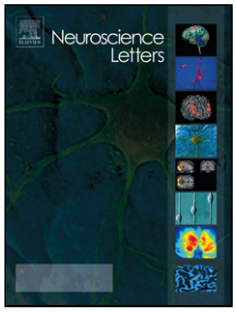


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PII: S0304-3940(16)30959-4
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2016.12.024
Reference: NSL 32493

To appear in: *Neuroscience Letters*

Received date: 10-11-2016
Revised date: 5-12-2016
Accepted date: 12-12-2016

Please cite this article as: Y.Campos-Jurado, L.Martí-Prats, T.Zornoza, A.Polache, L.Granero, M.J.Cano-Cebrián, REGIONAL DIFFERENCES IN MU-OPIOID RECEPTOR-DEPENDENT MODULATION OF BASAL DOPAMINE TRANSMISSION IN RAT STRIATUM, Neuroscience Letters http://dx.doi.org/10.1016/j.neulet.2016.12.024

# REGIONAL DIFFERENCES IN MU-OPIOID RECEPTOR-DEPENDENT MODULATION OF BASAL DOPAMINE TRANSMISSION IN RAT STRIATUM

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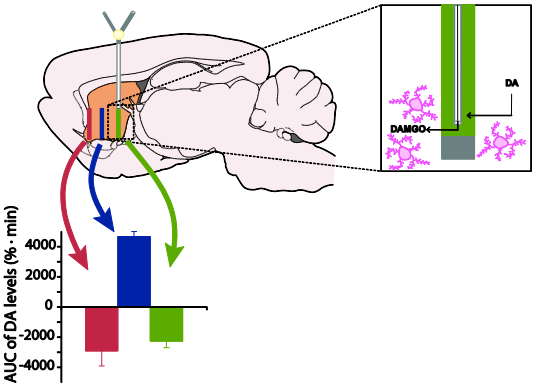
**Graphical abstract**




**Highlights:**

- MOR receptor is able to modulate Dopamine release in Dorsal Striatum
- Modulation of dopamine release could be exerted in a subregion-dependent manner
- Our work may support the existence of MOR receptors at presynaptical level onto dopaminergic neurons in rostral and caudal poles

**Abstract**

The nigrostriatal dopamine system is implicated in the regulation of reward and motor activity. Dopamine (DA) release in dorsal striatum (DS) is controlled by the firing rate of DA neurons in substantia nigra *pars compacta*. However, influences at terminal level, such as those involving activation of mu opioid receptors (MORs), can play a key role in determining DA levels in striatum. Nonetheless, published data also suggest that the effect of opioid drugs on DA levels may differ depending on the DS subregion analysed. In this study, *in vivo* microdialysis in rats was used to explore this regional dependence. Changes in basal DA levels induced by local retrodialysis application of DAMGO (selective MORs agonist) in three different subregions of DS along the rostro-caudal axis were studied. Our results indicate that whereas administration of 10µM DAMGO into the rostral and caudal DS significantly reduced DA levels, in medial DS an increase in DA levels was observed. These data reveal a regional-dependent MOR modulation of DA release in DS, similar to that described in the ventral striatum. Our findings may lead to a better understanding of the nigrostriatal DA system regulation.

**Keywords:** dorsal striatum, microdialysis, mu-opioid receptor, dopamine.

**Introduction**

The endogenous opioid system exerts an important neuromodulatory effect on the activity of the two main dopaminergic systems of the brain. On one hand, it is well demonstrated that opioid receptors regulate mesolimbic dopaminergic neuronal activity through mu-, delta- and kappa-opioid receptors [1-2] . This neuromodulatory effect is not only exerted at the proximal level of this projection; several investigations have also examined the involvement of the opioid receptors located at the terminal level in NAc in the opiate modulation of DA release [3-6]. In general, results of these studies show that application of different MORs and DORs agonists into the NAc by means of retrodialysis, alters DA extracellular levels in this brain area in a dose-dependent manner.

On the other hand, opioid receptors also modulate the activity of DA neurons in the nigrostriatal dopaminergic projection. As described for the mesolimbic pathway, opioid receptors within the substantia nigra *pars compacta* (SNpc) control the activity of DA neurons [7-9]. Similarly, MORs located at terminal level in the striatum are also involved in the control of the DA release from this projection [9-15]. It is not clear, however, if the consequences of local MORs activation in striatal DA levels are equivalent throughout the DS. For example, some authors report that activation of MORs enhances DA extracellular concentration in the dorsal striatum (DS) [8, 9, 11-13],

whereas others have shown the opposite effect [16, 17]. We hypothesize that these discrepancies could be the result of regional differences in the modulatory effect of MORs. The existence of such regional differences in the opioid control of DA release at terminal level has already been demonstrated in the NAc [6, 18]. Our group showed that local application of DAMGO ([D-Ala2, N-Me-Phe4, Gly5-ol]-encephalin] a selective agonist of MORs, caused a significant increase in DA levels in the NAc core, whereas the infusion of the same dose in the shell subregion provoked the opposite effect [6]. These differences in the neurochemical response could be indicative of important underlying shell/core differences in the anatomical organization and connectivity.

Several authors have described a different spatial organisation of MORs within the DS. Their results showed a heterogeneous distribution, being particularly abundant bordering and limiting the dorsolateral part of the striatum, mainly inside the patches at the level of the medial DS and the rostral pole of striatum, and forming a streak just below the corpus callosum [19-22]. If this region-specific distribution of MORs within the DS has specific consequences on DA release, has not been explored yet.

The present experiments were planned in order to shed light on this subject. Concretely, we focused on the analysis of possible differences in the modulatory role of MORs on DA release along the rostro-caudal axis of DS. By employing the selective agonist, DAMGO, the consequences of a local application of this drug on the DA extracellular levels in three areas of the DS, the *rostral pole*, the *medial area* and the *caudal pole* were tested. Our results show the existence of significant regional-dependent differences in the opioid modulation of the DA release in the DS.

## Materials and Methods

### *Animals and surgery*

Male albino Wistar rats (300–350 g) were used for the experiments. Rats were housed in plastic cages (27 x 50 x 14 cm) in groups of 4–6 with controlled humidity and temperature (22°C) and a 12/12 h light/dark cycle (on 08:00, off 20:00) with free access to food and water. All procedures were conducted in strict adherence to the EEC Council Directive 2010/63/UE, Spanish laws (Royal Decree 53/ 2013) and policies on animal protection. Experiments were approved by the Animal Care Committee of the University of Valencia and authorized by the Regional Government (*C*onselleria de Agricultura, Ganadería y Pesca).

On the day prior to the experiment, animals were anesthetized with ketamine/xylazine (95 mg/kg of ketamine and 10 mg/kg of xylazine, ip). Rats were stereotaxically (Stoelting) implanted with vertical concentric-style microdialysis probes with 3 mm of

active membrane (Hospal AN69; molecular cutoff 60,000 Da; Bologne, Italy), constructed according to Santiago and Westerink [23], targeting three different dorsal striatum (DS) subregions: the rostral pole, the medial DS and the caudal pole. Probes were implanted in the rostral pole with the tip targeted at AP = 2.5 mm, ML = ±2.5 mm from bregma and DV = 7.5 mm from dura [24]. For the medial DS, probes were also lowered vertically and targeted to AP = 2.0 mm, ML = ±2.5 mm from bregma and DV = 7.5 mm from dura. Finally, for the caudal pole, probes were also lowered vertically and targeted at AP = -0.2 mm, ML = ±3.5 mm from bregma and DV = 7.5 mm from dura. All surgical procedures were performed with the animal's head in 'flat skull' orientation. Probes were anchored to the skull with the aid of two stainless-steel screws and dental cement. After surgery, rats were housed individually in cylindrical cages (30 cm diameter) in which they had free access to food and water. Experiments were performed 20-24 hours after surgery.

*Microdialysis and analytical procedures*

PE10 inlet tubing was attached to a 2.5 mL syringe (Hamilton), mounted on a syringe pump (Harvard Instruments, South Natick, MA, USA) and connected to the probes. Probes were continuously perfused with artificial cerebrospinal fluid (aCSF) comprising 0.1 mmol/L aqueous phosphate buffer containing (expressed in mmol/L) 147 NaCl, 3.0 KCl, 1.3 $CaCl_2$ and 1.0 $MgCl_2$ (pH = 7.4) at a flow rate of 3.5 µL/min. Dialysate fractions were on-line analyzed for DA content every 20 min by HPLC with electrochemical detection, according to a previously reported procedure [25]. Drug delivery and sample collection time were corrected for the lag time resulting from the dead volume of the inlet and outlet tubes used to connect the rat to the HPLC system. The HPLC system consisted of a Waters 510 series pump in conjunction with an electrochemical detector (Mod. Decade, Antec, Leyden, The Netherlands). The applied potential was = 0.55 V (ISAAC cell, Antec, Leyden, The Netherlands). Dialysates were injected into a 2.1 mm RP-18 column (Phenomenex, Gemini-NX 3u 100x2.00 mm) via a VALCO valve fitted with a 65 µL sample loop. The mobile phase consisted of a sodium acetate/acetic acid buffer (0.1 mol/L, pH = 4.5) containing 150 mmol/L of sodium chloride, 85 mg/L of 1-octanesulfonic acid, 100 mg/L of EDTA and 105 mL/L of methanol. The mobile phase was pumped through the column at a flow rate of 0.06 mL/min. Chromatograms were analysed and compared with standards run separately on each experimental day, using the AZUR 4.2 software (Datalys, France).

The detection limit was defined by a signal-to-noise ratio of 2:1 which was approximately 6 fmol/sample (≈ 0.1 nmol/L).

*Drugs and experimental design*

Three experimental groups (initially n=7) were used for this experiment, one for each DS region studied. Rats were randomly assigned to each experimental group. On completion of the experiments and after the application of the inclusion/rejection criteria (see below), experimental groups for rostral pole and medial DS consisted of n=5 rats per group whereas caudal area consisted of n=6.

The MORs agonist, DAMGO, was purchased from Sigma–Aldrich Co. The drug was freshly dissolved in aCSF fluid and locally administered into the appropriate subregion of the DS via retrograde dialysis through the probes. Following an initial washing period (approximately 60 min), dialysate samples were collected at 20-min intervals. Once the DA basal level was established (defined as four consecutive samples with less than 10% variation in DA content) the DAMGO solution (10μM) was perfused for 40 min through the dialysis probe. The dose and time of application were selected from previously published data [6, 13, 18]. After the retrodialysis application of the drug, experiments were continued over an additional period of 120 min.

*Probe placement validation and inclusion/rejection criteria*

A careful evaluation of probe placements was histologically performed at the end of the experiments by a researcher who was unaware of the experimental group of animals that served to definitively include or reject animals in the experimental groups. To validate probe placement, rats were overdosed with isoflurane and brains were quickly removed, immediately frozen in isopentane and kept at -25$^{o}$ C until histological analysis. To this end, brains were cut in a cryostate (40 mm-thick), and were subjected to standard cresyl violet staining to verify the probe placement.

The atlas of Paxinos and Watson [24] was used as a reference for all probe location validations and to define the inclusion/rejection criteria. Three anatomical subregions were considered for inclusion along the anterior/posterior axis of the DS. Probe locations were considered in the rostral pole when the tract of the probe was located between the 2.5-2.2 mm AP planes from the bregma point. The probe was considered correctly located in the medial area when the tract was observed between the 2.1-1.5 mm A/P planes from the bregma point. Finally, probes were considered correctly located in the caudal pole when tracts were between 0.0-(-0.4) mm A/P planes from the bregma point.

*Data analysis*

Basal levels of DA in dialysates were expressed as nmol/L. Four basal values were averaged to obtain the baseline mean for each animal. These basal levels were then pooled to yield the group mean (one for each region analysed). Between-groups differences in the basal levels of DA in dialysates were evaluated using one-way analysis of variance (ANOVA). When significant differences were found, the multiple comparison Tukey test was applied.

Furthermore, DA levels obtained in each sample were transformed to percentages of each rat baseline. The effect of local application of DAMGO in every experimental subregion was analysed by a one-way repeated measures ANOVA. Whenever significant differences were found, *post-hoc* Dunnet's test were performed in order to identify the time points that differed significantly from its respective basal value (time 0 in figures). On the other hand, between-groups differences in each experimental time were assayed using a one-way ANOVA followed by the Tukey test when appropriate. The level of significance was always set at $p < 0.05$. Areas under the curve (AUC) for DA levels expressed as % of baseline from 80 to 240 minutes were estimated by means of the linear trapezoidal rule. The curve was subdivided into 8 different trapezoids. The base of each trapezoid was always 20 min and the respective height was the arithmetic mean of the contents of DA (as % of baseline) defining the two vertical sides of the trapezoid. AUCs values are, therefore, expressed in units of (min x %). AUCs were analysed by means of one-way ANOVA and the post-hoc Tukey test.

**Results**

*Probe placements*

Typical probe placements within the rostral pole, medial area and caudal pole of DS are shown in Fig. 1. Probe placements within the DS were confirmed using histological analysis. All probe placements are schematically shown in Fig.2D.

Only data from the rats in which probes were correctly placed inside the acceptance areas (see *Probe placement validation and inclusion/rejection criteria*) were included in the statistical comparisons (as indicated above: n= 5 for the rostral pole and medial area experimental groups, and n=6 for caudal pole group). A total of five animals were finally excluded from the groups based on the inclusion/rejection criteria.

[Insert "Figure 1"]

*Differences in basal dialysate concentrations of DA in regions of DS*

Mean baseline levels of DA in dialysates corresponding to each DS subregion are provided in **Table 1**. Statistical analysis revealed significant differences in extracellular DA basal levels ($F(2,13) = 6.470$, $p = 0.011$). Tukey test showed differences between the medial area and the caudal pole (p=0.011).

As can be seen in Figure 2, retrodialysis application of 10 µM DAMGO provoked changes in DA levels in the DS that apparently differed depending on the subregion analyzed. Statistical analysis showed that DA levels in the rostral pole of DS significantly decreased after DAMGO application (average 72 % of baseline) 40 min after the beginning of DAMGO administration ($F(8,32)= 3.952$, $p=0.002$), whereas in the medial area, DAMGO caused a persistent increase which was statistically significant from 80 min post-administration ($F(8,32)= 4.902$, $p=0.001$). In the caudal pole of DS, DAMGO administration provoked a significant decrease in extracellular DA levels to 76% of baseline ($F(8,40)= 3.514$, $p=0.004$), although the DA levels in this area showed a slight tendency to recover basal levels at the end of the experiment (Fig.2). As indicated above, DA levels in the medial DS increased up to 156% of the baseline at the last time interval analyzed and remained significantly high at the end of the experiment.

AUCs for DA content corresponding to each DS subregion are provided in **Table 2**. Statistical analysis revealed the existence of significant differences in AUCs ($F(2,13) = 38.48$, $p < 0.0001$). Tukey test showed differences between the medial area and the caudal pole (p=0.0001) and between the medial area and rostral pole (p=0.0001).

[Insert “figure 2”]

**Discussion**

The present study shows that intrastriatal administration (via retrodialysis) of the MORs agonist DAMGO alters DA extracellular levels in a regional-dependent manner. Whereas the application of this drug in the medial DS induces a significant and persistent increase in the DA levels, the administration of the same dose in the rostral and caudal poles produces a significant decrease. To our knowledge, this is the first time that a regional-dependent modulation of dopaminergic activity has been demonstrated at the terminal level in DS by MORs. Our data suggest that circuits modulating dopaminergic activity in the medial area could differ from those existing in the rostral and caudal areas of the DS.

Our results are compatible with previous anatomical findings [20, 22], revealing a higher density of MORs in the rostral and caudal areas of DS. Unfortunately, these authors did not specify if MORs are located at terminal level of DAergic projections or in soma of the different cell types present in DS. Our results in rostral and caudal poles of DS seem to suggest that MORs could be presynaptically located at the DAergic terminals in these subregions. Activation of MORs by DAMGO could provoke a hyperpolarization that would reduce DA release of DA at the synapsis. You and collaborators, in a set of experiments performed in the caudal area of DS, analyzed the effect of local perfusion via retrodialysis of 1μM and 10μM CTOP, a selective MORs antagonist, on DA release in the caudal area of striatum. Their results showed a dose dependent increase of extracellular dopamine in the caudal area of DS, suggesting that DA release could be under tonic inhibitory modulation [15].

Our results also show that administration of 10 μM DAMGO into the rostral pole of DS provokes a significant decrease of DA extracellular levels. In interpreting our results, we must take into account that in some animals, 20-30% of the active portion of the microdialysis probe was located in the caudal portion of the medial region between the shell and core of NAc. This fact could have affected our data, so we should be cautious in the interpretation of the results from the rostral pole of DS, especially in relation to DA basal levels. Nonetheless, it is interesting to highlight that a pronounced change in the connectivity and/or the organization of the striatum was observed. To the best of our knowledge, the modulation of DA release in the rostral extreme of DS by MORs has so far not been analyzed. According to our data, DAMGO produces similar effects in this subregion and in the caudal pole, thus it seems plausible to assume again the presence of MORs presynaptically in the DAergic terminals. Nevertheless, further experiments using both competitive antagonists of these receptors and local retrodialysis application of them into these two subregions, will contribute to confirm our hypothesis.

On the other hand, our data also indicate that opioid circuits modulating dopaminergic activity in the rostral and caudal poles could differ from those in the medial area of striatum, since the administration of 10 μM DAMGO into the medial area of striatum induced a persistent increase of extracellular DA (up to 156% of baseline). Again, our data are in agreement with previous studies on the role of opioid receptors in the modulation of DA in striatum using very similar probe locations and experimental protocols to those used by us. For example, Dourmap and collaborators demonstrated that local administration of 10 μM DAMGO via retrodialysis into the so-called medial DS provoked an increase of 140% of extracellular DA [9, 12, 13]. These authors suggested a direct implication of cholinergic interneurons in the control of DA release through a $M_2$-mediated mechanism, since the injection into the medial DS of AF 64-A, a toxin for cholinergic neurons, or the concomitant infusion of the $M_2$-muscarinic antagonist methoctramine, abolished the effect of DAMGO on the DA release.

However, other research groups obtained opposite results in this subregion of DS. Thus, Piepponen and collaborators [16] showed that both morphine and 1μM DAMGO, intrastriatally-administered by retrodialysis, were able to dose-dependently decrease the extracellular concentration of DA. This discrepancy could be due to the different criteria employed by these authors to define the medial DS subregion. A detailed observation of probe locations shown in their experiments indicates that effects of MOR agonists on dopaminergic activity were analyzed in an intermediate region (approximately, AP: 2.16; ML: 1.5-2.5; DV: 7) between our caudal and medial DS areas. Since, as shown in the present work, it is plausible to assume the existence of clear-cut differences along the rostro-caudal axis of DS on MORs modulation of DA release, in the same manner it has been demonstrated in NAc [26]. The different region analyzed in both studies could explain this apparent contradiction, but further experiments using different methodological procedures are necessary to corroborate this issue.

According to our results, MORs do not appear to be presynaptically located on the DAergic terminals in medial DS. Since activation of MORs, as indicated above, provokes a hyperpolarization of the membrane, our data in the medial DS make it possible to rule out the presence of MORs directly on the DA terminals of this area of study. As far as we know, however, there is not anatomical or electrophysiological evidence supporting or rejecting of this affirmation, therefore, future experiments are imperative to corroborate this issue. A plausible hypothesis could involve the presence of MORs in the cholinergic interneurons of the medial DS, as suggested by Dourmap and colleagues [13], whose results showed the suppression of DAMGO effect on DA release when the cholinergic activity was blocked. These cholinergic interneurons are able to control the release of DA in striatum through a mechanism mediated by muscarinic $M_2$ receptors [13, 27]. Since, stimulation of $M_2$ receptors, as occurs with MORs, generally leads to inhibitory-type effects, we hypothesised that local administration of MORs agonists could lead to the inactivation of the cholinergic interneurons, which, in turn, would result in a decrease of striatal acetylcholine release. Therefore, the lower activation of postsynaptic muscarinic-$M_2$ receptors located on the DAergic terminals would result in an augment of DA levels in the medial DS. This hypothesis seems to be supported by data indicating that retrodialysis infusion of methoctramine, a $M_2$ selective antagonist, in the medial DS results in a–dose-dependent increase in DA release [27].

Our present data show an intriguing parallelism with others previously published by our research group in the ventral part of the striatum [6], although they also showed some differences. As commented above, in our previous experiments in NAc we showed a conspicuous region-dependency in the modulatory control that DORs and MORs exert on DA levels, although this dependency was expressed along the medio-lateral axis of the NAc. Although, this dependency on the medio-lateral axis has not been exhaustively analyzed in the present work, in our opinion, it does not seem to

exist in DS. Hence, a deep analysis of our data reveals that animals belonging to the medial DS group and showing probe locations clearly distant along the medio-lateral coordinate, all responded with increased DA levels after DAMGO administration (although some probes are located in the lateral part of the medial DS and others are located in the medial zone, near to the third ventricle, see figure 2). We do not know, at present, the implication, if any, of these differences, but future experiments will address this issue.

**Conclusion**

Our results confirm that opiate modulation of the DA release in the dorsal striatum by MORs, is clearly region-dependent (along the rostro-caudal axis). Whereas application of DAMGO increases DA extracellular levels in the medial DS, the application of the same drug, both in the rostral and caudal areas of DS, significantly reduces DA levels. These findings may lead to a better understanding of the nigrostriatal DA system regulation.

**Funding**

The authors herewith disclose receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by grants from Ajudes per a Accions Especials d'Investigació, Universitat de València (UV-INV-AE13-136776) Universitat de València, (Spain). YC-J is recipient of a predoctoral fellowship from the "VALi+d" program, Generalitat Valenciana, Spain.

**Declaration of conflicting interests**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data are mean ± SEM. Significant differences between medial and caudal pole ($p<0.0001$) and between medial and rostral pole ($p=0.0001$) were detected.

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**Figure 1**. Photomicrographs of coronal sections of the rat brain stained with cresyl violet showing typical microdialysis probe placements within rostral pole (**A**), medial region (**B**) and caudal pole (**C**) of DS. Abbreviatures: **cc**: corpus callosum; **ac**: anterior commissure; **acp**: anterior commissure posterior part; **LV**: lateral ventricle.

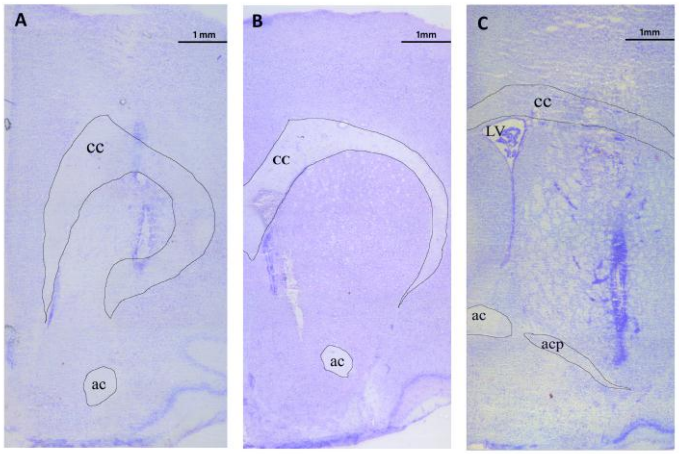
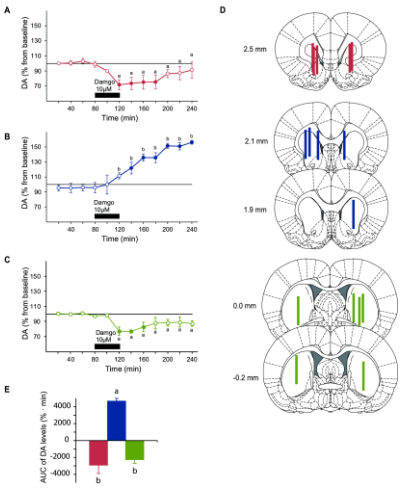


**Figure 2. Left**: Effect of retrodialysis application of the selective mu-opoid receptor agonist DAMGO (10µM) on DA levels in rostral pole (n=5) (**A**), medial area (n=5) (**B**) and caudal pole (n=6) (**C**) of DS. Data are mean ± SEM. The horizontal black bar indicates the time period of administration of DAMGO. Filled symbols indicate significant differences from the respective baseline (Dunnet's test, $p<0.05$). $^{a,b}$Time points with different superscript denote statistical significant differences between regions (Bonferroni's test). (**E**) Area under the curve for DA content (calculated as % from baseline x min by trapezoidal rule) in the three different regions analized. (a,b) denote statistical significant differences between regions (Tukey test). **Right:** Diagram of coronal sections from rat brain indicating placement of microdialysis probes within the three subregions of DS analyzed in this study **(D)**.



## LEGENDS OF FIGURES AND TABLE

**Table 1.** Basal dialysate levels of dopamine in DS subregions

Data are mean ± SEM, considering the different length of active membrane in each area of DS studied. Significant differences between medial and caudal pole were detected (one-way ANOVA test followed by Tukey test ($p<0.05$)). There were no significant differences in DA baseline levels in dialysates between the rostral and the caudal poles of DS.

| Subregion | DA (nmol/L) |
|---|---|
| Rostral pole (n=5) | 1.23±0.72 |
| Medial area (n=5) | 0.59±0.19 |
| Caudal pole (n=6) | 1.79±0.59 |

**Table 2.** Area under the curve of DA content from 60 to 240 min expressed as percentage x minute in the three DS subregions explored.

| Subregion | AUC |
|---|---|
| Rostral pole (n=5) | -2911,91 ± 992,50 |
| Medial area (n=5) | 4673,12 ± 327,73 |
| Caudal pole (n=6) | -2260,99 ± 533,98 |