

## ORIGINAL ARTICLE

# The effect of increased NaCl intake on rat brain endogenous $\mu$ -opioid receptor signalling

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**Funding information**

National Research Development and Innovation Office, Grant/Award Number: TÉT 10-1-2011-0085; National Minister of Science and Technology of Argentina, Grant/Award Number: HU/10/03; CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas); SECYT (Secretaría de Ciencia y Tecnología, Universidad nacional de Córdoba)

Numerous studies demonstrate the significant role of central  $\beta$ -endorphin and its receptor, the  $\mu$ -opioid receptor (MOR), in sodium intake regulation. The present study aimed to investigate the possible relationship between chronic high-NaCl intake and brain endogenous MOR functioning. We examined whether short-term (4 days) obligatory salt intake (2% NaCl solution) in rats induces changes in MOR mRNA expression, G-protein activity and MOR binding capacity in brain regions involved in salt intake regulation. Plasma osmolality and electrolyte concentrations after sodium overload and the initial and final body weight of the animals were also examined. After 4 days of obligatory hypertonic sodium chloride intake, there was clearly no difference in MOR mRNA expression and G-protein activity in the median preoptic nucleus (MnPO). In the brainstem, MOR binding capacity also remained unaltered, although the maximal efficacy of MOR G-protein significantly increased. Finally, no significant alterations were observed in plasma osmolality and electrolyte concentrations. Interestingly, animals that received sodium gained significantly less weight than control animals. In conclusion, we found no significant alterations in the MnPO and brainstem in the number of available cell surface MORs or *de novo* syntheses of MOR after hypertonic sodium intake. The increased MOR G-protein activity following acute sodium overconsumption may participate in the maintenance of normal blood pressure levels and/or in enhancing sodium taste aversion and sodium overload-induced anorexia.

**KEYWORDS**

$\beta$ -endorphin,  $\mu$ -opioid receptor signalling, brainstem, G-protein activation, sodium ingestion

## 1 | INTRODUCTION

Several lines of evidence suggest that central endogenous opioid peptides and receptors are involved in the regulation of salt ingestion.  $\beta$ -endorphin, comprising one of these, plays a key role in the modulation of salt hedonic palatability and sodium appetite, as well as in dietary-sodium-overload induced sympathetic and pressor responses.<sup>1-5</sup> Previous results from our laboratory indicated

that  $\beta$ -endorphin knockout mice and heterozygous mutant mice consume approximately 50% less 2% NaCl solution than wild-type mice after sodium depletion, suggesting that  $\beta$ -endorphin facilitates induced sodium appetite.<sup>3</sup> On the other hand, our results also showed that endogenous  $\beta$ -endorphin is implicated in the compensatory response to body sodium overload.<sup>6,7</sup> This was demonstrated by  $\beta$ -endorphin knockout mice displaying increased systolic blood pressure, urinary epinephrine excretion and median preoptic nucleus (MnPO) neural activity (as shown by Fos-immunoreactivity), when submitted to a high-sodium diet (4%

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NaCl) for 2 weeks, whereas no effect was registered in wild-type and heterozygous mice. Additionally, Caeiro and Vivas (2008)<sup>7</sup> showed that  $\beta$ -endorphin-MnPO administration produces a decrease in blood pressure and heart rate in normotensive animals and inhibits the pressor response evoked by an acute increase in plasma osmolality.

Numerous studies demonstrate the significant role of the  $\mu$ -opioid receptor (MOR) in sodium intake regulation. The MOR, together with two other classical types of opioid receptors,  $\kappa$  and  $\delta$ , belongs to the G-protein coupled receptor (GPCR) superfamily. All of these receptors predominantly couple to  $G_{\alpha i/o}$  type inhibitory G-proteins,<sup>8</sup> which inhibits adenylyl cyclase activity,<sup>9</sup> decreases calcium ion entry<sup>10</sup> and increases potassium ion efflux.<sup>11</sup> It has been demonstrated that central injection of the selective MOR agonist [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin (DAGO) significantly increased the intake of saline solution (at both 0.6% and 1.7% NaCl) in nondeprived rats.<sup>12</sup> Moreover, systemic injection of morphine increased the preference of mice and rats for normally aversive hypertonic NaCl solutions (1.5%–30% NaCl).<sup>13,14</sup> Increased MOR signalling along the nucleus accumbens, ventral pallidum and central amygdala nucleus (CeA) has been mainly associated with the hedonic palatability of NaCl when it is tasted.<sup>5,15–17</sup> MOR activity along the brainstem, within the lateral parabrachial nucleus (LPBN) and the nucleus of the solitary tract (NTS), mainly modulates motivated sodium/food intake and blood pressure.<sup>18–21</sup>

However, experiments such as these fail to determine the mechanisms by which endogenous MOR signalling acts to modulate sodium appetite and blood pressure regulation. The present study aimed to investigate the effect of increased salt intake (2% NaCl solution) in rats during a short period of time (4 days) on MOR mRNA expression along the MnPO and the NTS. We also explored brainstem and MnPO MOR G-protein activity (efficacy and potency), well as brainstem MOR binding capacity, to determine whether sodium overload induced any changes in the signalling and binding properties of the MOR in these brain regions. Finally, we also examined the changes in plasma concentrations of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> and body weight of the animals after the sodium overload.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

Tris-HCl, ethylene glycol tetraacetic acid (EGTA), NaCl, MgCl<sub>2</sub> × 6H<sub>2</sub>O, GDP and the GTP analogue GTP $\gamma$ S were purchased from Sigma-Aldrich (Budapest, Hungary). The highly selective MOR agonist enkephalin analog, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO), was obtained from Bachem Holding AG (Bubendorf, Switzerland). The nonselective opioid receptor antagonist, naloxone, was kindly provided by Endo Laboratories of DuPont de Nemours (Wilmington, DE, USA). Ligands were dissolved in water and were stored in 1 mmol L<sup>-1</sup> stock solution at -20°C. The radiolabelled GTP analogue [<sup>35</sup>S]GTP $\gamma$ S (specific activity: 1250 Ci mmol<sup>-1</sup>) was purchased from PerkinElmer (Budapest,

Hungary). [<sup>3</sup>H]DAMGO (specific activity: 38.8 Ci mmol<sup>-1</sup>) was radiolabelled in the Isotope Laboratory of BRC (Szeged, Hungary) and has been characterised previously.<sup>22</sup> The Ultima Gold MV aqueous scintillation cocktail was purchased from PerkinElmer (Budapest, Hungary).

### 2.2 | Animals

As a result of collaboration, we used two rat strains provided by each local research institute. Male, Wistar-derived rats (350–400 g) from the colony of the Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET, Universidad Nacional de Córdoba, Córdoba, Argentina) were used for the relative MOR mRNA expression and plasma osmolality and electrolyte analysis. The MOR binding experiments were carried out in male Sprague-Dawley rats (200–300 g) obtained from the animal house of the Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged (Szeged, Hungary).

Animals were kept under a 12:12 hour light/dark cycle, in a temperature-controlled environment, with food and water available *ad libitum* until the initiation of the experiments. Each animal was used in only one experimental condition. All of the experimental protocols in Wistar rats were approved and carried out in accordance with the guidelines of the Ferreyra Institute Ethical Committee for the use and care of laboratory animals, as well as the guidelines of the International Public Health Service Guide for the Care and Use of Laboratory Animals. Housing and experiments performed in Sprague-Dawley rats were in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII. tv. 32.§, registration number: IV./141/2013.). All efforts were made to minimise animal suffering and to reduce the number of animals used.

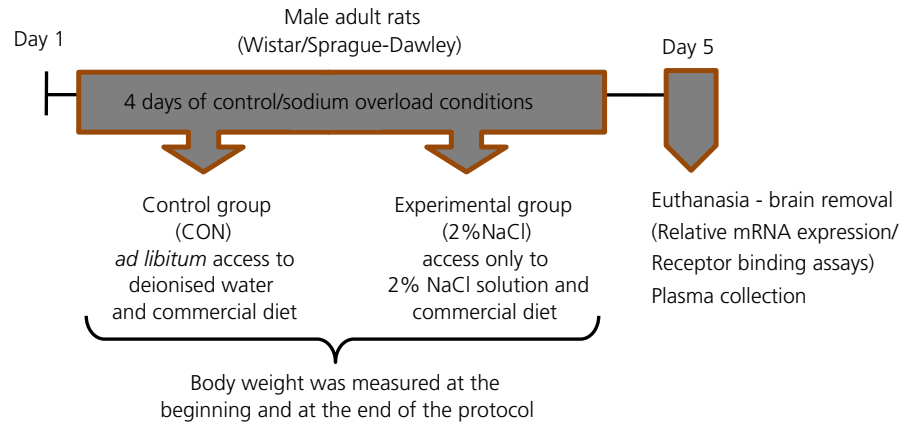
### 2.3 | Experimental design

As indicated in Figure 1, the experimental group had access only to 2% NaCl solution (2% NaCl), whereas the control group (CON) had *ad libitum* access to deionised water during the 4-day protocol period, after which the animals were used for the appropriate experimental set-up. Both experimental and control groups had normal access to commercial diet. Plasma was collected from both groups (CON and 2% NaCl) at the end of the treatment to measure plasma osmolality and electrolyte concentrations. Body weight was measured in both groups at the beginning and at the end of the protocol.

### 2.4 | Relative mRNA expression of MOR (*Oprm1*)

#### 2.4.1 | Brain microdissection, tissue collection, RNA extraction and calibration of primers

After 4 days of control or sodium overload conditions, the animals were decapitated and brains immediately excised and stored at -80°C for mRNA determination. Coronal sections of 540  $\mu$ m in the



**FIGURE 1** Schematic diagram showing the experimental design and protocols

MnPO and of 780  $\mu\text{m}$  in the nucleus of the solitary tract (NTS) were obtained from the frozen brains in a microtome with a stainless-steel punch needle. The brain nuclei were identified and delimited in accordance with a rat brain atlas.<sup>23</sup> The mRNA was isolated from micropunches of specific brain areas, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions with some modifications: RNA precipitation with isopropanol was performed overnight at  $-20^{\circ}\text{C}$ . The RNA was treated with DNase (Fermentas, Glen Burnie, MD, USA) and quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (NanoDrop, Wilmington, DE, USA) and then reverse-transcribed into cDNA (enzyme RTM-MLV) (Promega, Madison, WI, USA).

Brain *Oprm1* gene expression was determined using Syber Green Real-Time PCR Master Mixes (Applied Biosystems, Foster City, CA, USA) in the Step One Real-Time equipment (Applied Biosystems). PCR amplification efficiency was established by means of 5-point 1:10 calibration curves. The selected dilution for the samples was 1:10 from the initial RT PCR concentration. The  $C_t$  values of the samples fell into the linear dynamic range of the calibration curve. All primer-pairs were confirmed to be 90%-110% efficient by means of the calibration curve, with efficiencies  $E = 2 \pm 0.1$  ( $E = 10^{[-1/\text{slope}]}$ ) and amplified a single product determined by melting curve analysis. The primer sequences are provided in Table 1.

#### 2.4.2 | Calculations of relative gene expression

The relative quantification was determined by the  $\Delta\Delta\text{Ct}$  method with STEPONE, version 2.2 (Thermo Fisher Scientific Inc., Waltham, MA, USA), where the fold change of mRNA content in the unknown sample relative to control group was determined by calculating a

ratio =  $(E^{\text{target}})^{\Delta\text{Ct}(\text{target})} / (E^{\text{Gapdh}})^{\Delta\text{Ct}(\text{Gapdh})}$ , where  $E$  is the efficiency of the primer set and  $\Delta\text{Ct} = \text{Ct}_{(\text{control})} - \text{Ct}_{(\text{experimental})}$ . For each sample, the  $\text{Ct}$  was determined and normalised to the average of the house-keeping gene, *Gapdh*. Relative quantifications of the target gene (*Oprm1*) were normalised to each control group. Data are presented as mRNA expression relative to the control calibrator group. The  $2^{-\Delta\text{CT}}$  method was used to determine the expression of *Gapdh* between treatments (CON vs NaCl 2%). The relative amounts of *Gapdh* were calculated using the  $2^{-\Delta\text{CT}}$  equation, where  $\Delta\text{CT} = \text{CT treated} - \text{CT control}$ .<sup>24</sup>

## 2.5 | Receptor binding assays

### 2.5.1 | Preparation of brain samples for binding assays

After the final day of treatment, 12 rats for the experimental and control conditions were decapitated and their brains were quickly removed. The brainstems were prepared for membrane preparation as described by Benyhe et al.<sup>25</sup> and partly used for saturation binding experiments and partly further prepared for the [<sup>35</sup>S]GTP $\gamma$ S binding protocols, as described by Zádor et al.<sup>26</sup> The brainstem and MnPO were separated in accordance with the rat brain atlas of Paxinos and Watson<sup>23</sup> and were homogenised and suspended in Tris-HCl, EGTA and  $\text{MgCl}_2$  buffer for [<sup>35</sup>S]GTP $\gamma$ S binding assays.

### 2.5.2 | Functional [<sup>35</sup>S]GTP $\gamma$ S binding experiments

The functional [<sup>35</sup>S]GTP $\gamma$ S binding experiments were performed as described previously,<sup>27,28</sup> with modifications. Briefly, the

**TABLE 1** Primer pairs for *Oprm1* and *Gapdh* mRNAs

	<i>Oprm1</i>	<i>Gapdh</i>
GenBank access number	NM_001304737.1	NM_017008.4
Primer forward 5'- to 3'	CTGTCTGCCACCCAGTCAAA	TGTGAACGGATTGGCCGTA
Primer reverse 5'- to 3'	TGCAATCTATGGACCCCTGC	ATGAAGGGTTCGTTGATGGC
Product size (bp)	150	93
Annealing temperature ( $^{\circ}\text{C}$ )	60 $^{\circ}\text{C}$	59 $^{\circ}\text{C}$

membrane fractions of brainstem and MnPO homogenates were incubated at 30°C for 60 minutes in Tris-EGTA buffer (pH 7.4) comprising 50 mmol L<sup>-1</sup> Tris-HCl, 1 mmol L<sup>-1</sup> EGTA, 3 mmol L<sup>-1</sup> MgCl<sub>2</sub> and 100 mmol L<sup>-1</sup> NaCl, containing 20 MBq/0.05 cm<sup>3</sup> [<sup>35</sup>S]GTPγS (0.05 nmol L<sup>-1</sup>) and increasing concentrations (10<sup>-10</sup> to 10<sup>-5</sup> mol L<sup>-1</sup>) of DAMGO. The experiments were performed in the presence of excess GDP (30 μmol L<sup>-1</sup>) in a final volume of 1 mL. Total binding was measured in the absence of test compounds, determining nonspecific binding in the presence of 10 μmol L<sup>-1</sup> unlabelled GTPγS. The difference represents basal activity. The reaction was terminated by rapid filtration under vacuum (M24R Cell Harvester; Brandel, Boca Raton, FL, USA) and washed three times with 5 mL of ice-cold 50 mmol L<sup>-1</sup> Tris-HCl (pH 7.4) buffer through Whatman GF/B glass fibres (GE Healthcare, Little Chalfont, UK). The radioactivity of the dried filters was detected in an UltimaGold MV aqueous scintillation cocktail (Perkin Elmer, Waltham, MA, USA) with a Tricarb2300TR liquid scintillation counter (Packard, Palo Alto, CA, USA). [<sup>35</sup>S]GTPγS binding experiments were performed in triplicate and repeated at least three times.

### 2.5.3 | Saturation binding experiments

Aliquots of frozen rat brainstem membrane homogenates were centrifuged (36288 g for 20 minutes at 4°C) to remove sucrose and the pellets were suspended in 50 mmol L<sup>-1</sup> Tris-HCl buffer (pH 7.4). Membranes were incubated in the presence of [<sup>3</sup>H]DAMGO in increasing concentrations (1.06 to 24.32 nmol L<sup>-1</sup>) at 35°C for 45 minutes. Both nonspecific and total binding were determined in the presence and absence of 10 μmol L<sup>-1</sup> unlabelled naloxone, respectively. The reaction was terminated by rapid filtration under vacuum (M24R Cell Harvester) and washed three times with 5 mL of ice-cold 50 mmol L<sup>-1</sup> Tris-HCl (pH 7.4) buffer through Whatman GF/C glass fibres. The radioactivity of the dried filters was detected in an UltimaGold MV aqueous scintillation cocktail with a Tricarb 2300TR liquid scintillation counter. The saturation binding assays were performed in duplicate and repeated at least three times.

## 2.6 | Plasma osmolality and electrolyte analysis

For the assay of plasma osmolality and electrolyte concentrations, we used groups of animals different from those used in the relative mRNA expression and receptor binding studies. The animals from both groups (CON and 2% NaCl) were decapitated and bled at the end of the 4 days of treatment. Trunk blood was collected in chilled tubes containing ethylenediaminetetraacetic acid (final concentration 2 mg mL<sup>-1</sup> blood) for centrifugation at 1008 g for 10 minutes at 4°C. Plasma electrolyte concentrations were measured with a Beckman Lablyte system (model 810; Beckman Instruments, Brea, CA, USA), plasma osmolality was determined from duplicate 8-μL plasma samples using vapor pressure osmometry (VAPRO 5520; Wescor Inc., Logan, UT, USA).

## 2.7 | Statistical analysis

### 2.7.1 | Relative mRNA expression of MOR (*Oprm1*), plasma osmolality, weight and electrolyte concentration data

Relative mRNA expression of MOR (*Oprm1*), plasma osmolality, weight and electrolyte concentration data were subjected to a *t* test, and the loci of significant effects were further analysed using a one-way ANOVA Tukey's test (type I error probability was set at .05). All experimental data are presented as the mean ± SEM.

### 2.7.2 | Receptor binding data

The specific binding of the radiolabelled compounds ([<sup>35</sup>S]GTPγS, [<sup>3</sup>H]DAMGO) was calculated by subtracting the nonspecific binding values from total binding values and expressed as a percentage. Data were normalised to total specific binding, which was set at 100%, which, in the case of [<sup>35</sup>S]GTPγS binding assays, also represents the level of G-protein basal activity. Experimental data are presented as the mean ± SEM as a function of the applied ligand concentration range, which, in the case of [<sup>35</sup>S]GTPγS binding assays, was indicated in logarithm form. Points were fitted with PRISM, version 5.0 (GraphPad Prism Software Inc., San Diego, CA, USA), using nonlinear regression. In the [<sup>35</sup>S]GTPγS binding assays, the 'Sigmoid dose-response' fitting was used to establish the maximal stimulation or efficacy ( $E_{max}$ ) of the G-protein coupled receptors and the ligand potency ( $EC_{50}$ ). Stimulation was given as a percentage of the specific [<sup>35</sup>S]GTPγS binding observed over basal activity, which was set at 100%. In saturation binding assays, the 'one site - specific binding' fitting equation was applied to establish the concentration of the radioligand that produced 50% of the maximal binding capacity or, in other words, the dissociation constant ( $K_d$ ) and the maximum binding capacity of the receptor ( $B_{max}$ ). The amount of receptors that specifically bound [<sup>3</sup>H]DAMGO is presented in fmol mg<sup>-1</sup>, as calculated by the total protein content and the amount of radioligand in the appropriate concentration point, as well as by the specific activity of the radioligand. An unpaired *t* test with a two-tailed *P* value was performed to determine the significance level, using PRISM, version 5.0. *P* < .05 level was considered statistically significant.

## 3 | RESULTS

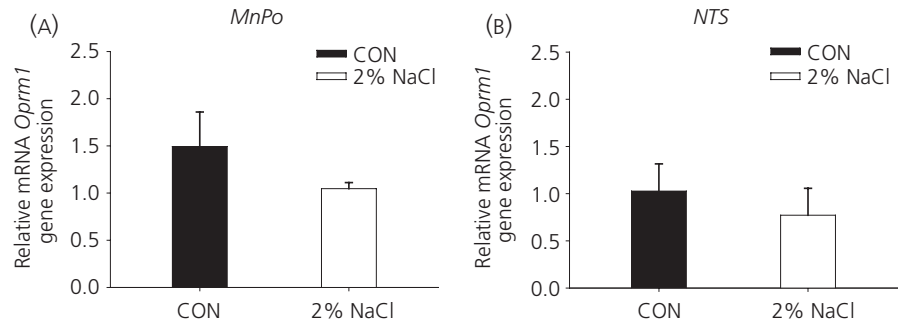
### 3.1 | Relative gene expression of the MOR (*Oprm1*)

After treatments (CON and 2% NaCl), no significant differences were observed in the relative gene expression of the μ-opioid receptor (*Oprm1*) in any of the brain nuclei analysed (Figure 2).

### 3.2 | MOR G-protein activity measurements

To test the effect of high sodium intake treatment on MOR activity (efficacy and potency), we performed functional [<sup>35</sup>S]GTPγS binding

**FIGURE 2** Relative mRNA expression of opioid receptor *Mu1* (*Oprm1*) at the (A) median preoptic nucleus (MnPO) and (B) nucleus of the solitary tract (NTS). Bar graphs show relative *Mu1* mRNA gene expression in control (CON) (open bars) and sodium overload (filled bars) rats. Values are the mean  $\pm$  SEM ( $n = 3$ –4 per group)



assays. This type of assay can monitor the GDP $\rightarrow$ GTP exchange of the  $G_{\alpha i/o}$  protein with the radioactive, nonhydrolysable GTP analogue [ $^{35}$ S]GTP $\gamma$ S during agonist-mediated receptor activation. The MOR was stimulated with increasing concentrations of the highly MOR-selective pure agonist peptide DAMGO. The assays were performed in control and experimental groups of rat brainstem membrane homogenate and in MnPO homogenates.

In the control group brainstem membrane homogenates, the agonist DAMGO increased the specific binding of [ $^{35}$ S]GTP $\gamma$ S in MOR  $G_{i/o}$  protein in a concentration-dependent manner. The increased specifically bound [ $^{35}$ S]GTP $\gamma$ S reached a 32% maximum over basal activity (100%), thus demonstrating a total of  $132 \pm 2.6\%$  maximum efficacy ( $E_{max}$ ) for the MOR G-protein, with a  $238.1 \text{ nmol L}^{-1}$  ( $pEC_{50}$ :  $6.62 \pm 0.2 \text{ mol L}^{-1}$ ) potency ( $EC_{50}$ ) of the agonist DAMGO (Figure 3A). The 2% sodium intake significantly enhanced MOR agonist-mediated maximum G-protein efficacy ( $143.7 \pm 2.3\%$ ) (Figure 3A), whereas the potency of DAMGO remained unaltered ( $pEC_{50}$ :  $6.81 \pm 0.13 \text{ mol L}^{-1}$ ) (Figure 3A).

In the MnPO homogenates, DAMGO activated MORs G-protein over the basal activity more effectively than in the brainstem because the activation resulted in  $168.6 \pm 3.6\%$  MOR maximum efficacy, which is approximately 30% more than in the brainstem (Figure 3B). In the MnPO, the potency of DAMGO was slightly lower than in the brainstem, at  $271.4 \text{ nmol L}^{-1}$  ( $pEC_{50}$ :  $6.56 \pm 0.12 \text{ mol L}^{-1}$ ) (Figure 3B). However, 2% sodium chloride consumption did not cause significant changes either in G-protein efficacy ( $163.5 \pm 5.7\%$ ) (Figure 3B) or agonist potency ( $pEC_{50}$ :  $6.41 \pm 0.2 \text{ mol L}^{-1}$ ) (Figure 3B).

### 3.3 | MOR binding capacity measurements

In the next step, we investigated the correlation of the enhanced G-protein activity of MOR and the higher levels of available MORs in the brainstem after chronic sodium exposure. We performed saturation binding experiments, in which we saturated MORs with increasing concentrations of [ $^3$ H]DAMGO. Thus, we could calculate the maximal binding capacity ( $B_{max}$ ) of the MOR and the dissociation constant ( $K_d$ , binding affinity) of [ $^3$ H]DAMGO after normal sodium or high sodium intake in brainstem membrane homogenates. The high sodium intake treatment did not change the maximal binding capacity of the MOR ( $96.2 \pm 7.9 \text{ fmol g}^{-1}$  vs  $86.3 \pm 5.5 \text{ fmol g}^{-1}$ ) (Figure 3) or the  $K_d$  value of the [ $^3$ H]DAMGO ( $5.3 \pm 1 \text{ nmol L}^{-1}$  vs  $5 \pm 0.8 \text{ nmol L}^{-1}$ ) (Figure 4).

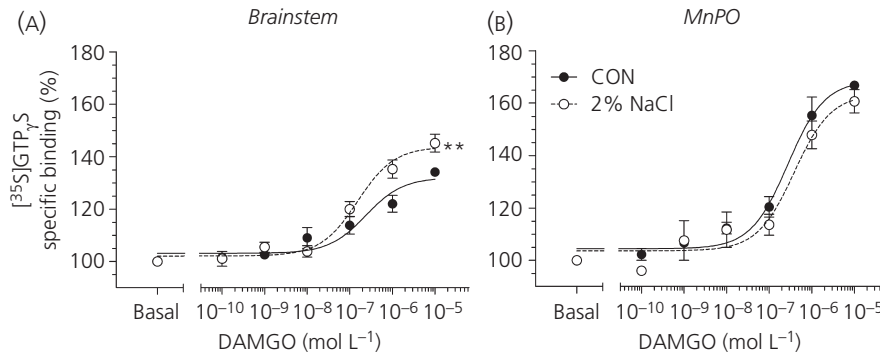
### 3.4 | Plasma osmolality, electrolyte and body weight analysis

No differences were observed in plasma osmolality and plasma electrolyte concentrations in control and experimental groups (Table 2). However, the 4 days of 2% NaCl ingestion reduced weight gains in both Wistar and Sprague-Dawley rats compared to their respective control groups (Table 3).

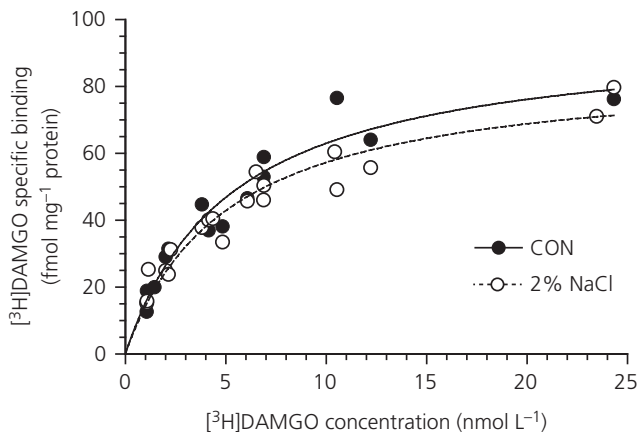
## 4 | DISCUSSION

Based on previous data demonstrating the importance of MOR signalling in body sodium homeostatic responses, the present study aimed to investigate whether increased salt intake in rats may induce changes in MOR mRNA expression, G-protein activity and MOR binding capacity in brain regions previously involved in salt intake and blood pressure regulation. Although, after 4 days of obligatory hypertonic sodium chloride intake, there was no difference in the binding properties of the MOR system, there were clearly evident changes in the maximal efficacy of MORs G-protein, increasing the receptor activity for signalling within the brainstem.

Our results also showed that, at least along the MnPO and at brainstem level, MOR binding capacity and mRNA receptor expression did not change and thus did not explain the  $\beta$ -endorphin modulation after sodium loading conditions. If we hypothesise that more receptors are available on the surface membrane along the brainstem, then more G-proteins could couple to them, thus increasing their maximum efficacy. However, our saturation binding experiments exclude this hypothesis: the MORs were saturated to an almost equal level in the control and experimental groups in this brain region, indicating no alteration in the number of available MORs on the cell surface. This was confirmed by our G-protein activity measurements, in which the control and experimental brain samples had similar radioactivity values of specifically bound [ $^{35}$ S]GTP $\gamma$ S (data not shown). We can also rule out the possibility of improved binding capacities of MORs after the high salt intake because neither the potency, nor the dissociation constant was altered in the brainstem, comprising parameters that describe the binding properties of DAMGO and [ $^3$ H]DAMGO, respectively. The most likely explanation of our results is that the amount of  $G_{i/o}$ -protein available for coupling was increased after



**FIGURE 3**  $\mu$ -opioid receptor G-protein activity in DAMGO-stimulated [ $^{35}$ S]GTP $\gamma$ S binding assays in rat (A) brainstem membrane and (B) median preoptic nucleus (MnPO) homogenates during sodium overload. Specifically bound [ $^{35}$ S]GTP $\gamma$ S is indicated as a percentage in the presence of increasing concentrations ( $10^{10}$  to  $10^{-5}$  mol L $^{-1}$ ) of DAMGO in control (CON) and after 2% NaCl treatment in rats in the indicated brain areas.\*Significant alteration in  $E_{\max}$  value after treatment (unpaired  $t$  test, two-tailed  $P$  value). Points and columns represent the mean  $\pm$  SEM for at least three experiments performed in triplicate.  $\mu$ -Opioid receptor (MOR) basal activity was set as 100%. \* $P < .01$



**FIGURE 4**  $\mu$ -Opioid receptor (MOR) maximal binding capacity during sodium overload in rat brainstem membrane homogenate in saturation binding experiments. Specifically bound fixed [ $^3$ H]DAMGO is indicated in cpm in increasing ( $0.7$ – $50.3$  nmol L $^{-1}$ ) concentrations in control (CON) and following 2% NaCl treatment.  $B_{\max}$ , maximum binding capacity; cpm, counts per minute

**TABLE 2** Plasma osmolality and electrolyte concentrations after 4 days of sodium overload

	CON	2% NaCl
OSM (mOsm kg $^{-1}$ H $_2$ O)	309 $\pm$ 1.97	314.14 $\pm$ 4.27
Na $^+$ (mEq L $^{-1}$ )	140.12 $\pm$ 1.08	140.88 $\pm$ 1.35
K $^+$ (mEq L $^{-1}$ )	8.03 $\pm$ 0.11	9.06 $\pm$ 0.31
Cl $^-$ (mEq L $^{-1}$ )	99.90 $\pm$ 2.32	99.76 $\pm$ 2.27

Data are the mean  $\pm$  SEM.

4 days of hypertonic sodium intake, perhaps activating this system and counterbalancing the sodium-overload increase in sympathetic activity and blood pressure and provoking the well-known sodium overload-induced anorexia.

Sodium ion has long been known to inhibit opioid agonist binding at near physiological concentrations ( $100$ – $140$  mmol L $^{-1}$ ) *in vitro*,<sup>29,30</sup>

and, moreover, a distinct binding pocket on the MOR has recently been described for the ion.<sup>31</sup> It has also been demonstrated that lower sodium concentrations increased basal G-protein coupling, which reduced the DAMGO-mediated MOR G-protein maximum stimulation.<sup>32</sup> The sodium ion can also promote the activation of the MOR by enhancing the movement of water molecules toward the allosteric site, as indicated by molecular dynamics simulations.<sup>33</sup> Although there were no differences in plasma sodium concentration between control and experimental groups when monitored after 4 days of 2% NaCl ingestion, sodium concentrations may change during the 4-day study, contributing to the enhancement of G-protein activity. Thus, a “sodium effect” on MOR binding and signaling cannot be entirely excluded.

Another interesting observation of this model was that, despite the plasma sodium concentration and osmolality remaining at the physiological level, reflecting the different types of efficient renal compensatory mechanisms, the body weight of animals after 4 days of 2% NaCl ingestion was significantly lower than that of the control group (Table 3). This may reflect a loss of body fluid stimulated by renal mechanisms that attempt to compensate for the high plasma sodium concentration, and particularly the natriuresis-driven diuretic water loss mechanism. On the other hand, our preliminary food intake data (not shown) indicate that there is a comparatively reduced food intake in sodium overload animals, which may also explain their body weight loss. However, another mechanism triggered by dietary high salt intake during long-term studies ( $100$ – $200$  days) has been described recently.<sup>34</sup> In this case, high salt intake may reprioritise osmolality and energy metabolism for body fluid conservation (mainly by urea production by liver and skeletal muscle), which provokes a reduction in body weight. This stage may be still not reached in our short-term study.

The brainstem pathways leading to the perception of salt involve a circuitry in which the NTS and LPBN are the key sites that receive and integrate both homeostatic salt and satiety signals. The LPBN, for example, receives direct inputs from central osmo-sodium receptors located in the sensory circumventricular organs of the

**TABLE 3** Body weights (initial, final and delta) in the control and experimental groups of Wistar and Sprague-Dawley rats after 4 days drinking water or 2% NaCl solution, respectively (n = 10-12)

	Wistar		Sprague-Dawley	
	CON	2% NaCl	CON	2% NaCl
Initial body weight (g)	376.67 ± 11.15	373.71 ± 12.63	301.58 ± 4.11	304.92 ± 3.87
Final body weight (g)	431.33 ± 8.97	399.86 ± 12.46	342.83 ± 2.99	333.5 ± 3.59
Gain in weight (g)	54.67 ± 4.22	26.14 ± 9.72*	41.25 ± 3.37	28.58 ± 1.77*

\*Significant difference compared to control (CON) (t test,  $P < .05$ ).

Data are the mean ± SEM.

lamina terminalis, and indirect inputs from peripheral osmoreceptors through the vagus nerve.<sup>35</sup> Many neurotransmitters/neuro-modulators regulate its responses (serotonin, angiotensin, GABA, noradrenaline) and opioid signalling has been shown to modulate salt appetite.<sup>20,21,36</sup> The LPBN contains neurones that express enkephalins and MORs<sup>37</sup> and pharmacological activation of MOR within this nucleus increases salt consumption.<sup>20,21</sup> A second major pathway within the brainstem by which the brain detects body sodium status is via neurones residing in the dorsocaudal subregion of the NTS that has fenestrated capillaries and expresses 11 $\beta$ -hydroxysteroid dehydrogenase type 2.<sup>38</sup> This nucleus contains the second-order salt-sensitive neurones that receive gustatory information coming from the first-order sensory neurones within the lingual branch of the glossopharyngeal nerve, the superficial petrosal branch of the facial nerve and the laryngeal branch of the vagus nerve.<sup>39</sup> In addition to receiving gustatory information about salt, it is important to take into account that a bi-directional opioid-opioid signalling pathway exists between the rostral NTS and the CeA, which influences appetitive behaviours such as food intake via MORs.<sup>40</sup> A similar opioid circuitry exists within the LPBN that projects to the CeA, which then activates the mesolimbic reward system involved in the motivation to consume salt and its rewarding palatability.<sup>5,17,41</sup>

In conclusion, the results of the present study have revealed that acute sodium overconsumption increases the maximal efficacy of MORs G-protein, increasing the receptor activity for signalling within the brainstem. This probably reflects the influence of the endogenous brainstem  $\mu$ -opioid receptor system with respect to regulating the maintenance of normal blood pressure levels and/or enhancing sodium taste aversion and sodium overload-induced anorexia in response to central and visceral homeostatic inputs. For further studies, it would be interesting to examine whether the enhanced MOR G-protein activity further affects Gi/o-mediated signalling, such as adenylate cyclase activity.

## ACKNOWLEDGEMENTS

The present study was supported by Bilateral Scientific and Technological Joint Project provided by the National Research Development and Innovation Office (NKFI; grant number: TÉT 10-1-2011-0085) and the National Minister of Science and Technology of Argentina (MINCYT-NKTH, grant code HU/10/03). It

was also supported in part by grants of CONICET and SECYT to LV. LV and XEC are members of CONICET. FMD holds a CONICET fellowship. The authors thank Dr Sándor Benyhe (Biological Research Centre, Hungarian Academy of Sciences) for critically reading the manuscript and Adrienn Seres and Árpád Márki (Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Szeged) for their assistance. They also thank Joss Heywood for revising the language of the final version of the manuscript submitted for publication.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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**How to cite this article:** Dadam F, Zádor F, Caeiro X, et al. The effect of increased NaCl intake on rat brain endogenous  $\mu$ -opioid receptor signalling. *J Neuroendocrinol*. 2018;30:e12585. <https://doi.org/10.1111/jne.12585>