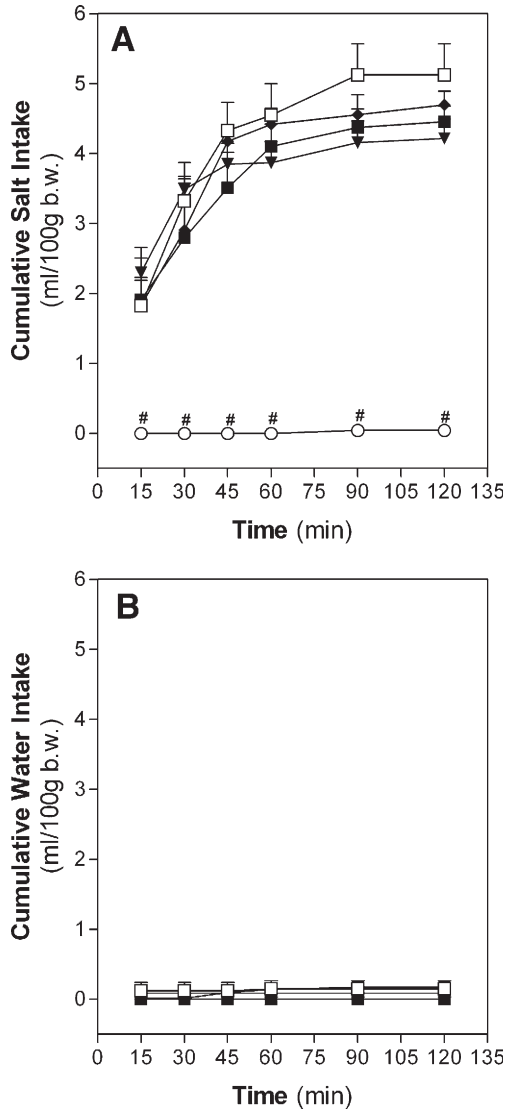

Fig. 4 – Cumulative salt (A) and water (B) intakes (ml/100 g body weight) of sodium-depleted animals treated with bilateral injections of ondansetron, at various doses, into the MeA. The following groups are presented: saline (□; *n* = 06); ondansetron 40 nmol/rat (♦; *n* = 06); ondansetron 80 nmol/rat (▼; *n* = 06); ondansetron 160 nmol/rat (■; *n* = 07). An additional control group of animals not submitted to sodium depletion and receiving injections of saline into the MeA is also shown (○; *n* = 09). Data are presented as mean ± SEM. # indicates a statistically significant difference (two-way ANOVA followed by Newman–Keul's test; *P* < 0.05) when the group of animals not submitted to sodium depletion is compared to all other groups. Each curve in the graph has been obtained from a naive group of animals.

$F(20,145) = 4.54$, $P < 0.0001$, respectively]. Here, as in the previous experimental sets, there was a significantly greater salt intake in sodium-depleted rats receiving bilateral MeA injections of isotonic saline solution when compared to normonatremic rats also receiving central administration of isotonic saline solution. At all doses used, ondansetron failed to modify the high salt intake exhibited by sodium-depleted rats.

Fig. 4B shows the effect of treatment with ondansetron alone at various doses (40, 80 and 160 nmol) on water intake in sodium-depleted rats. Analysis of variance indicated no significant treatment main effects but a significant treatment × time interaction [$F(4,29) = 0.74$; $P = 0.5691$; $F(5,20) = 5.37$, $P = 0.0001$; $F(20,145) = 3.36$, $P < 0.0001$, respectively]. Here, as in the previous experiment, water intake was negligible in sodium-depleted rats and remained unaltered by any of the treatments.

Fig. 5A shows the effect of bilateral MeA injections of the selective 5-HT_{2C} receptor agonist *m*-CPP, at different doses (40, 80 and 160 nmol), on salt intake in sodium-depleted rats. Analysis of variance indicated significant treatment and time main effects and significant treatment × time interaction [$F(4,29) = 64.90$; $P < 0.0001$; $F(5,20) = 50.82$, $P < 0.0001$; $F(20,145) = 5.30$, $P < 0.0001$, respectively]. As expected, salt intake increased significantly in sodium-depleted control animals receiving MeA injections of isotonic saline solution when compared with animals not submitted to sodium depletion also receiving injections of saline into the MeA. Bilateral injections of the selective 5-HT_{2C} receptor agonist *m*-CPP into the MeA failed to modify the high salt intake presented by sodium-depleted rats at any of the doses used.

Fig. 5B shows the effect of bilateral MeA injections of the selective 5-HT_{2C} receptor agonist *m*-CPP, at different doses (40, 80 and 160 nmol), on water intake in sodium-depleted rats. Analysis of variance indicated no significant treatment and time main effects and no significant treatment × time interaction [$F(4,29) = 0.877$; $P = 0.4895$; $F(5,20) = 2.325$, $P = 0.0458$; $F(20,145) = 0.625$, $P = 0.8891$, respectively]. As expected, water intake was negligible in sodium-depleted rats and was not modified by any of the treatments.

Fig. 6A shows the effect of bilateral MeA injections of the selective 5-HT_{2C} antagonist SDZ SER 082 alone (40, 80 and 160 nmol) on salt intake in sodium-depleted rats. Analysis of variance indicated significant treatment main effects and significant treatment × time interaction [$F(4,33) = 47.27$; $P < 0.0001$; $F(5,20) = 69.25$, $P < 0.0001$; $F(20,165) = 7.58$, $P < 0.0001$, respectively]. Here, as in the previous experimental sets, there was a significant increase in salt intake in sodium-depleted rats receiving bilateral MeA injections of isotonic saline solution when compared to normonatremic rats also receiving central administration of isotonic saline solution. At the lowest dose used, SDZ SER 082 failed to modify the high salt intake of sodium-depleted rats. At the intermediate dose used, SDZ SER 082 significantly decreased salt intake in sodium-depleted rats in the first 90 min of the experiment. At the highest dose injected, SDZ SER 082 significantly reduced salt intake in sodium-depleted rats for the entire duration of the experiment.

Fig. 6B shows the effect of treatment with SDZ SER 082 alone at various doses (40, 80 and 160 nmol) on water intake in sodium-depleted rats. Analysis of variance indicated no significant treatment main effects and no significant treatment × time interaction [$F(4,33) = 0.62$; $P = 0.6481$; $F(5,20) = 4.44$, $P = 0.0008$; $F(20,165) = 0.905$, $P < 0.5807$, respectively]. Here, as in the previous experimental set, water intake was negligible in sodium-depleted rats and was not altered by any of the treatments.

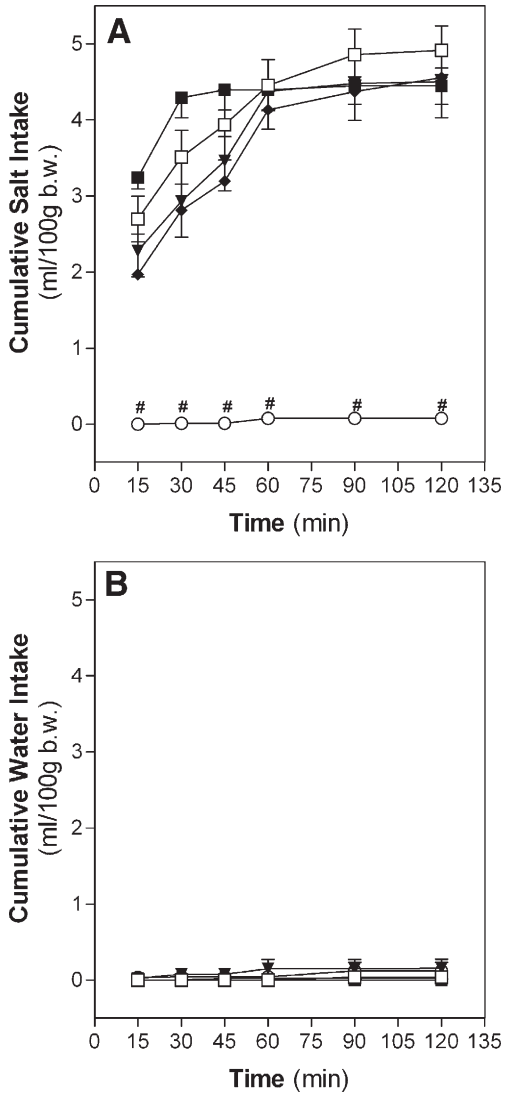

Fig. 5 – Cumulative salt (A) and water (B) intakes (ml/100 g body weight) of sodium-depleted animals treated with bilateral injections of *m*-CPP, at various doses, into the MeA. The following groups are presented: saline (□; *n* = 06); *m*-CPP 40 nmol/rat (♦; *n* = 06); *m*-CPP 80 nmol/rat (▼; *n* = 06); *m*-CPP 160 nmol/rat (■; *n* = 06). An additional control group of animals not submitted to sodium depletion and receiving injections of saline into the MeA is also shown (○; *n* = 09). Data are presented as mean ± SEM. # indicates a statistically significant difference (two-way ANOVA followed by Newman–Keul's test; *P* < 0.05) when the group of animals not submitted to sodium depletion is compared to all other groups. Each curve in the graph has been obtained from a naive group of animals.

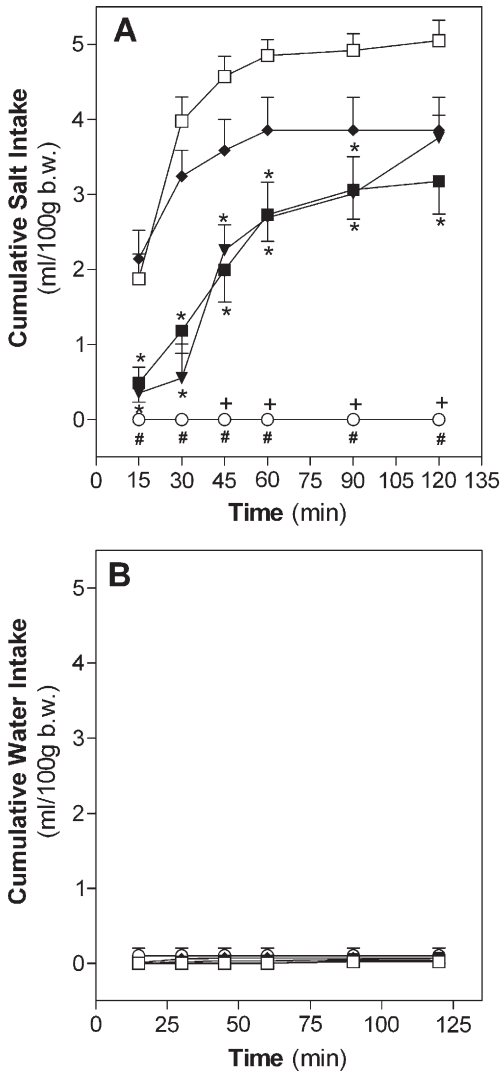

Fig. 6 – Cumulative salt (A) and water (B) intakes (ml/100 g body weight) of sodium-depleted animals treated with bilateral injections of SDZ SER 082, at various doses, into the MeA. The following groups are presented: saline (□; *n* = 09); SDZ SER 082 40 nmol/rat (♦; *n* = 07); SDZ SER 082 80 nmol/rat (▼; *n* = 06); SDZ SER 082 160 nmol/rat (■; *n* = 05). An additional control group of animals not submitted to sodium depletion and receiving injections of saline into the MeA is also shown (○; *n* = 08). Data are presented as mean ± SEM. Asterisks indicate a statistically significant difference (two-way ANOVA followed by Newman–Keul's test; *P* < 0.05) when the distinct groups of sodium-depleted animals are compared to control sodium-depleted animals receiving injections of saline into the MeA. # indicates a statistically significant difference when the group of rats not submitted to sodium depletion is compared to sodium depletion animals receiving saline or SDZ SER 082 at the dose of 40 nmol. + indicates a statistically significant difference when the group of rats not submitted to sodium depletion receiving injections of saline into MeA was compared to the groups of sodium-depleted animals receiving MeA injections of SDZ SER 082 at the doses of 80 and 160 nmol. Each curve in the graph has been obtained from a naive group of animals.

Table 3 shows the effect of bilateral injections of ondansetron (160 nmol) and *m*-CPP (160 nmol) into the MeA on water and salt intake in normonatremic animals. In this condition, the administration of these compounds failed to produce any modification in those parameters compared to saline-treated controls.

Fig. 7A depicts the results of the avoidance test performed to verify whether the antinatriorexic effects of *m*-CPBG could be attributed to any "illness-like" side effects. Analysis of

Table 3 – Cumulative salt and water intakes (ml/100 g body weight) of sodium-repleted animals receiving bilateral injections of *m*-CPP and ondansetron at various doses into the MeA

Time	Intakes	15	30	45	60	90	120	ANOVA	
Treatment								Water	Salt
Saline (6)	Water	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	Factor A — drug $F(1,14) = 1.48$; $P = 0.2435$ Factor B — time $F(5,5) = 14.48$; $P < 0.0001$ Factor A × Factor B $F(5,70) = 13.02$; $P < 0.0001$	Factor A — drug $F(1,14) = 0.00$; $P = 1.000$ Factor B — time $F(5,5) = 0.00$; $P = 1.000$ Factor A × Factor B $F(5,70) = 0.00$; $P = 1.000$
	Salt	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
Ond	Water	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2		
160 nmol (4)	Salt	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
Saline (9)	Water	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	Factor A — drug $F(1,14) = 0.0724$; $P = 0.7918$ Factor B — time $F(5,5) = 7.4647$; $P < 0.0001$ Factor A × Factor B $F(5,70) = 3.6307$; $P = 0.0056$	Factor A — drug $F(1,14) = 0.127$; $P = 0.7272$ Factor B — time $F(5,5) = 2.333$; $P = 0.0510$ Factor A × Factor B $F(5,70) = 0.919$; $P = 0.4738$
	Salt	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1		
m-CPP	Water	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1		
160 nmol (8)	Salt	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1		

Results are shown as mean ± SEM. There were no statistically significant differences among the groups. The number of animals used in each experimental set is indicated in the parenthesis.

variance indicated a significant treatment difference between the groups [$F(2,18) = 27.2$; $P < 0.0001$]. As expected, there was a significant reduction in saccharin intake on the following day in animals establishing a previous association between lithium chloride and saccharin as compared to saline-treated controls. In contrast, the previous association of *m*-CPBG with saccharin failed to produce any significant reduction in saccharin intake the next day, which suggests that it is unlikely that illness-like effects could explain the results observed here after the injection of these compounds into the medial amygdala.

Fig. 7B shows the results of the dessert test. Here, saccharin intake was similar in saline-treated control animals and animals receiving bilateral MeA injections of *m*-CPBG (160 nmol), indicating that the hedonic behavior represented by the preferential intake of a "tasty" solution was not modified by central injections of *m*-CPBG ($t = 0.303$; $df = 13.0$; $P = 0.7669$).

Fig. 7C shows the result of the avoidance test performed to verify whether the antinatriorexic effects of SDZ SER 082 could be ascribed to some "illness-like" side effects. Analysis of variance indicated a significant treatment difference between the groups [$F(2,25) = 80.8$; $P < 0.0001$]. In animals establishing a previous association between lithium chloride and saccharin, there was a significant decrease in saccharin intake on the following day when compared to saline-treated controls. Conversely, the previous association of SDZ SER 082 with saccharin was unable to modify saccharin intake the next day, indicating that the results observed here after the injection of this compound into the medial amygdala are not due to some illness-like effect.

Fig. 7D presents the results of the dessert test. In this case, saccharin intake was comparable in saline-treated control animals and animals receiving bilateral MeA injections of SDZ SER 082 (160 nmol), indicating that the hedonic behavior represented by the preferential intake of a "tasty" solution was not modified by central injections of SDZ SER 082 ($t = 0.794$; $df = 14.0$; $P = 0.4404$).

3. Discussion

The present data show that the pharmacological stimulation of 5-HT_3 receptors located in the medial amygdala by the selective agonist *m*-CPBG significantly decreases salt intake in sodium-depleted rats. This antinatriorexic effect of *m*-CPBG seems to be due to its action on 5-HT_3 receptors since it is abolished by pretreatment with the 5-HT_3 receptor antagonist ondansetron. In sodium-depleted animals, a 5-HT_3-receptor-dependent inhibitory drive on salt intake seems to be absent since treatment with ondansetron alone failed to modify salt intake in this group of animals. 5-HT_3 receptors located in the medial amygdala exert neither inhibitory nor stimulatory drive on salt intake in normonatremic rats (animals not submitted to sodium depletion) since injections of ondansetron into the MeA also failed to modify salt intake in this group of animals. The present data also show that pharmacological administration of the selective 5-HT_{2C} receptor agonist *m*-CPP was unable to modify salt intake in sodium-depleted rats. However, a 5-HT_{2C}-receptor-dependent stimulatory drive on salt intake seems to exist in sodium-depleted rats since the treatment of animals in this condition with the selective 5-HT_{2C} receptor antagonist SDZ SER 082 significantly blunted salt intake in this group of animals. In addition, the pharmacological activation of 5-HT_{2C} receptors located within the MeA failed to induce any modification in salt intake in rats not submitted to sodium depletion. The inhibitory effects of *m*-CPBG and SDZ SER 082 on salt intake cannot be attributed to sickness-like effects induced

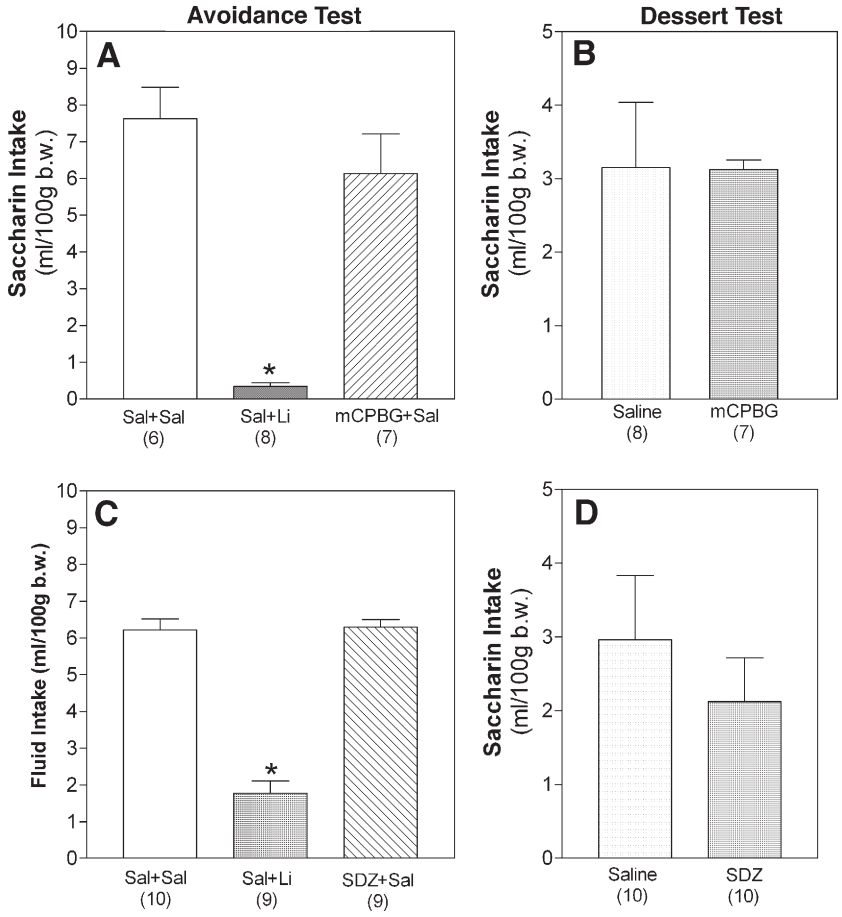

Fig. 7 – Avoidance tests: saccharin solution (0.25%) consumption (ml/100 g body weight) over 15 min at a second offering in animals receiving injections of *m*-CPBG (160 nmol) or saline into the MeA (A) and SDZ SER 082 (160 nmol) or saline (C). The sequence of injections used during the first offering of saccharin and the number of animals used are indicated in the figure. The first injection was into the MeA and the second via intraperitoneal route. The asterisk indicates a statistically significant difference ($P < 0.001$) between that particular group and controls (saline + saline). Dessert tests: saccharin intake (ml/100 g body weight) during 2 h in the test cage in rats receiving MeA injections of isotonic saline solution (controls), *m*-CPBG at the dose of 160 nmol (B) and SDZ SER 082 at the dose of 160 nmol (D). The treatment received by each group and the number of animals used are indicated in the graph. There was no significant difference in the ingestion of saccharin between groups treated with saline and the serotonergic agents tested. Data are expressed as mean ± SEM.

by these compounds since aversion tests excluded this possibility. Furthermore, the reduction in salt intake induced by *m*-CPBG and SDZ SER 082 appears to be a selective antinatriorexic effect, and not a general inhibition of ingestive behaviors, since the intake of a palatable solution of saccharin remained unaffected when these compounds were bilaterally injected into the medial amygdala.

The amygdala receives many inputs from other prosencephalic regions involved in the regulation of sodium appetite and thirst, such as the subfornical organ (SFO) and the anteroventral third ventricle region (AV3V), mainly through angiotensinergic pathways (Johnson et al., 1999). The amygdala and the bed nucleus of the stria terminalis (BST) may have had a common ontogenic origin, and this led to the concept of an extended amygdala, formed by several structures including the central and the medial amygdala as well as their extensions to the lateral and medial parts of the BST (Johnson et al., 1999).

The extended amygdala is strongly involved in the control of sodium appetite. Indeed, surgical lesions of the MeA inhibit salt intake induced by mineralocorticoid administration (Nitabach et al., 1989; Schulkin et al., 1989) and the same type of lesions in the central amygdala (CeA) as well as in the BST inhibit sodium appetite in several experimental protocols (Galaverna et al., 1991; Zardetto-Smith et al., 1994). Immunocytochemical studies indicate that 5-HT_3 receptors are extensively located throughout the rat brain (Bloom and Morales, 1998), including some areas related to the control of water intake and salt appetite, such as the hypothalamus, the amygdaloid complex and the septal region (Tecott et al., 1993). 5-HT_{2C} receptor, a serotonin receptor subtype that seems to exist only in the central nervous system, is ubiquitously found, being present in limbic areas that participate in the mechanisms controlling water intake (Barnes and Sharp, 1999; Clement et al., 2000; Giorgetti and Tecott, 2004).

In the present study, the pharmacological stimulation of 5-HT_3 receptors located within the MeA by a selective 5-HT_3 receptor agonist leads to a significant reduction in salt intake in sodium-depleted rats. This indicates that a local 5-HT_3 receptor-dependent circuitry, when pharmacologically activated, leads to salt intake inhibition. However, the administration of a selective 5-HT_3 receptor antagonist, ondansetron, into the MeA failed to increase salt intake either in sodium-depleted or sodium-repleted rats. This suggests that (1) suppression of the endogenous serotonergic activity on 5-HT_3 receptors located within the MeA is not capable of modifying the high intensity of salt intake that results from the activation of several salt-intake-inducing mechanisms normally triggered during sodium depletion, at least for the high level of sodium appetite induced by the experimental protocol we have used here, and (2) in sodium-repleted rats, it seems that an endogenous inhibitory drive on salt intake depending on the functional integrity of 5-HT_3 receptors located into the MeA is not a major feature in the central mechanisms controlling sodium appetite in rats. However, it is important to note that central 5-HT_3 receptors play a role in anxiolytic, antipsychotic and cognitive-enhancing events (Farber et al., 2004) and that drugs acting on 5-HT_3 receptors are extensively used in clinical therapeutics, mainly as antiemetic agents during chemotherapy (Aapro, 2005). Therefore, any information concerning pharmacological effects of that class of therapeutical agents is relevant.

In the present study, the pharmacological activation of 5-HT_{2C} receptors situated in the MeA by *m*-CPP, a selective agonist, failed to modify salt intake in sodium-depleted rats. Conversely, the blockade of 5-HT_{2C} receptors located in the MeA by the selective 5-HT_{2C} receptor antagonist SDZ SER 082 significantly reduced salt intake in sodium-depleted animals. This indicates that the endogenous serotonergic activity on 5-HT_{2C} receptors within the MeA is essential for the full expression of sodium appetite in sodium-depleted rats. The fact that the pharmacological activation of 5-HT_{2C} receptors located within the MeA did not produce any further increase in salt intake in sodium-depleted rats may simply mean that, during sodium depletion, the other salt intake-inducing mechanism(s) normally activated under this condition are able to promote a maximal salt intake response.

The pharmacological agents used in the present study were adequate tools for clarifying the questions raised here. *m*-CPBG is a well-documented 5-HT_3 receptor agonist (Sepúlveda et al., 1991; Van Hooft and Vijverberg, 1997), ondansetron is a well-recognized 5-HT_3 receptor antagonist (Gaster and King, 1997), *m*-CPP is a selective 5-HT_{2C} agonist (Simansky et al., 2004) and SDZ SER 082 is a selective 5-HT_{2C} receptor antagonist (Hernandez et al., 2003). *m*-CPP may display some affinity for other serotonin receptors. However, it binds to the 5-HT_{2C} receptor with much greater affinity than to any other serotonin receptor subtype and, in the absence of a drug that could be considered a strictly selective 5-HT_{2C} agonist, *m*-CPP is considered the prototypical pharmacological tool for studying 5-HT_{2C} function (Hajos et al., 2003; Jakus et al., 2003; Mitchell et al., 2003; Simansky et al., 2004). Therefore, the results obtained in the present study have to be considered a consequence of *m*-CPP-induced 5-HT_{2C} receptor stimulation.

There is sparse information regarding the role of the various neurotransmitters in the MeA in the control of sodium appetite. Most studies have used experimental protocols in which anatomical lesions of this structure induced alterations in salt intake in different conditions. We failed to find any pharmacological studies especially designed to clarify the role of the different serotonin receptor subtypes located in the MeA in the regulation of salt intake. Hence, the present results contribute new and relevant data concerning the physiology and pharmacology of brain serotonin receptors.

We have previously demonstrated that the pharmacological activation of brain 5-HT_3 receptors by third ventricle injections of *m*-CPBG reduces water intake induced by three different physiological thirst-inducing stimuli: hyperosmolarity due to an acute intragastric salt load, hypovolemia induced by subcutaneous administration of polyethylene glycol and dehydration provoked by an overnight period of water deprivation (Castro et al., 2002b). We have also shown that third ventricle injections of the same compound (*m*-CPBG) inhibit salt intake in sodium-depleted rats (Castro et al., 2003). Brain circuitries whose activation triggers water intake normally exert a positive drive on salt intake (Johnson and Thunhorst, 1997; McKinley and Johnson, 2004; Stricker and Verbalis, 1990). In previous studies, in which we used third ventricle injections of *m*-CPBG, we were able to confirm that pharmacological activation of 5-HT_3 receptors located at circumventricular structures elicited a significant antinatriorexic effect. With the present results, we add new data indicating that the same antinatriorexic effect is also produced when 5-HT_3 receptors located in a particular central region involved in the regulation of water and salt intake are activated.

In a previous study, we demonstrated that third ventricle injections of *m*-CPP, the same 5-$HT_{2B/2C}$ receptor agonist used in the present study, promoted a dose-dependent reduction in salt intake in sodium-depleted rats (Castro et al., 2003). Data reported here indicate that 5-HT_{2C} receptors located within the MeA are essential for the full expression of salt intake in sodium-depleted rats since the pharmacological blockade of these receptors reduced sodium appetite induced by sodium depletion. The large family of serotonin receptors operates multiple functions in the brain, and the activation of the same receptor subtype may generate distinct effects depending on the central area in which the receptor is located (Uphouse, 1997). Considering both our previous data and the data presented here, it would appear that the activation of 5-HT_{2C} receptors located in circumventricular areas reduces salt intake, whereas the activation of these receptors in the MeA is a necessary step to the increase in sodium appetite in sodium-depleted rats.

The inhibition of ingestive behaviors observed in experimental protocols in animals may be due to actions on brain sites induced by the specific measures employed in those protocols. On the other hand, such inhibitory actions may be the result of aversive effects associated with those procedures. We have previously shown that third ventricle injections of both *m*-CPBG and *m*-CPP do not generate aversive effects (Castro et al., 2002a,b). In the present paper, we demonstrate that injections into the MeA of the compounds that induced a reduction in sodium appetite (*m*-CPBG and SDZ SER 082) do not produce aversive effects that could explain the reduction in

salt intake observed here. We have also shown that both drugs failed to disrupt hedonic ingestive behaviors since they do not modify the ingestion of saccharin offered as a dessert meal. This clearly indicates that the drugs are specifically inhibiting salt intake instead of producing a general inhibition of all ingestive behaviors.

In the present study, no effect was seen when the drugs were injected into sites located outside the medial amygdala. This clearly points out that the effects shown here are specifically due to the pharmacological manipulation of 5-HT_3 and 5-HT_{2C} receptors located in the MeA.

In summary, the present data suggest that the pharmacological activation of 5-HT_3 receptors located within the MeA inhibits salt intake in sodium-depleted rats and that, in this same brain region, the functional integrity of 5-HT_{2C} receptors is required for the full expression of sodium appetite in sodium-depleted rats.

4. Experimental procedure

4.1. *Animals*

In the present study, we used male Wistar rats weighing 280 ± 20 g. They were housed in individual cages and kept under controlled light (lights on from 7 AM to 7 PM) and temperature (22–24 °C) conditions. In all experimental protocols, central injections of saline (controls) and each individual dose of the serotonergic agents were tested in a naive group of animals. All experiments were conducted between 7 AM and 12 PM. The experimental protocols were conducted according to the regulations established by the National Institutes of Health (USA) and were approved by a local committee regulating the use of animals in research laboratories.

4.2. *Surgical procedures*

Cannulation of the MeA was performed under pentobarbital anesthesia (50 mg/kg i.p.). Five days before the experimental sessions, a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) was used to implant a 15 mm, 28-gauge, stainless steel cannula. The following coordinates were used: anteroposterior = 2.8 mm behind bregma; lateral = 6.8 mm; vertical = 8.6 mm below the skull. The animals were placed in the stereotaxic apparatus with their heads in the horizontal position. The cannulas were cemented to the skull bone with dental acrylic, and an obturator (22-gauge) was provided to avoid obstruction. After surgery, the animals in all the study groups had free access to two different bottles, one containing distilled water and the other containing 1.5% saline solution. In order to minimize the stress of the experimental maneuvers, the animals were handled every day. At the end of the experiments, the animals were anesthetized with ether and submitted to transcardiac perfusion with isotonic saline solution followed by 10% formalin. The brains were then removed and fixed in 10% formalin. They were frozen and cut into 40 μm sections. To confirm the injection sites in relation to the MeA, the slices were stained with cresyl violet and analyzed by light microscope. Data from animals in which the cannulas were strictly inside the medial amygdala were analyzed and taken into consideration for the interpretation of the effects of the pharmacological agents on water and salt intake. A special table condenses the data from animals in which the cannulas were off target.

4.3. *Drugs and microinjections*

The following drugs were used: *m*-chlorophenylbiguanide hydrochloride (1-(3-cholrophenyl)biguanide; *m*-CPBG), a selective 5-HT_3 agonist (Sepúlveda et al., 1991; Van Hooft and Vijverberg, 1997), *m*-CPP (1-(3-Chlorophenyl)piperazine), a 5-HT_2 agonist (Simansky et al., 2004), and SDZ SER 082 [(+)-*cis*-4,5,7a,8,9,10,11,11a-octahydro-7*H*-10-methylindolo[1,7-bc] (Barnes and Sharp, 1999; Castro et al., 2002a)-naphthyridine], a selective 5-HT_{2C} receptor antagonist (Hernandez et al., 2003), were all purchased from Tocris Cookson, Inc. Ballwin, MO. Ondansetron, a specific 5-HT_3 antagonist, was kindly donated by GlaxoWellcome Research and Development Limited, UK (Gaster and King, 1997). Lithium chloride was acquired from Sigma Chemical, Co., St. Louis, MO. Furosemide, a loop diuretic, was purchased from Aventis Pharma Ltd., São Paulo, Brazil. Central injections were performed using a Hamilton microsyringe connected to a Myzzy-Slide-Pak needle through polyethylene tubing. The injectors we have used extended 1 mm beyond the end of the guide cannulas. All drugs were dissolved in isotonic saline solution. The final volume injected was 0.5 μl over a period of 60 s.

4.4. *Sodium depletion*

To induce sodium depletion, the animals were submitted to an experimental protocol in which they had simultaneous access to two bottles (distilled water and 1.5% saline solution) and standard rat chow from the period immediately after MeA cannulation until the moment of furosemide administration. To provoke the renal sodium loss that induces sodium depletion, the rats received a subcutaneous injection of furosemide (20 mg/kg) 24 h prior to the experimental sessions. Access to 1.5% saline ceased immediately after the furosemide injection. From that moment on, the animals continued to have free access to distilled water, and normal rat chow was replaced by a low sodium diet (0.001% Na^+ and 0.33% K^+). Control animals not submitted to sodium depletion received subcutaneous injections of isotonic saline solution instead of furosemide. We have previously shown that furosemide administration, at the dose used here, effectively increases urine output and renal sodium excretion and produces hyponatremia (Castro et al., 2003). To test the participation of central 5-HT_{2C} and 5-HT_3 receptors in water and salt intake in sodium-depleted rats, different groups of sodium-depleted animals received bilateral injections of the serotonergic agents at different doses into the MeA. Sodium-depleted control animals received injections of isotonic saline solution into this same area. The bottles containing 1.5% saline solution were reintroduced into the cages 30 min after the injections into the MeA. The first measurement of fluid intake was recorded 15 min after this and measurements continued for the next 120 min. All groups were also compared to a control group of normonatremic animals.

4.5. Avoidance test

An avoidance test was carried out to verify whether the central administration of the serotonergic agents *m*-CPBG and SDZ SER 082 was devoid of non-specific, inhibitory, "illness-like" effects on water intake. An experimental protocol based on the original design proposed by Nachman (1970) was adopted. This protocol uses a temporal association between the novel taste of a 0.25% saccharin solution and the distress induced by lithium chloride administration. Five days after the cannulation of the MeA, the animals had their access to water restricted to 15 min/day (between 12 and 12:15 PM) for 4 consecutive days. Under these conditions, rats drank water rapidly and reliably. On the fifth day, they were divided into 4 different groups that, after being submitted to the different pharmacological protocols, had access to bottles containing saccharin (no water was offered on this day). The first group (controls) received two consecutive injections of isotonic saline solution, one immediately following the other, the first being intraperitoneal and the second into the MeA. In the second group of animals, 0.15 M lithium chloride intraperitoneal injections (0.6% b.w.) were followed by injections of isotonic saline solution into the MeA. In this group, the lithium-induced, illness-like effects, a condition that generally disrupts ingestive behaviors in rats, were associated with the novel taste of saccharin. The third and the fourth groups of animals received intraperitoneal injections of saline solution in the same volume used in the previous group followed by injections of *m*-CPBG (third group) or SDZ SER 082 (fourth group). Both drugs were injected at the dose of 160 nmol. In these groups of animals, we investigated whether the administration of the serotonergic agents *m*-CPBG and SDZ SER 082 into the MeA provokes any degree of discomfort leading to a general reduction in ingestive behavior that the animals could associate with the novel taste of saccharin. On the sixth day, at the same time that the bottles had been available on the previous days (12 to 12:15 PM), saccharin-containing bottles were placed in all cages and the amount ingested recorded. No drugs were injected on this day.

4.6. Dessert test

To investigate whether the serotonergic agents used in the present study were able to modify water and salt intake through a non-specific general inhibition of the central nervous system or through a locomotor deficit, we investigated the effect of their injection into the MeA on the intake of 0.1% saccharin solution, a well-established example of hedonic behavior in rats (Johnson and Schwob, 1975). In this experiment, after the cannulation of MeA, two different groups of animals, kept in the usual individual cages where the only fluid available was water, were transferred (for 2 h each day for seven consecutive days) to a different cage (the test cage) in which two bottles, one containing water and the other containing a 0.1% saccharin solution, were accessible. After this period of training, two different groups of fluid-deprived animals received injections of *m*-CPBG (160 nmol), SDZ SER 082 (160 nmol) or saline (controls) into the MeA, 30 min before being transferred to the test cage. The intake of saccharin was then recorded during the following 120 min.

4.7. Statistical analysis

A computer software package (SigmaStat for Windows, Jandel Scientific, San Rafael, CA) was used to carry out two-way analysis of variance for repeated measures. The post hoc Student–Newman–Keuls test was used for comparison of each treatment with its corresponding time in the control groups. One-way ANOVA was used to analyze the data resulting from the avoidance test. Data resulting from the dessert test were analyzed using Student's *t* test. Data are presented as mean ± SEM. The effects were considered significantly different when $P < 0.05$.

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