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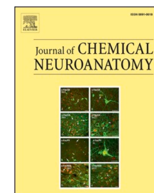

# Changes in hypothalamic mu-opioid receptor expression following acute olanzapine treatment in female rats: Implications for feeding behavior

Maiken Krogsbaek [a,*], Nick Yao Larsen [a,b], Anne M. Landau [c,d], Connie Sanchez [d,e], Jens Randel Nyengaard [a,f]

[a] *Core Centre for Molecular Morphology, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark*
[b] *Center of Functionally Integrative Neuroscience, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark*
[c] *Department of Nuclear Medicine and PET Centre, Aarhus University Hospital, Denmark*
[d] *Translational Neuropsychiatry Unit, Department of Clinical Medicine, Aarhus University, Denmark*
[e] *Alkermes Inc., Waltham, MA, USA*
[f] *Department of Pathology, Aarhus University Hospital, Aarhus, Denmark*

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ABSTRACT

Advances have been made in recent years in using opioid receptor antagonists as an adjunct therapy to psychotropic medication to reduce debilitating weight gain and metabolic adverse effects associated with in particular second generation antipsychotics. However, it is unknown whether second generation antipsychotics produce a change in opioid receptor expression in the brain. The present study investigated early changes in opioid receptor expression in the female rat hypothalamus, a master controller of hunger and metabolic regulation, after acute treatment with olanzapine, a commonly used second generation antipsychotic. Using quantitative spatial in situ hybridization and receptor autoradiography, expression levels of the three opioid receptors; kappa, mu and delta, were determined at mRNA and protein level, respectively, in the five hypothalamic areas: paraventricular nucleus, arcuate nucleus, ventromedial nucleus, dorsomedial nucleus and lateral hypothalamus. After 48 h of olanzapine treatment at clinically relevant plasma concentration weight gain and food intake changes, and increased plasma glucose were observed in female rats. Olanzapine treatment also led to a significant increase in mu opioid receptor availability in the arcuate nucleus, which contains both satiety and hunger controlling neurons. No other areas showed any opioid receptor expressional changes with olanzapine treatment on neither at mRNA nor protein level. Technical difficulties made it impossible to analyze mRNA levels in the lateral hypothalamus and overall binding of delta opioid receptors. Thus, the present study provided insights in to how olanzapine at clinically relevant plasma levels already at an early stage modulated the opioid system in the hypothalamus.

## 1. Introduction

Olanzapine, is a second generation antipsychotic that is associated with a substantial risk of weight gain and metabolic dysregulation such as decreased glucose tolerance and free fatty acids, and increased insulin resistance and blood triglycerides (Albaugh et al., 2011a). Weight gain is observed in up to 80% of patients receiving olanzapine with approximately 30% developing obesity (Seeman, 2009). The same adverse effects are observed in rats dosed with olanzapine, and in both rats and humans the weight gain observed with olanzapine is in part due to hyperphagia (Albaugh et al., 2011a, 2012, 2006, 2011b).

The hypothalamus controls the homeostatic sensation of hunger and satiety. The control of hunger and energy expenditure is highly complex, and the regulation involves both the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the ventromedial nucleus (VMH), the dorsomedial nucleus (DMH) and the lateral hypothalamus (LH) (Timper and Brüning, 2017; Saper and Lowell, 2014). These nuclei are intrinsically connected, regulated by a large number of different peptides and transmitters, and receive both central and peripheral circulatory information (Saper and Lowell, 2014; Roh and Kim, 2016; Hill, 2012). The orexigenic neurons of the hypothalamus elicit a hunger-inducing signal and stimulate increased activity, e.g. neuropeptide Y (NPY) expressing

* Correspondence to: Aarhus University Hospital, Palle Juul Jensens Boulevard 99. Level 1, C112/C113, 8200 Aarhus N, Denmark.
*E-mail address:* mkm@clin.au.dk (M. Krogsbaek).

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neurons of ARC and melanin concentrating hormone (MCH) expressing neurons of LH. The anorexigenic neuron types stimulate satiety and decreased activity, e.g. proopiomelanocortin (POMC) expressing neurons of ARC (Saper and Lowell, 2014; Berthoud, 2002; Menyhért et al., 2006; Stuber and Wise, 2016; Pritchard et al., 2002). Olanzapine has various effects on the hypothalamus. Sub-chronic olanzapine treatment in rats led to increased expression of NPY mRNA and decreased POMC and cocaine- and amphetamine regulated transcript mRNA in the ARC (Fernø et al., 2011). Moreover, olanzapine led to decreased leptin and increased ghrelin, orexin A and MCH mRNA expression in whole hypothalamic homogenates (Lazzari et al., 2017).

In both rats and humans, the weight gain observed with olanzapine treatment is in part mediated by hyperphagia (Albaugh et al., 2011a, 2012, 2006, 2011b). It is primarily female rats that gain weight and have increased food intake, along with other metabolic effects (Albaugh et al., 2006; Goudie et al., 2002; Pouzet et al., 2003; Arjona et al., 2004; Cooper et al., 2005). Some studies in female rats have shown hyperphagia as early as 24 h after initiation of treatment with olanzapine, and after chronic treatment, rats similar to humans develop obesity and/or metabolic abnormalities (Albaugh et al., 2012, 2006, 2011b; Fernø et al., 2011). Male rats, however, experience increased fat tissue mass along with dysmetabolic effects such as impaired glucose and insulin tolerance, but without significant weight gain. Increased food intake is sometimes observed in male rats and often their physical activity is decreased while on olanzapine (Albaugh et al., 2006, 2011b; Pouzet et al., 2003; Thornton-Jones Z et al., 2002; Cooper et al., 2007; Lee and Clifton, 2002; Hartfield et al., 2003; Benvenga and David Leander, 1997; Minet-Ringuet et al., 2006).

Opioid receptors and peptides participate in the regulation of both hunger- and satiety stimulating neurons and circuits (Bouret et al., 1999; Feng et al., 2012). Although in situ hybridization studies, autoradiography and electrophysiological studies in rodents have shown variable distribution patterns, they all support that mu, delta and kappa opioid receptors are expressed in the hypothalamic nuclei (Bouret et al., 1999; Feng et al., 2012; Tempel and Zukin, 1987; Mansour et al., 1987; Clarissa Desjardins et al., 1990; DenBleyker et al., 2009; Kelly et al., 1990; Zheng et al., 2005; Mansour et al., 1994; Merrer, 2009; Chu Sin Chung and Kieffer, 2013; Peckys and Landwehrmeyer, 1999; Weems et al., 2016). In addition, feeding triggers opioid release independently of hedonia, suggesting that the opioidergic system may act via the hypothalamic homeostatic feeding regulation (Tuulari et al., 2017).

Here, we investigated the effect of 48 h of olanzapine treatment in female rats in order to determine changes in opioid receptor expression in the hypothalamus using high resolution spatial, quantitative experiments.

## 2. Experimental procedures

### 2.1. Chemicals

Long acting injectable form of olanzapine (olanzapine LAI, olanzapine pamoate, Zyprexa Adhera®), was kindly provided by Alkermes Inc.

### 2.2. Animals

The study was performed adhering to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and approved by the Alkermes Institutional Animal Care and Use Committee. Twenty female Sprague-Dawley rats (12 weeks old) were purchased from Charles River. The rats were housed in pairs and maintained at a 12:12 h light:dark cycle in a temperature and humidity controlled environment (22 ± 2 °C, 45 ± 10% relative humidity). After one week of acclimation, the rats were divided into two groups of ten rats randomized based on body weight: one group injected with a 100 mg/kg s.c. dose of olanzapine, and the other group treated was injected only with the vehicle containing 2% CMC (carboxymethyl cellulose) and 0.2% PS20 (polysorbate 20) in 5 mM phosphate buffer. This dose of olanzapine should ensure exposure of clinically relevant steady state plasma concentrations (~40 ng/ml olanzapine), throughout the 48 h study period. The animals were fed ad libitum with standard chow (Harlan Teklad Global 2018) and tap water. The drugs were administered at day 0, and the rats were euthanized 48 h later. Food, water intake, and body weight was monitored.

Rats were euthanized through $CO_2$ inhalation. A blood sample was taken from the heart and blood glucose concentration was measured with a glucometer. The heads were removed with a guillotine, and the brains were taken out and frozen in Isopentane (2-methylbutane) cooled to − 40 °C. All brains were stored at − 80 °C. Fresh frozen brains were cut in 20 μm thick serial sections (14 series, 12–14 sections per series) on a cryostat (Microm Cryo Star HM 560), collected on Superfrost Plus slides (for in situ hybridization) or Poly-L-lysine slides (for receptor autoradiography) and stored at − 80 °C. The first section of the first series was collected approximately at Bregma − 0.4 mm. to Bregma − 4.36 mm (The Rat Brain in Stereotaxic Coordinates, 5th Edition, Paxinos and Watson), thus spanning the hypothalamus from the rostral part of PVN to the caudal part of ARC.

### 2.3. RNAscope in situ hybridization and image capture

*In situ* hybridization was performed as previously described (Krogsbaek et al., 2023). The standard Multiplex fluorescent RNAscope protocol from Advanced Cell Diagnostics was used. Briefly, series of 20 μm sections were chosen for RNAscope (Advanced Cell Diagnostics) in situ hybridization targeting *Oprk1* (Probe-Rn-Oprk1, C1 channel, cat. 452551, ACDBio), *Oprm1A* (Probe-Rn-Oprm1, C2 channel, cat. 410691-C2, ACDBio) and *Oprd1* (Probe-Rn-Oprd1, C1 channel, cat. 457011, ACDBio). Negative control probe targeting *dapB* (Cat. 320871, ACDBio) and positive control probes (Cat. 320891, ACDBio) were used in each experiment. The attached fluorophores were: Alexa Fluor-488 for *Oprk1* and *Oprm1A*, and Atto-550 for *Oprd1*. The following fluo cubes were used for imaging: A4 for DAPI staining (Ex/Em 350/470 nm), L5 for Alexa Fluor 488 (Ex/Em 493/516 nm) and TxRed for Atto-550 (Ex/Em 596/615).

*In situ* hybridization images were captured using a Leica microscope (DM6000 B, Leica Microsystems GmbH, Wetzlar, DE) equipped with a Ludl motorized x–y specimen stage (99S121, LUDL Electronic Products LTD., Hawthorne, New York, U.S.A.), and an Olympus DP72 digital color camera (12.8 megapixel, Olympus Denmark, Ballerup, Denmark). Low magnification images were acquired using DAPI nucleus staining for anatomical overview (10x objective, NA=0.25, HCX FL PLAN, Leica, Germany). High magnification images with a resolution of 6.2 pixels/μm were obtained that included the four hypothalamic areas PVN, ARC, VMN and DMN (2189x with 63x oil lens, NA=1.25, HCX PL FLUOTAR, Leica, Germany).

### 2.4. Image analysis

The image analysis for this study was carried out using the procedure described by Erben et al., 2019 (Erben and Buonanno, 2019) and Krogsbaek et al., 2023 (Krogsbaek et al., 2023). The anatomical delineations from "The Rat Brain in Stereotaxic Coordinates" (5th Edition, Paxinos and Watson) were used to identify the hypothalamic regions of the PVN, ARC, VMN, and DMN in ImageJ (version 1.51j8). The total fluorescent dot expression of the delineated fluorescent in situ hybridization images were analyzed in MATLAB using open access scripts from the referenced GitHub-repository (Larsen and Krogsbaek, 2022). Small objects and artifacts were removed from the images while keeping the size and shape of the fluorescent dot expression. Watershed segmentation was used to separate fluorescent dot expressions that were touching or overlapping one another. CellProfiler was used to perform post-profiling of expression patterns of corresponding nuclear DAPI images and followed the same procedure as in study (Krogsbaek et al.,

2023).

Here were the DAPI and RNA images first converted into grayscale and a global threshold was estimated for the DAPI images using the Otsu thresholding method (two-class thresholding). Data obtained include information on total RNA expression within each area, total number of cells, number of dot positive cells, and number of dots within each positive cell. Original RNAscope image, MATLAB rendered image, DAPI image and CellProfiler overlay image are seen in Fig. S1.

### 2.5. In vitro receptor autoradiography

Autoradiography was performed as previously described by Krogsbaek et al (Krogsbaek et al., 2023). Briefly, fresh frozen 20 µm brain sections on poly-L-lysine coated glass slides were thawed at room temperature, washed in assay buffer (50 mM Tris-HCl (pH 7.4) (MOR) or 50 mM Tris-HCl, 100 mM NaCl, 1 mg/ml BSA, 5 mM $MgCl_2$ (pH 7.4) (KOR+DOR) and incubated with radioactive tracer alone or in combination with the non-specific ligand Naloxone (10 mM, N7758, Sigma Aldrich) for 1 h. The following tracers and incubations times were used: [$^3$H]-DAMGO (4 nM, NET902, lot 2564533, Perkin Elmer) targeting MOR, [$^3$H]-U69,593 (8 nM, NET952, lot 2586932, Perkin Elmer) targeting KOR and [$^3$H]-DPDPE (10 nM, NET922, lot 2573313, Perkin Elmer) targeting DOR. Slides were then washed in assay buffer for 2 × 1 min, dipped in $dH_2O$ and air-dried for 1–2 h. Bound tracer was detected in the BeaQuant v. 1.14 (ai4r, France) for 6 h ([$^3$H]-DAMGO) or 22 h ([$^3$H]-U69,593 and [$^3$H]-DPDPE). Usually polymer standards are used, but because of high voltage artefacts on polymer slides in the BeaQuant an in-house standard slide was made by mixing blood with known concentrations of tritium, put as droplets on a polysine slide, dried and stored at 4 °C. The standard slide was included in every read.

When analyzing the data, a standard curve was made using the signal measured in the BeaQuant and the known concentration of each droplet on the standard slide. The radioactive tracer concentration was calculated using this standard curve and the concentration from the non-specific control was subtracted from the total binding slide to obtain the specific tracer binding (radioactive decay in the standard slide was accounted for in calculations). Hypothalamus as a whole was used as the area measured in the non-specific control.

Following Nissl stain with toluidine blue, the same tissue slides processed for autoradiography were scanned with a Nanozoomer 2.0HT (Hamamatsu) for detailed anatomical orientation.

### 2.6. Image analysis

BeaQuant scanned images are typically quantified by the dedicated Beamage program; however, because we were working with very small nuclei, we needed to overlay the images with Nissl stain for higher resolution and accurate delineation. To enable overlay, the autoradiography images (DAF files) were first exported as 32-bit TIFF files from the Beamage program and pixels were converted to voxels in ImageJ. The NDPITools plugin for ImageJ was used to convert Nissl images (NDPI files) to TIFF files. The Nissl-stained images were overlaid in ImageJ with the matching autoradiography images and the correct overlay alignment was verified for each brain slice. The Nissl stain was used to delineate the hypothalamic nuclei of the PVN, ARC, VMN and DMN, and the mean gray intensity value was subsequently measured in the corresponding autoradiography delineation. The smallest measured area had a minimum of 500 pixels. Mean gray value was converted to tracer binding activity (uCi/ml) using the blood standard. The equations in Griem-Krey et al. (2019) can be used to convert the units from uCi/ml to ng/mol.

### 2.7. Tissue exclusion

Data were excluded for one or more areas if the tissue was damaged during tissue freezing, cutting or laboratory experiments. Tissue that was not qualified was identified in the DAPI or Nissl-stained sections.

### 2.8. Statistics

The statistical software GraphPad Prism 9 was used for the statistical testing. Weight, food and water intake data were tested using repeated measures ANOVA followed by Sidak's multiple comparison test if significant differences were found. Plasma concentrations were tested using unpaired t-test. Group sizes varied because some animals were excluded in the in situ hybridization and autoradiography studies. When this was the case, a mixed effect model was used instead, followed by Sidak's multiple comparison test if significant differences were found. Gaussian distribution and variance tests for normality and log normality tests (D′Agostino and Pearson, Anderson-Darling, Shapiro-Wilk and Kologorov-Smirnov) showed normal distribution and variance homogeneity of the datasets. Data is presented as mean (SD).

## 3. Results

### 3.1. Effect of olanzapine on weight, food intake and plasma metabolites

Olanzapine treatment led to an increase in body weight after only 48 h of treatment with 71% higher weight gain in olanzapine treated rats (10.6 (4.7) g) compared to the vehicle treated (6.24 (4.90) g) ($p < 0.0001$), see Figs. 1A and S2. At euthanasia, the average olanzapine concentration in plasma was 21 (8.7) ng/ml. Olanzapine treated rats increased their food intake rapidly after only 24 h, see Fig. 1B. Although non-significant, after 48 h of olanzapine treatment rats showed an 18% higher intake of food per day compared to vehicle control, 22.9 (3.9) g vs 19.4 (2.8) g respectively ($p = 0.07$). There was a slight tendency towards increased water intake in the olanzapine treated rats, however no significant difference was observed between the two groups (Fig. S3). Olanzapine led to a 10% increase in blood glucose levels at euthanasia, 11.1 (1.5) mmol/L (olanzapine) vs 10.1 (1.4) mmol/L (vehicle) ($p < 0.05$).

### 3.2. Opioid receptor RNA expression and receptor binding in the rat hypothalamus

Images of each opioid receptor mRNA target can be seen in Fig. 2. Fig. 3 shows digital readings of the three opioid receptor tracers and their respective non-specific naloxone control. The specific binding of the [$^3$H]-DPDPE tracer was too low to measure in the hypothalamus of most brains and too low to make any real expressional definition in this study, and thus delta opioid receptor binding is not shown. From this dataset, there are some similarities between opioid receptor mRNA and protein expression in the hypothalamus, but also some discrepancies. For kappa opioid receptors, identical expression patterns are observed from mRNA to receptor, with high expression in PVN and DMN, followed by VMN and lowest in the ARC. For nu opioid receptors, *Oprm1A* expression patterns differs from [$^3$H]-DAMGO binding patterns in the ARC, which is markedly lower compared to the other areas at the receptor level, which is not observed at the *Oprm1A* level. It was too difficult to delineate LH from the DAPI images, because of its many diffuse borders with others areas, which meant that LH was not included in the RNA analysis.

All data numbers from the in situ hybridization image analysis are available in Table S1, and visualized in Fig. S4. There is a general overall pattern between the opioid receptor mRNA with highest expression of *Oprk1* (Figs. 4A and S4 left column), followed by *Oprm1A* (Figs. 4B and S4 mid column) and *Oprd1* (Figs. 4C and S4 right column). *Oprd1* expression in the VMN is the exception, where expression matches the highest *Oprk1* expressing areas. This pattern is observed both when looking at total RNA expression (Fig. S4A-C), RNA per total cell number (Fig. S4D-F), number of RNA per positive cell (Fig. 4A-C) and number of RNA positive cells (Fig. S4G-I). The number of RNA positive cells per

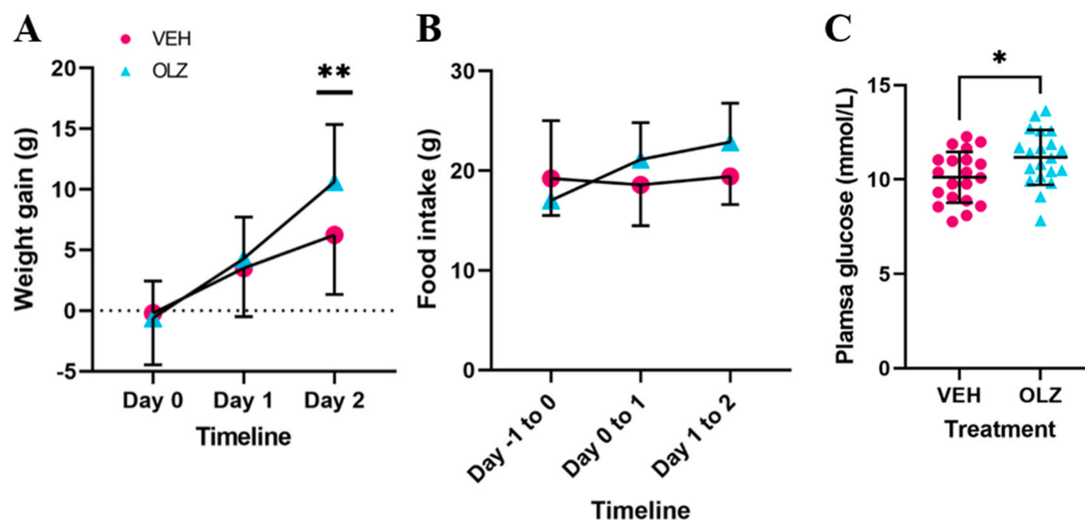


**Fig. 1.** Weight gain, food intake changes and plasma glucose concentrations following acute olanzapine treatment. A) Olanzapine treatment led to weight gain after only two days of treatment. B) Olanzapine treatment led to a tendency towards increased food intake, although non-significant ($p = 0.07$). C) Plasma glucose at day two showed a significant increase in olanzapine treated rats. OLZ: olanzapine, VEH: vehicle (control). * $p < 0.05$, ** $p < 0.0001$.

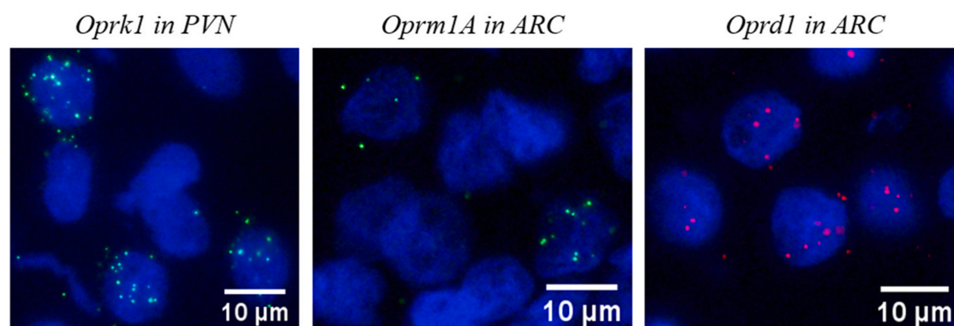


**Fig. 2.** High resolution images of Oprk1, Oprm1A and Oprd1 in PVN, ARC and VMN respectively from a representative brain. Images have been enhanced for better visualization here. All analysis were done on raw image files. PVN: paraventricular nucleus, ARC: Arcuate nucleus, VMN: ventromedial nucleus. Scale bar: 10 µm.

total cell number provides a good idea of the RNA distribution (how many cells are expressing the mRNA), as shown in Fig. S4J-L, while the RNA per total cell number and the number of RNA per positive cell illustrates density. We have recently shown that kappa opioid receptor data is translatable between mRNA density and receptor density (Krogsbaek et al., 2023). With a focus on mRNA counts per positive cell numbers, the lowest *Oprd1* expression is observed in PVN, ARC and DMN, with higher expression observed in the VMN (Fig. 4C). The *Oprm1A* expression per positive cell number showed the lowest expression in ARC, VMN and DMN and higher expression in the PVN (Fig. 4B). A similar more profound pattern is observed with *Oprk1* expression per positive cell number, where ARC, VMN and DMN show the lowest expression, with markedly higher expression in PVN (Fig. 4A).

Detailed analysis of the [$^3$H]-U69,593 tracer binding in the hypothalamus showed the lowest kappa opioid receptor binding density in the ARC and LH, followed by VMN, DMN and the highest binding density in the PVN, when correcting for area size, see Fig. 4D. [$^3$H]-DAMGO tracer binding showed the lowest mu opioid receptor binding density in ARC, followed by the DMN, VMN, PVN and highest in the LH, see Fig. 4E. Acute olanzapine treatment resulted in an increased availability and activity of mu opioid receptors as observed through the increased [$^3$H]-DAMGO binding. Following 48 h of olanzapine treatment a 41% increase in [$^3$H]-DAMGO binding activity in the ARC was observed compared to vehicle ($p < 0.05$) (olanzapine 0.27 (0.07) uCi/ml/mm$^2$, vehicle 0.19 (0.06) uCi/ml/mm$^2$). This increased availability of mu opioid receptors in cells of the ARC following olanzapine treatment is not observed on mRNA level.

## 4. Discussion

Olanzapine binds to multiple targets, and several have been investigated in relation to unwanted metabolic side effects. The effects observed are most likely caused by a combination of dysregulation in peripheral tissues and brain involved in metabolic and food intake regulation. This study has focused on opioid receptor expressional changes in the hypothalamus, specifically with a focus on the metabolism and food intake regulating regions – ARC, PVN, VMN, DMN and LH. The rationale for studying the opioid receptors in the hypothalamus following olanzapine treatment, comes from an increased interest in the use of an opioid receptor antagonist as co-treatment with psychotropic drugs to alleviate weight and metabolic related adverse effects (Billes et al., 2014; Silverman et al., 2017; Chaudhary et al., 2019; Martin et al., 2019; Cunningham et al., 2019). Recently, the FDA approved a combination of the mu opioid receptor antagonist Samidorphan and olanzapine for the treatment of schizophrenia and bipolar disorder (Silverman et al., 2017; Chaudhary et al., 2019; Martin et al., 2019; Potkin et al., 2019; Sun et al., 2019). In rats, this co-treatment normalized body composition in both sexes, attenuated weight gain in female rats and restored glucose utilization to normal, when compared to olanzapine treatment alone (Cunningham et al., 2019). Behavioral studies have shown that olanzapine suppressed effects induced by morphine (Torigoe

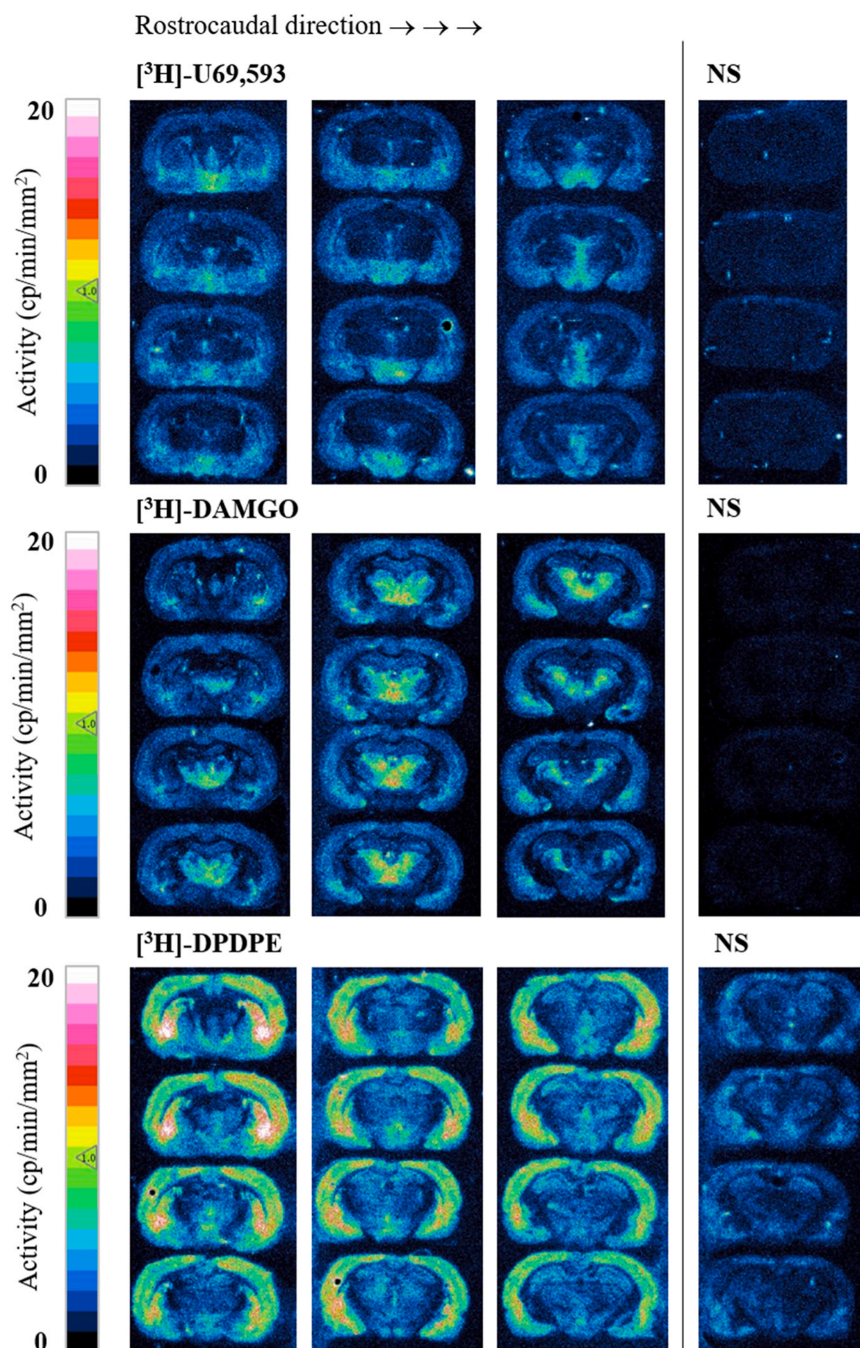


**Fig. 3.** Opioid receptor tracer binding in coronal rat brain sections. Total binding activity in rostrocaudal rat brain sections (Section 1 to 12, approximately Bregma −0.4 mm to −4.36 mm) of the [$^{3}$H]-U69,593 tracer (top), [$^{3}$H]-DAMGO tracer (middle) and [$^{3}$H]-DPDPE tracer (bottom). Resolution 60 µm/pixel. Naloxone is used to assess non-specific (NS) binding and any tracer bound in the hypothalamus is subtracted from the total binding to determine specific binding activity.

et al., 2012; Davis and Sanger, 2021), however to our knowledge, it has not previously been shown that olanzapine affects opioid receptor expression. Since olanzapine does not bind directly to opioid receptors, this effect would be secondary, but important nonetheless, as it provides a mechanistic rationale for the use of drugs like Samidorphan and naltrexone to counteract these important adverse effects of olanzapine.

From this and previous studies (Albaugh et al., 2012, 2006; Fernø et al., 2011; Skrede et al., 2014; Ersland et al., 2015), it is clear that steady state, clinically relevant, concentrations of olanzapine produced a rapid effect on food intake in female rats, and that the weight promoting effect of olanzapine is, at least in part, dependent on its orexigenic effects. Increased weight gain is observed after only 48 h of olanzapine treatment, together with a tendency towards increased food intake, and without any short term weight related stress reaction from the injection (Fernø et al., 2011; Skrede et al., 2014). It should be noted that previous studies with the same treatment method did not show weight gain as rapidly as observed here, and the 48 h end date was set based on these studies, with the goal of analyzing the hypothalamus prior to the weight gain (Skrede et al., 2014; Ersland et al., 2015).

There is sexual dimorphism in both the effects of olanzapine and in opioid receptor expression. While olanzapine induced weight gain and increased food intake is typically always observed in studies using female rats (Albaugh et al., 2012, 2006; Fernø et al., 2011; Thornton-Jones Z et al., 2002), it is not consistently observed in male rats, as they show more lipid alterations (Albaugh et al., 2006, 2011b; Pouzet et al., 2003; Cooper et al., 2007; Weston-Green et al., 2012). As this study aimed at

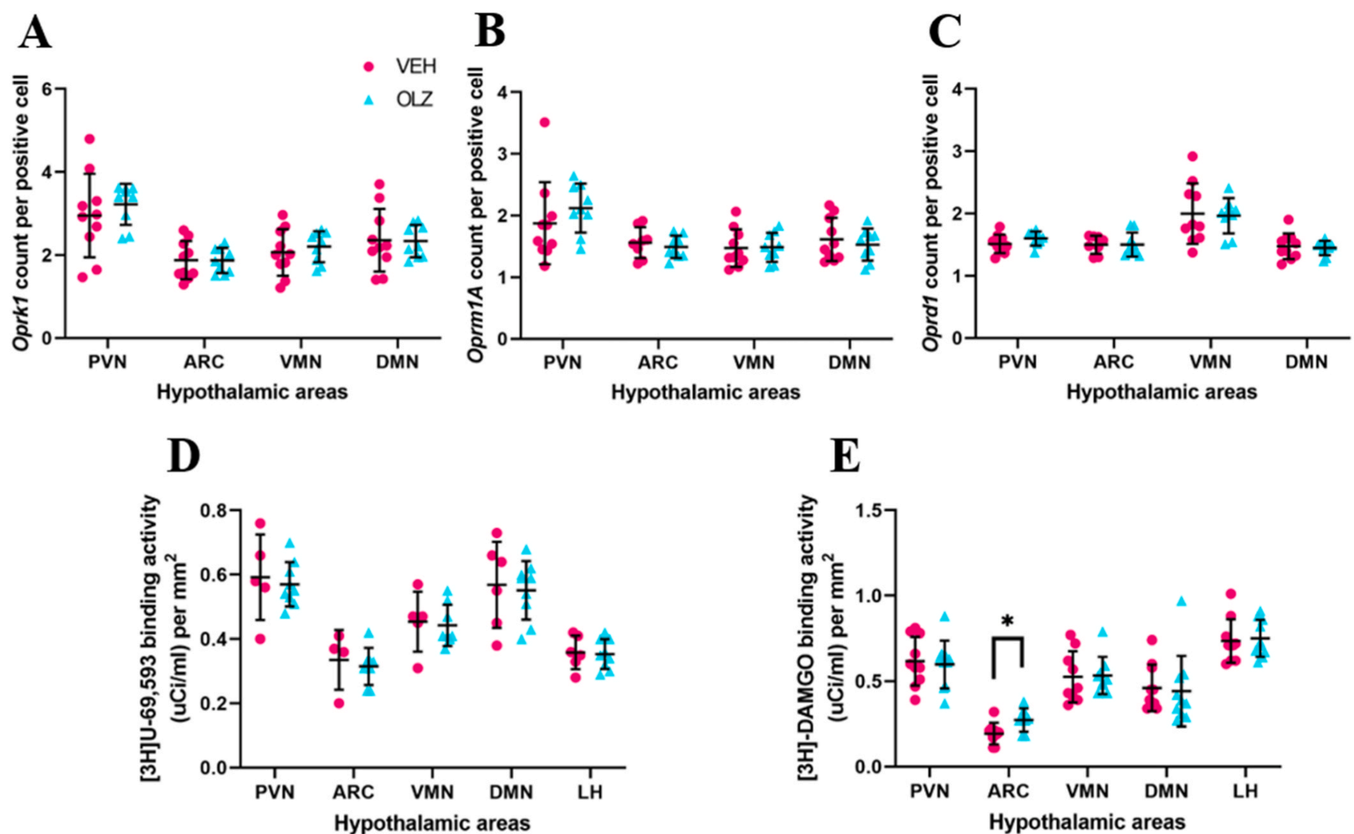


**Fig. 4.** Quantitative mRNA data from in situ hybridization and autoradiography data from all hypothalamic areas in olanzapine and vehicle treated groups. A) Oprk1, B) Oprm1A and C) Oprd1 mRNA count per positive cell in the PVN, ARC, VMN and DMN. D) [$^3$H]-U69,593 tracer targeting kappa opioid receptor. E) [$^3$H]-DAMGO tracer targeting mu opioid receptor. [$^3$H]-DAMGO binding activity in the arcuate nucleus shows a 41% increased mu opioid receptor binding in olanzapine treated rats when compared to vehicle (* $p < 0.05$). OLZ: olanzapine, VEH: vehicle, (control), PVN: paraventricular nucleus, ARC: Arcuate nucleus, VMN: ventromedial nucleus, DMN: dorsomedial nucleus, LH: Lateral hypothalamus, Oprk1: opioid receptor kappa mRNA, Oprm1A: opioid receptor mu 1A mRNA, Oprd1: opioid receptor delta mRNA, KOR: kappa opioid receptor, MOR: mu opioid receptor.

discovering hypothalamic opioid receptor alterations concurrent with olanzapine induced hyperphagia, only female rats where used. It is important to state that the opioid receptor expression patterns found in this study on the female rat hypothalamus can not necessarily be translated into the male rat brain, especially because it has been discovered that there are sex differences in the response to pain and analgesics (Hurley and Adams, 2008). A potential caveat when looking at hypothalamic opioid receptor expression in female rats is the estrous cycle. While a study across multiple strains of female rats showed no interaction between nociception and the estrous cycle (Terner et al., 2005), other studies do find cycle specific differences in the MOR expression (Loyd et al., 2008; Maggi et al., 1993). In order to account for this potential effect in future studies, the rats would have to be within the same estrous cycle phase during the study execution.

Opioid receptor expression was investigated in five areas of the hypothalamus that are related to metabolic and hunger regulation; ARC, PVN, VMN, DMN and LH. After 48 h of olanzapine treatment, we found an increased expression of mu opioid receptors in the ARC, but only at the receptor level. This indicates that the changes observed are a response of rapid changes in protein expression and receptor availability on the cell surface, and not an effect of increased opioid receptor mRNA expression and increased production of new receptor protein. No other hypothalamic areas were found to have changes in receptor mRNA expression or receptor availability. We have previously demonstrated a strong correlation between mRNA expression and receptor availability using these methods when targeting kappa opioid receptors in vehicle-treated rats, as done here. In addition, the expression patterns fit quite well with what has previously been shown (extensive comparison in (Krogsbaek et al., 2023)). However, the same direct correlation does not seem to be present when considering the mu opioid receptors. Mu opioid receptor mRNA has many different splice variants and although *Oprm1A* is the most common, this discrepancy between mu opioid receptor mRNA and receptor availability might be due to a different distribution of the mu opioid receptor mRNA splice variants in the tissue. Alternatively, there is simply not the same correlation between mu opioid receptor mRNA and receptor expression patterns as observed with kappa opioid receptor. Therefore, for mu opioid receptors, the mRNA levels cannot be used as a measure of mu opioid receptor availability. The mu, kappa and delta opioid receptor levels detected in this study are in agreement with other studies, with slight tracer-related and possibly sensitivity-related variance (Mansour et al., 1994; Merrer, 2009; Mansour et al., 1987).

Studies show that mu opioid receptors are located on different cell types in the ARC, among others the anorexigenic POMC neurons (Koch et al., 2015; Carr and Lovering, 2000). It is suggested that mu opioid receptors on POMC neurons acts as natural autoregulation (Bouret et al., 1999), since POMC neurons themselves produce β-endorphin, the endogenous ligand for mu opioid receptors. A mechanistic explanation could be that the increased number of mu opioid receptors observed after olanzapine treatment, results in increased inhibition of POMC neurons and thus decreased satiety signalling through the PVN, leading to the rapidly increasing food intake and weight gain. An alternative explanation could be an increased mu opioid receptor driven inhibition of GABAergic neurons that regulate the activity of NPY neurons, thus in second order leading to an increased NPY signal and increased hunger signalling (Millington, 2007; Lambert et al., 1993).

The primary pharmacologic targets of olanzapine are dopamine $D_2$ and 5-hydroxytryptamine 2 A ($5\text{-}HT_{2A}$) and 2 C ($5\text{-}HT_{2C}$) receptor antagonism. However, olanzapine also binds to many other receptors with differing intrinsic activity (Bymaster et al., 1996; Tollefson and Taylor, 2000). The $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors are among others located on the satiety stimulating POMC neurons (Martin-Gronert et al., 2016; Zhang et al., 2002). It is suggested that binding of olanzapine to $5\text{-}HT_{2A/2C}$ receptors on neurons of the hypothalamus plays an important role as the primary pharmacological mechanism of these unwanted side effects (Lord et al., 2017). Specifically, $5\text{-}HT_{2A}$ but not $5\text{-}HT_{2C}$ receptor mRNA has been shown to be decreased significantly in the ARC, VMN and LH two hours after olanzapine treatment (Huang et al., 2006). In addition, multiple studies have shown a synergistic interaction between 5-HT receptors and opioid receptors (Marek, 2003; Rangel et al., 2014; Li et al., 2011; Pang et al., 2016). One of these studies showed that mu but not delta opioid receptors modulates behavioural effects mediated by $5\text{-}HT_{2A}$ receptors in the rat prefrontal cortex. Thus, if there is a reduced function of $5\text{-}HT_{2A}$ receptors we would have expected to observe fewer mu opioid receptors in the ARC. Moreover, if there was a reduced expression of opioid receptors, there would not be a clear rationale for using an opioid receptor antagonist like Samidorphan.

In conclusion, we propose that the increased mu opioid receptor signalling in the ARC creates a decreased satiety signal or increased hunger signal, thus driving the observed increase in weight gain and food intake. Future studies should aim at establishing a mechanistic connection between olanzapine and mu opioid receptors thereby determining whether $5\text{-}HT_{2A}$ receptor antagonism by olanzapine is the connecting link. This could be investigated using cFos staining to evaluate downstream activation in cells that also bind opioid receptor antibodies. Unfortunately, at this point, good reliable opioid receptor antibodies for use in rats are not available. In addition, it would be important to investigate which neuron types are experiencing increased mu opioid receptor expression and whether e.g. $5\text{-}HT_{2A/2C}$ or dopamine $D_2$ receptor expression is also altered in these neurons, thereby determining if the weight gain is an effect of decreased satiety signalling or increased hunger signalling.

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## Ethical statement

*Considerations regarding animal use, conflicts of interest and funding source.*

## CRediT authorship contribution statement

All authors have contributed in a meaning full and substantial way in each their area. **Maiken Krogsbaek**: Conceptualization, Methodology, Investigation, Software, Validation, Formal analysis, Writing – original draft, Visualization, Project administration. **Nick Yin Larsen**: Software, Writing – original draft. **Anne M. Landau**: Resources, Writing – review & editing. **Connie Sanchez**: Conceptualization, Resources, Writing – review & editing. **Jens Randel Nyengaard**: Conceptualization, Supervision, Project administration, Writing – review & editing.

## Declaration of Competing Interest

We have no declarations of interest or competing interest.

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### Animal use

All rats were housed, managed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and the experiment was approved by the Alkermes Institutional Animal Care and Use Committee. We attempted to follow the three R's (Replacement, Reduction, Refinement), calculating how many animals where needed at a minimum (reduction) and making sure that each animal could be used for many experiments by planning well ahead, serial sampling the brain and storing for future studies (refinement).

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jchemneu.2023.102324.

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