

Deficiency of Orexin Receptor Type 1 in Dopaminergic Neurons Increases Novelty-Induced Locomotion and Exploration

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint posted

November 1, 2023 (this version)

Sent for peer review

August 21, 2023

Posted to bioRxiv

August 7, 2023

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Abstract

Orexin signaling in the ventral tegmental area and substantia nigra promotes locomotion and reward processing, but it is not clear whether dopaminergic cells directly mediate these effects. We show that dopaminergic neurons in these areas mainly express orexin receptor subtype 1 (Ox1R). In contrast, only a minor population in the medial ventral tegmental area express orexin receptor subtype 2 (Ox2R). To analyze the functional role of Ox1R signaling in dopaminergic neurons, we deleted Ox1R specifically in dopamine transporter-expressing neurons of mice and investigated the functional consequences. The deletion of Ox1R increased locomotor activity and exploration during exposure to novel environments or when intracerebroventricularly injected with orexin. Spontaneous activity in home cages, anxiety, reward processing, and energy metabolism did not change. Positron emission tomography imaging revealed that Ox1R signaling in dopaminergic neurons affected distinct neural circuits depending on the stimulation mode. In line with an increase of neural activity in lateral paraventricular nucleus (LPGi) of Ox1R^{ΔDAT} mice, we found that dopaminergic projections innervate the LPGi in regions where the inhibitory dopamine receptor subtype D2 but not the excitatory D1 subtype resides. These data suggest a crucial regulatory role of Ox1R signaling in dopaminergic neurons in novelty-induced locomotion and exploration.

eLife assessment

This manuscript describes **valuable** findings on the expression pattern of orexin receptors in the midbrain and how manipulating this system influences several behaviors, such as context-induced locomotor activity and exploration. The overall strength of evidence - which includes anatomical, viral manipulation studies, and brain imaging - is **solid** and broadly supports claims in the paper, however, there are several areas in which the conclusions are only partially supported by the data provided. These results have implications for understanding the neural underpinnings of reward and will be of interest to neuroscientists and cognitive scientists with an interest in the neurobiology of reward.

Introduction

Orexin neurons are exclusively located in the lateral hypothalamus (LH) and adjacent regions in the brain, including perifornical area, and dorsomedial and posterior hypothalamus (Soya & Sakurai, 2020 [↗](#)). Abundant orexin fibers innervate the ventral tegmental area (VTA) and substantia nigra (SN), which together contain the majority of dopaminergic neurons in humans and rodents (Fadel & Deutch, 2002 [↗](#); Hrabovszky et al., 2013 [↗](#); Peyron et al., 1998 [↗](#)). There are also some dopaminergic neurons in other brain regions, although not all of these neurons express the dopamine transporter (DAT) (Koblinger et al., 2018 [↗](#); Sharples et al., 2014 [↗](#); Turiault et al., 2007 [↗](#)). Both the orexin and dopaminergic systems control food intake, locomotor activity, reward processing as well as energy metabolism (Howe & Dombek, 2016 [↗](#); Inutsuka & Yamanaka, 2013 [↗](#); Narayanan et al., 2010 [↗](#); Palmiter, 2007 [↗](#); Tsujino & Sakurai, 2013 [↗](#)). The nigrostriatal dopaminergic system, containing the dopaminergic projections from SN to the dorsal striatum, predominantly regulates action selection and exploratory behaviors, while the mesolimbic and mesocortical dopaminergic systems, which consist of the dopaminergic pathways from the VTA to ventral striatum and prefrontal cortex, predominantly regulate motivation, cognition, reward, and aversion signaling (Roeper, 2013 [↗](#)). Orexin A injection into the VTA increases cocaine- and morphine-related reward processing (Mahler et al., 2012 [↗](#)), and intra-SN injection of orexin A induces hyperlocomotion and stereotypic behaviors (Liu et al., 2018 [↗](#); Nakamura et al., 2000 [↗](#)). On the other hand, systemic injection of dopamine receptor antagonists reduces orexin A-induced increase of hyperlocomotion, stereotypic and grooming behaviors (Nakamura et al., 2000 [↗](#)). However, whether the above effects of orexin signaling in the VTA and SN is mediated by dopaminergic or non-dopaminergic cells is not clear yet.

The orexin system consists of the two peptides orexin A and B, which are derived from the precursor, prepro-orexin (de Lecea et al., 1998 [↗](#); Sakurai et al., 1998 [↗](#)). Both are endogenous ligands for two G-protein-coupled receptors, orexin receptor subtype 1 (Ox1R), and 2 (Ox2R) (Sakurai et al., 1998 [↗](#)). Orexin A activates both receptors, while orexin B predominantly activates Ox2R (Sakurai et al., 1998 [↗](#)). The mRNA detection by *in situ* hybridization revealed the expression of both receptors in VTA and SN (Marcus et al., 2001 [↗](#)). However, the cell-type specific expression of Ox1R and Ox2R in the VTA and SN is not well understood. Electrophysiological studies showed that orexin receptors are present in dopaminergic VTA neurons (Baimel et al., 2017 [↗](#); Korotkova et al., 2003 [↗](#); Tung et al., 2016 [↗](#)), whereas data for the SN seem controversial (Korotkova et al., 2002 [↗](#); Liu et al., 2018 [↗](#)).

Here, we aim to map the expression patterns of Ox1R and Ox2R in dopaminergic VTA and SN neurons and investigate the functional role of Ox1R using conditional knockout mice.

Ox1R is the predominant orexin receptor in dopaminergic neurons

To analyze the expression patterns of orexin receptors in dopaminergic VTA and SN neurons, we performed fluorescent *in situ* hybridization (RNAscope) of tyrosine hydroxylase (*Th*), Ox1R (*Hcrtr1*) and Ox2R (*Hcrtr2*) in control (Ox1R flox/flox, DAT wt/wt) mice and Ox1R^{ΔDAT} (Ox1R fl/fl, DAT-Cre tg/wt) mice, from the same breeding crossing mice carrying a loxP-flanked Ox1R-allele (Ox1R fl/fl) with DAT-Cre mice (Ekstrand et al., 2007 [\[1\]](#)).

In control mice, Ox1R was expressed in 44 - 58% of dopaminergic neurons in all VTA and SN sub-regions, including the interfascicular (IF), paranigral (PN), parainterfascicular (PIF) and parabrachial (PBP) subnuclei of the VTA and SN. In contrast, Ox2R-positive dopaminergic neurons were mainly located in the medial VTA, including IF, PN, and PIF, with 25 to 42% of dopaminergic neurons expressing the Ox2R (**Figure 1A, C, D, G, H** [\[1\]](#); Figure 1—figure supplement 1A). While the above data were calculated as the mean percentages in the investigated mice, we also calculated the mean dopaminergic neuron numbers, separately for those expressing both or exclusively one of the orexin receptor types. Thus, we could analyze the distribution of Ox1R-, Ox2R-, Ox1R and Ox2R-, and none (of orexin receptors)-expressing dopaminergic neurons in each VTA subregions and SN, which was shown in the schematic illustration with pie charts (**Figure 1I** [\[1\]](#)). It showed similar results to the above: 47 - 59% of dopaminergic neurons expressed Ox1R in all VTA and SN sub-regions, and Ox2R was mainly expressed by 26 - 41% of dopaminergic neurons in the medial VTA (**Figure 1I** [\[1\]](#)). 19 - 25% of dopaminergic neurons in the medial VTA expressed both Ox1R and Ox2R (**Figure 1I** [\[1\]](#)). Of note, abundant Ox1R and Ox2R expression was present in Th-negative neurons in the VTA and SN and neighboring regions (**Figure 1A, C, D** [\[1\]](#), Figure 1—figure supplement 1A).

Since the vast majority of dopaminergic neurons express Ox1R, we focused on the functional analysis of Ox1R signaling in dopaminergic neurons. Ox1R was specifically inactivated in dopaminergic (DAT-expressing) neurons of mice (Ox1R^{ΔDAT}). In Ox1R^{ΔDAT} mice, Ox1R expression in dopaminergic neurons was significantly reduced and remained unchanged in Th-negative cells. In contrast, the Ox2R expression patterns remained similar to that in the control mice, indicating the successful and specific knockout of Ox1R in dopaminergic neurons (**Figure 1B, E-H** [\[1\]](#); Figure 1—figure supplement 1B). To test if orexin activates dopaminergic SN neurons and to functionally validate the deletion of Ox1R signaling, we performed Ca²⁺ imaging on dopaminergic SN neurons from control and Ox1R^{ΔDAT} mice using the genetically encoded Ca²⁺ indicator GCaMP6 expressed in dopaminergic (DAT expressing) neurons (**Figure 1J-L** [\[1\]](#), Figure 1—figure supplement 2). During the experiments, GABAergic and glutamatergic synaptic input was blocked. The orexin A-induced Ca²⁺ responses were expressed as a percentage of the maximum fluorescence induced by KCl (**Figure 1K, L** [\[1\]](#), Figure 1—figure supplement 2). Bath-applied 100 nM orexin A significantly increased [Ca²⁺]_i in dopaminergic SN control neurons (p < 0.0001, n = 71, one-sample t-test). An analysis on the level of individual cells (3 sigma criterion) showed that ~60% of dopaminergic SN neurons responded to 100 nM orexin A. In dopaminergic SN neurons from Ox1R^{ΔDAT} mice, 100 nM orexin A had no effect (p = 0.5, n = 86, one-sample t-test) (**Figure 1K, L** [\[1\]](#)).

In conclusion, Ox1R is the main orexin receptor expressed in dopaminergic VTA and SN neurons and mediates their activation by orexin. Therefore, we wanted to determine further the functional role of Ox1R signaling in dopaminergic neurons. By comparing data from control and Ox1R^{ΔDAT} mice, we first identified behaviors modulated by orexin via dopaminergic neurons. In a second step, we used PET imaging to define and map neuronal pathways modulated by orexin..

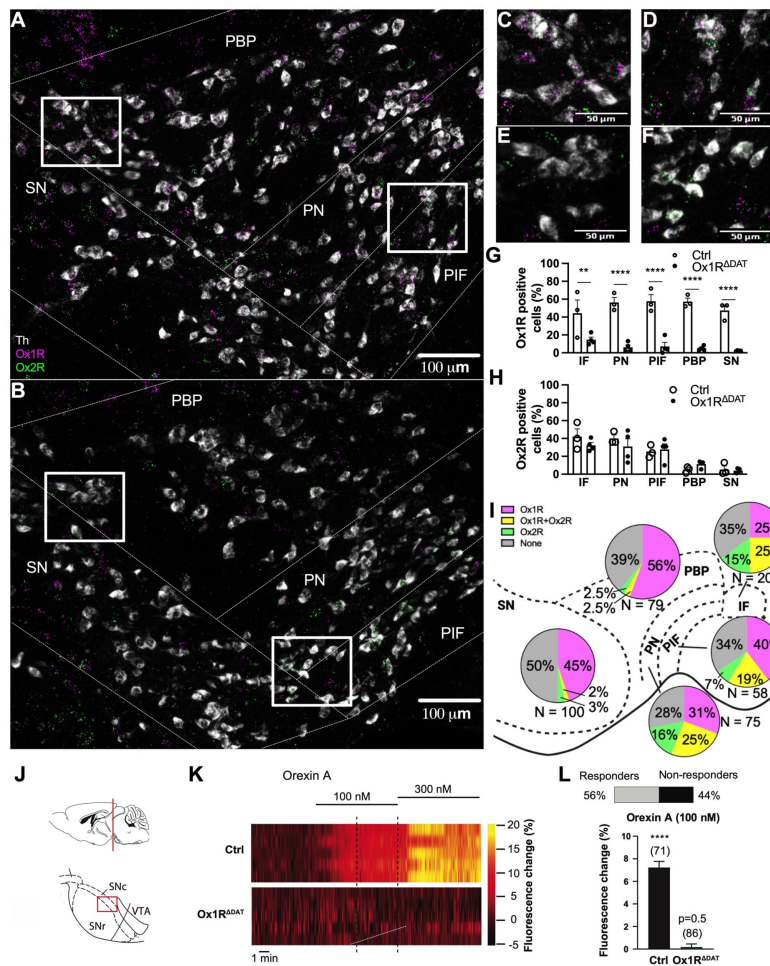


Figure 1.

Ox1R predominantly mediates orexin-induced activation of dopaminergic neurons in the substantia nigra (SN).

Representative images of RNAscope *in situ* hybridization in SN and paranigral (PN), parainterfascicular (PIF) and parabrachial (PBP) subnuclei of the ventral tegmental area (VTA), of (A) a control and (B) an *Ox1R^{ADAT}* mouse. Amplifications of the (C) left and (D) right marked areas in white squares in (A). Amplifications of the (E) left and (F) right marked area in white squares in (B). White, tyrosine hydroxylase (Th); magenta, Ox1R; green, Ox2R. Scale bar: 100 μ m (A, B) or 50 μ m (C-F). Representative images of an overview in SN and VTA were shown in figure 1—figure supplement 1. Percentages of (G) Ox1R and (H) Ox2R positive neurons in Th positive neurons. IF, interfascicular subnuclei of VTA. Control, n = 3; *Ox1R^{ADAT}*, n = 4. Data are represented as means $\pm$ SEM. **p < 0.01; ****p < 0.0001; as determined by two-way ANOVA followed by Sidak's post hoc test. (I) Schematic illustration of Ox1R and Ox2R expression in dopaminergic neurons in the VTA and SN. 3 control mice were analyzed and N indicates the mean dopaminergic neuron numbers in the respective subregions. Magenta, dopaminergic neurons only expressing Ox1R; green, dopaminergic neurons only expressing Ox2R; yellow, dopaminergic neurons expressing both Ox1R and Ox2R; grey, dopaminergic neurons expressing neither Ox1R or Ox2R. (J-L) Effect of orexin A on dopaminergic SN neurons analyzed by Ca^{2+} imaging with GCaMP6. Recordings were performed in brain slices from control and *Ox1R^{ADAT}* male mice with GCaMP6 expressed in dopaminergic SN neurons for Ca^{2+} imaging. (J) Schematic illustration of the SN. The red frame indicates the region where calcium imaging was performed. (K, L) Orexin A effect on $[Ca^{2+}]_i$ of dopaminergic SN neurons. (K) Heat maps of 5 individual dopaminergic SN neurons from control (top) and *Ox1R^{ADAT}* mice. The recordings show the responses to 100 nM orexin A. The dashed lines indicate the range where the responses were quantified. (L) Mean Ca^{2+} responses upon 100 nM orexin A application. Data are shown as the percentage of the maximal response to high K^+ saline. Bar graphs represent means $\pm$ SEM. The stacked bar shows the percentage of individual dopaminergic neurons in control mice in which the increase in $[Ca^{2+}]_i$ was larger than 3 x SD of the control, thus defining them as orexin A responsive (see Materials and Methods). The significance of mean changes in $[Ca^{2+}]_i$ during orexin A application were compared using one-sample t-tests. **** p < 0.0001. n values are given in brackets.

Ox1R in dopaminergic neurons regulates novelty-induced locomotion and exploration

The behavioral test included an analysis of locomotor behavior, novelty exploration, reward processing, anxiety, and energy homeostasis. Since sex differences exist in orexin- and dopamine-mediated behaviors (Duraija & Fendt, 2020 [DOI](#); Freeman et al., 2021 [DOI](#); Lippert et al., 2020 [DOI](#)), we analyzed behavioral phenotypes in both males and females. Compared to controls, Ox1R^{ADAT} mice did not show significant changes in spontaneous locomotor activity in home cages (Figure 2—figure supplement 1). When exposed to a novel open field, both male and female Ox1R^{ADAT} mice exhibited an increase in locomotion and exploration, with increased traveling distance, ambulating time, and vertical activity in the open field test (Figure 2A–C, E–G [DOI](#)). Female Ox1R^{ADAT} mice also showed increased stereotypic activity (Figure 2D, H [DOI](#)). Furthermore, intracerebroventricular (ICV) injection of orexin A (1 nmol) induced a more pronounced increase of locomotor activity in both female and male Ox1R^{ADAT} mice, compared to control mice (Figure 2I–L [DOI](#)).

In a hole-board test, female Ox1R^{ADAT} mice showed increased nose pokes into the holes in early (1st and 2nd) sessions compared to control mice (Figure 2—figure supplement 2B). This effect disappeared later when mice became familiar with the environment (3rd – 8th session) (Figure 2—figure supplement 2B). In the novel object recognition test, female Ox1R^{ADAT} mice spent more time with each of the two identical objects in an open field compared to control mice (Figure 2—figure supplement 2E). One hour later, when female mice were exposed to the same environment except that one object was replaced by a novel object, there was no significant difference between control and Ox1R^{ADAT} mice in exploring the novel object (Figure 2—figure supplement 2F). In contrast, male Ox1R^{ADAT} mice did not behave differently in the hole-board and novel object recognition tests from controls (Figure 2—figure supplement 2A, C, D). This indicates that Ox1R signaling in dopaminergic neurons is more important for females in novelty-induced exploration, which was independent of novel object discrimination.

The behavioral changes seemed not related to alterations in anxiety, since there were no detectable changes in anxiety-related behaviors in an open field test, dark/light box test or 0-maze test (Figure 2—figure supplement 3).

Ox1R deletion in dopaminergic neurons did not change energy metabolism and reward processing

The orexin and dopaminergic systems are well known to regulate energy metabolism and reward processing (Howe & Dombeck, 2016 [DOI](#); Inutsuka & Yamanaka, 2013 [DOI](#); Narayanan et al., 2010 [DOI](#); Palmiter, 2007 [DOI](#); Tsujino & Sakurai, 2013 [DOI](#)). Therefore, we measured the impact of Ox1R deletion in dopaminergic neurons on metabolism and reward-related behaviors. We found that, compared to control mice, neither male or female Ox1R^{ADAT} mice showed significant changes in the two-bottle preference test (comparing sweet solution and water drinking), conditional place preference test (comparing cocaine and saline injection) or energy metabolism, including body weight, fat mass, respiratory exchanging ratio, energy expenditure, feeding behaviors, glucose tolerance and insulin sensitivity (Figure 2—figure supplement 4 and 5).

Ox1R in dopaminergic neurons regulates specific neural pathways under different conditions

To explore and map the underlying neuronal pathways of the orexin-mediated behaviors unbiasedly, we performed positron emission tomography (PET) imaging of control and Ox1R^{ADAT} mice upon ICV injection of orexin A or saline (control).

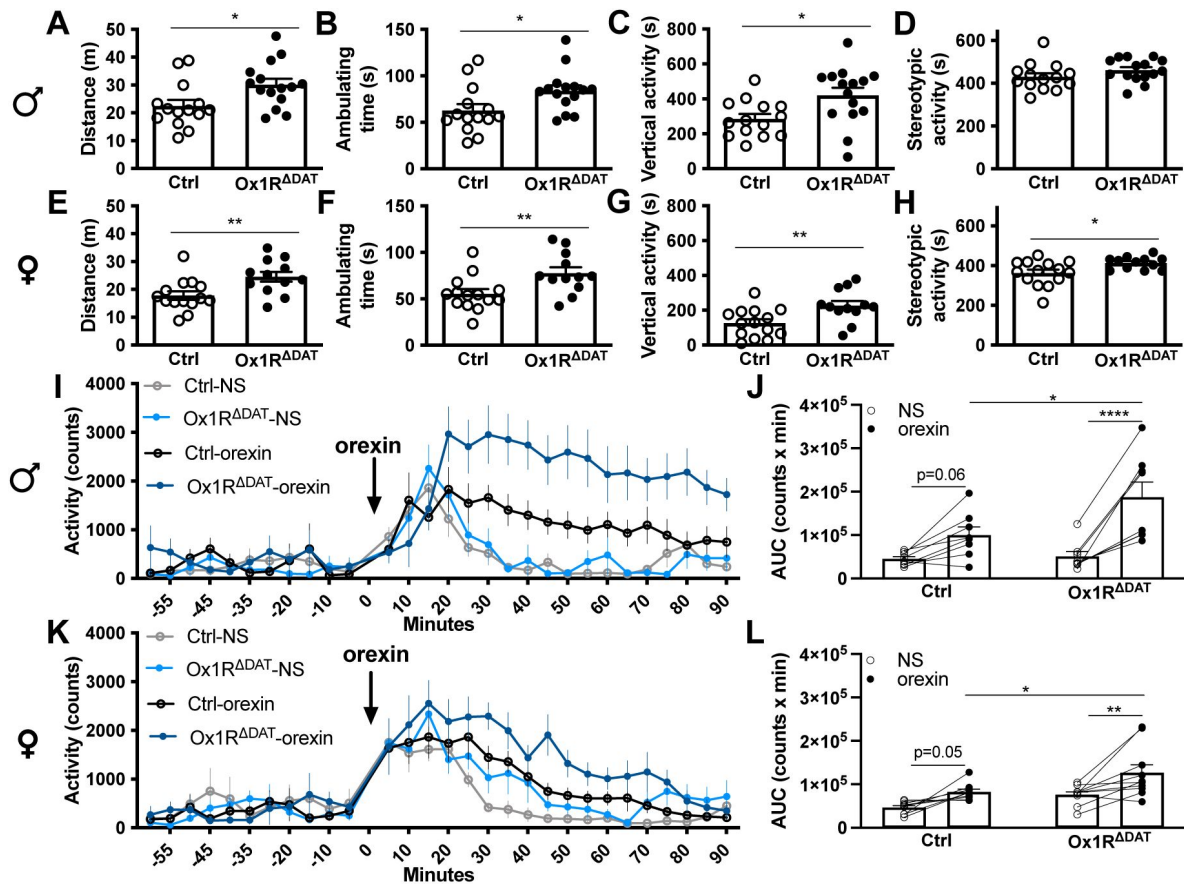


Figure 2.

Deficiency of Ox1R in dopaminergic neurons increases locomotion and exploration behaviors.

(A) Total traveling distance, (B) ambulating time, (C) vertical activity and (D) stereotypic activity of male mice in the open field test. Control, $n = 14$; Ox1R^{ΔDAT}, $n = 15$. (E) Total traveling distance, (F) ambulating time, (G) vertical activity and (H) stereotypic activity of female mice in the open field test. Control, $n = 14$; Ox1R^{ΔDAT}, $n = 12$. Locomotor activity upon intracerebroventricular (ICV) injection of saline (NS) or orexin A in (I) male and (K) female control and Ox1R^{ΔDAT} mice. (J, L) Quantification of the area under curve (AUC) after orexin injection to (J) male and (L) female mice. Male, $n = 8$; female-control, $n = 9$; female-Ox1R^{ΔDAT}, $n = 10$. Data are represented as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; as determined by unpaired two-tailed Student's t -test (A-C, E-H), or two-way ANOVA followed by Sidak's post hoc test (J, L).

In $Ox1R^{ADAT}$ mice under control conditions (saline injection), we observed higher neuronal activity around the medial preoptic area (MPA), piriform cortex (Pir), endopiriform claustrum, lateral stripe of the striatum (LSS), ventral part of subcoeruleus nucleus (SubCV), spinal trigeminal nucleus, spinal trigeminal tract (sp5), the intermediate reticular zone (IRt) and the gigantocellular reticular nucleus (Gi) than control mice (**Figure 3A**, Figure 3—figure supplement 1). Compared to control mice, central application of orexin (1 nmol, ICV) to $Ox1R^{ADAT}$ mice induced higher neuronal activity around the lateral paragigantocellular nucleus (LPGi), nucleus of the horizontal limb of the diagonal band (HDB) and magnocellular preoptic nucleus (MCPO), and lower activity around secondary motor cortex (M2), the dorsal bed nucleus of the stria terminalis (BNST), the hindlimb, shoulder, and trunk regions and barrel field of primary somatosensory cortex (S1HL, S1Sh, S1Tr, and S1BF), the lateral area of secondary visual cortex (V2L), primary visual cortex (V1), Pir and dorsal endopiriform claustrum (DEn) (**Figure 3B**, Figure 3—figure supplement 1). PET imaging did not reveal significant changes in the VTA and SN (**Figure 3**).

Postmortem analysis of c-Fos staining revealed low c-Fos expression in dopaminergic neurons in the VTA and SN. In contrast, clear orexin-induced c-Fos activity was observed in non-dopaminergic cells (Figure 3—figure supplement 2). Further studies would be needed to investigate how orexin directly and indirectly affect the VTA and SN neurons in the brain.

Dopamine receptors in the dorsal BNST and LPGi

Based on the PET imaging findings, we analyzed the dopaminergic pathways into the dorsal BNST and LPGi that could have been affected by the $Ox1R$. To this end, we injected Cre-dependent AAV-EYFP bilaterally into the VTA and SN of DAT-Cre (DAT^{EYFP}) and $Ox1R^{ADAT}$ ($DAT^{AOx1R}; EYFP$) mice, and discovered dopaminergic projections in the dorsal BNST and LPGi (Figure 4—figure supplement 1), suggesting that VTA and SN dopaminergic neurons could directly modulate neuronal activity in the dorsal BNST and LPGi. To further explore this idea, we assessed expression of Fos and dopamine receptors in the dorsal BNST and LPGi by immunostaining and RNAscope experiments.

Orexin injection (1 nmol, ICV) induced higher c-Fos expression levels in LPGi of male $Ox1R^{ADAT}$ mice compared to control mice (**Figure 4A, C**). Only the inhibitory D2 but not excitatory D1 subtypes of dopamine receptors (DRD2, DRD1) could be detected in LPGi (**Figure 4D, E**). Both receptors were detected around the lateral ventricle (as positive control), whereas no signal was detectable in LPGi incubated with negative control probe for mice (**Figure 4F, G**). Th-positive fibers were detected in LPGi but not Gi (**Figure 4A, B**), consistent with previous reports (Kitahama et al., 2000). These data support the hypothesis that $Ox1R$ deletion in dopaminergic neurons results in the disinhibition of dopaminergic signaling in LPGi.

Orexin injection increased c-Fos expression in the dorsal BNST in control but not in $Ox1R^{ADAT}$ mice (**Figure 4H, J**). The expression of DRD1 was significantly reduced, and DRD2 expression levels showed a tendency towards a decrease in dorsal BNST of $Ox1R^{ADAT}$ mice (**Figure 4K–M**). The Th expression levels were not changed between genotypes (**Figure 4H, I**). This indicates that dorsal BNST could be less activated, due to the decrease of DRD1 and the lack of orexin-mediated activation of dopaminergic neurons.

Discussion

The main findings of this study are based on RNAscope and optophysiological experiments. These data show that $Ox1R$ is the most abundant orexin receptor in the midbrain and the only orexin receptor in the SN. Ca^{2+} imaging experiments on SN dopaminergic neurons from control and $Ox1R^{ADAT}$ mice functionally confirm these results. They show that orexin-induced activation of SN dopaminergic neurons is exclusively mediated by $Ox1R$. In addition, the behavioral experiments,

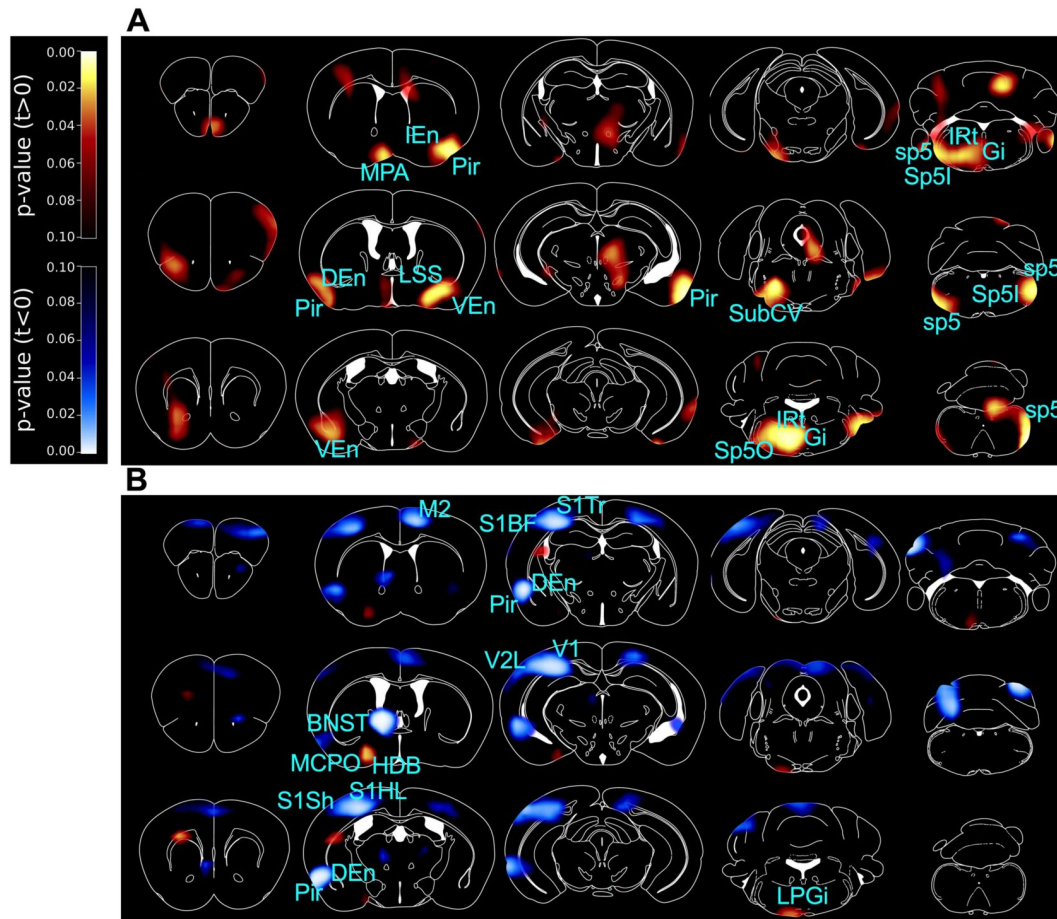


Figure 3.

PET imaging studies comparing Ox1R^{ADAT} and control mice.

3D maps of p-values in PET imaging studies comparing Ox1R^{ADAT} and control mice, after intracerebroventricular (ICV) injection of **(A)** saline (NS) and **(B)** orexin A. Brain areas with significant changes are indicated. Control-NS, n = 8; control-orexin, n = 6; Ox1R^{ADAT}, n = 8. M2, secondary motor cortex; MPA, medial preoptic area; Pir, piriform cortex; IEn, intermediate endopiriform claustrum; DEn, dorsal endopiriform claustrum; VEn, ventral endopiriform claustrum; LSS, lateral stripe of the striatum; BNST, the dorsal bed nucleus of the stria terminalis; HDB, nucleus of the horizontal limb of the diagonal band; MCPO, magnocellular preoptic nucleus; S1Sh, primary somatosensory cortex, shoulder region; S1HL, primary somatosensory cortex, hindlimb region; S1BF, primary somatosensory cortex, barrel field; S1Tr, primary somatosensory cortex, trunk region; V1, primary visual cortex; V2L, secondary visual cortex, lateral area; SubCV, subcoeruleus nucleus, ventral part; Gi, gigantocellular reticular nucleus; IRt, intermediate reticular nucleus; LPGi, lateral paragigantocellular nucleus; Sp5O, spinal trigeminal nucleus, oral part; Sp5I, spinal trigeminal nucleus, interpolar part; sp5, spinal trigeminal tract.

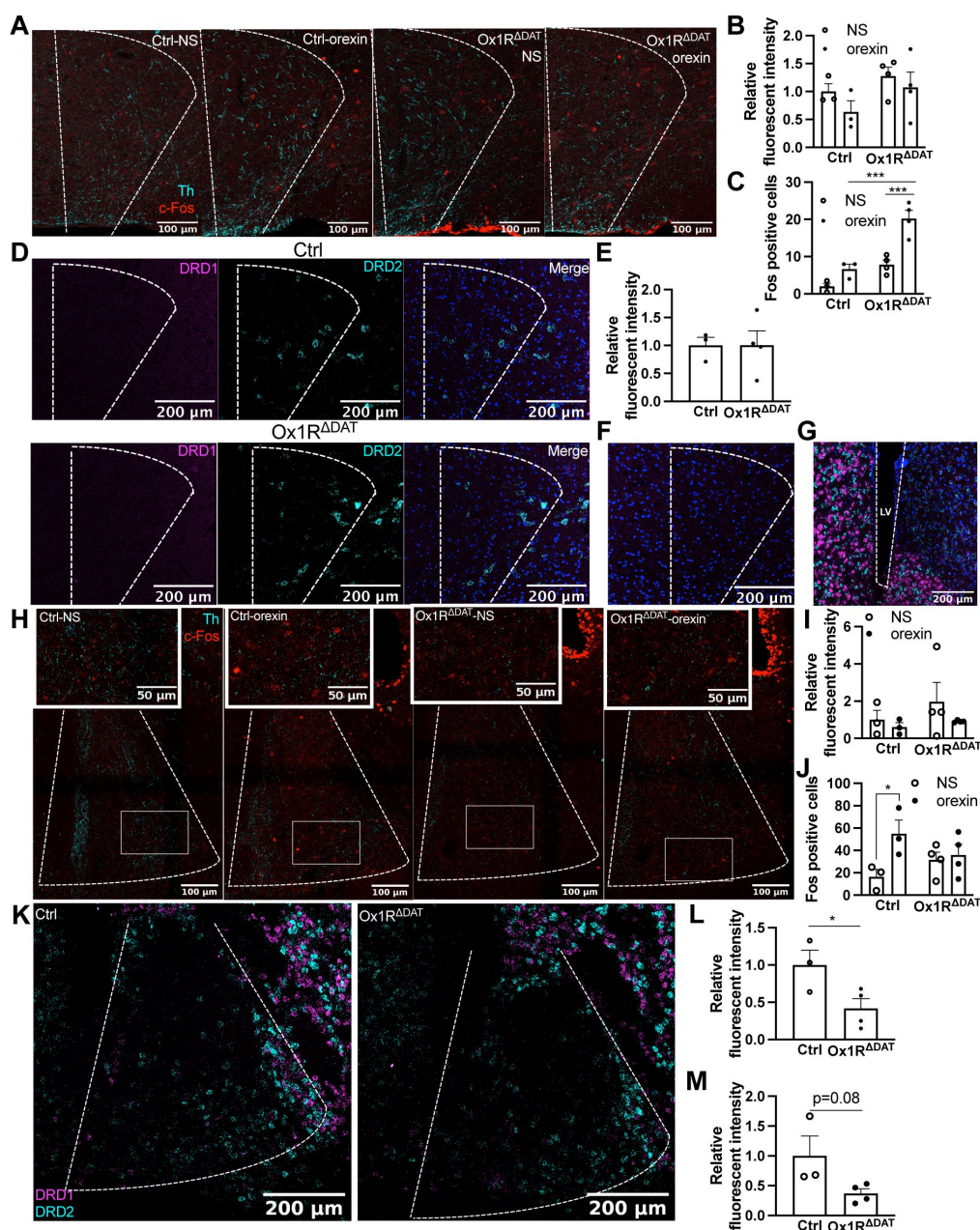


Figure 4.

c-Fos and dopamine receptors in the lateral paraventricular nucleus (LPGi) and the dorsal bed nucleus of the stria terminalis (BNST).

(A) Representative images of c-Fos and tyrosine hydroxylase (Th) staining in LPGi of control and $Ox1R^{ADAT}$ mice injected (ICV) with saline (NS) and orexin A. Quantification of (B) Th fluorescence and (C) c-Fos positive neurons in LPGi. (D) Representative images of D1 and D2 subtypes of dopamine receptor (DRD1 and DRD2) in LPGi of control and $Ox1R^{ADAT}$ mice. (E) Quantification of DRD2 fluorescence in LPGi. (F) Representative images of a negative control staining of DRD1 and DRD2 in LPGi of control mice, and (G) a positive control staining around the lateral ventricle (LV). (H) Representative images of c-Fos and Th staining in the dorsal BNST of control and $Ox1R^{ADAT}$ mice injected (ICV) with saline or orexin A. Quantification of (I) Th fluorescence and (J) c-Fos positive neurons in dorsal BNST. (K) Representative images of DRD1 and DRD2 in the dorsal BNST of control and $Ox1R^{ADAT}$ mice. Quantification of (L) DRD1 and (M) DRD2 fluorescence in dorsal BNST. Scale bar: 200 μm (D, F, G, K), 100 μm (A, H) or 50 μm (insertions in H). Control, $n = 3$; $Ox1R^{ADAT}$, $n = 4$. Cyan, Th; red, c-Fos (A, H). Magenta, DRD1; cyan, DRD2; blue, dapi (D, F, G, K). Data are represented as means $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$; as determined by unpaired two-tailed Student's t-test (L, M), or two-way ANOVA followed by Sidak's post hoc test (C, J).

PET imaging and Fos analyses provide important evidence of these data's systemic relevance. They should serve as a basis for developing testable working hypotheses on the systemic physiological role of orexin signaling. Ox1R deficiency in DAT-expressing neurons causes an increase in novelty-induced locomotion and exploration without altering energy metabolism and anxiety- or reward-processing-related behaviors. PET imaging, and Fos and DRD expression analysis provide some indications about the underlying mechanisms.

Earlier studies showed that orexin-induced activation of VTA dopaminergic neurons (Baimel et al., 2017 [DOI](#); Korotkova et al., 2003 [DOI](#); Tung et al., 2016 [DOI](#)). At the end of whole-cell patch clamp recordings, mRNA measurement of the individual recorded VTA neurons revealed fewer Ox2R- than Ox1R-positive dopaminergic neurons (Korotkova et al., 2003 [DOI](#)), which is in line with our findings. While our data clearly show an orexin A-induced activation of dopaminergic SN neurons when GABAergic and glutamatergic synaptic input is blocked, previous *in vitro* (Korotkova et al., 2002 [DOI](#)) or *in vivo* electrophysiological recordings (Liu et al., 2018 [DOI](#)) studies were controversial about whether orexin A could activate dopaminergic neurons in the SN. Since Ox1R and Ox2R are expressed by non-dopaminergic neurons around the SN, recordings in the absence of synaptic blockers, could detect a mixed direct and indirect orexin-induced response in dopaminergic neurons (Nair-Roberts et al., 2008 [DOI](#)). This may partially explain their controversial findings.

We found that Ox1R deficiency in dopaminergic neurons caused an increase in novelty-induced locomotion and exploration, which could indicate elevated arousal levels and hyperactivity. Interestingly, children with attention deficit and hyperactivity disorder (ADHD) have lower orexin A but unaltered orexin B levels in plasma compared to the healthy controls (Baykal et al., 2019 [DOI](#)). ADHD patients exhibit impaired dopaminergic functions, and medicine improving dopaminergic signaling is used to treat ADHD in clinics (Feldman & Reiff, 2014 [DOI](#); Volkow & Swanson, 2013 [DOI](#)). Ox1R signaling in dopaminergic neurons might be important to limit novelty-induced arousal and hyperactivity, which are related to task performance and learning capability.

We did not observe significant impact of Ox1R signaling in dopaminergic neurons on reward processing. Previous studies reported that Ox1R signaling in VTA promoted reward processing with pharmacological methods (Tsujino & Sakurai, 2013 [DOI](#)). The difference could have resulted from the involvement of non-dopaminergic neurons in VTA or different behavioral models. It would be interesting to identify the effect under other conditions, such as obesity and operant reward learning, in the future.

The PET imaging results suggest that Ox1R signaling in dopaminergic neurons affects separate and distinct neuronal circuits, depending on the mode of stimulation. Orexin and dopaminergic systems are known to increase arousal in mammals' sleep-wake transitions, locomotor activity, and motivation (Berke, 2018 [DOI](#); Berridge et al., 2010 [DOI](#); Furlong et al., 2009 [DOI](#); Mahler et al., 2014 [DOI](#)). The orexin system responds to various environmental stimuli, such as stress and novelty (Berridge et al., 2010 [DOI](#); Gonzalez et al., 2016 [DOI](#)). Projection-defined dopaminergic populations in the VTA exhibited different responses to orexin A (Baimel et al., 2017 [DOI](#)). Our PET imaging experiments, without orexin application, indicate that Ox1R signaling in dopaminergic neurons modulates brain regions (MPA, Pir, endopiriform claustrum, LSS, SubCV, spinal trigeminal nucleus, sp5, IRT and Gi) important for olfaction, eating, arousal and sleeping (Supplementary table 1). When central orexin levels were increased by orexin injection, Ox1R signaling in dopaminergic neurons regulates HDB, MCPO, Pir, DEn, S1, V2L, V1, BNST, LPGi and M2, which are important for sensation, emotion, exploration, and action selection (Supplementary table 1). In future studies, it would be interesting to investigate the direct and indirect downstream pathways of Ox1R-expressing dopaminergic neurons. Interestingly, in the fruitfly *Drosophila melanogaster*, it has been shown that the dopaminergic system oppositely regulates sleep-wake arousal and environmentally stimulated arousal, and the authors discussed the possibility to identify similar

patterns in mammals (Lebestky et al., 2009 [↗](#)). It was further suggested that the dopaminergic system differentially regulates arousal levels under different stimulating conditions (Lebestky et al., 2009 [↗](#); Lima & Miesenbock, 2005 [↗](#)).

We found a potential direct impact of dopaminergic neurons on LPGi and BNST. DRD1 and DRD2 in the BNST regulate anxiety, fear, and addiction (De Bundel et al., 2016 [↗](#); Eiler et al., 2003 [↗](#); Giardino et al., 2018 [↗](#); Krawczyk et al., 2013 [↗](#)). LPGi regulates exploratory behaviors and locomotor speed (Takato et al., 2013 [↗](#); Arber & Costa, 2022 [↗](#); Capelli et al., 2017 [↗](#)). There are few publications on the functional relevance of dopaminergic projections in the LPGi. Future studies would be very interesting to functionally analyze DRD2 signaling in the LPGi and to understand how orexin-interacts with DRD2 signaling.

Materials and Methods

Animal care

All animal procedures were conducted in compliance with protocols approved by the local government authorities (Bezirksregierung Cologne, Germany) and were in accordance with National Institutes of Health guidelines. Mice were housed in groups of 3 - 5 at 22°C - 24°C using a 12-hr light/12-hr dark cycle. Animals had *ad libitum* access to water and food at all times. Animals were fed a NCD (R/M-H, ssniff[®]), which contains 57% calories from carbohydrates, 34% calories from protein, and 9% calories from fat.

Generation of Ox1R^{ΔDAT} mice

Mice carrying a loxP-flanked Ox1R-allele (Ox1R fl/fl) were generated in our facility (Xiao et al., 2021 [↗](#)). Ox1R fl/fl mice were crossed with Dat-Cre mice (Ekstrand et al., 2007 [↗](#)) to obtain Ox1R fl/fl, Dat-Cre tg/wt mice, i.e. dopaminergic-specific Ox1R knockout mice on a C57/Bl6 N background (Ox1R^{ΔDAT}). Mice from the same breeding without the Cre transgene were used as controls (Ox1R fl/fl, Dat-Cre wt/wt).

Glucose and insulin tolerance tests

Glucose tolerance tests were performed on 12-weeks-old animals that had been fasted for 6 hours from 9 am. Insulin tolerance tests were performed on 11-weeks-old random-fed mice around 9 am. Animals received an intraperitoneal injection of 20% glucose (10 ml/kg body weight; KabiPac) or insulin (0.75 U/kg body weight; Sanofi-Aventis) into the peritoneal cavity, respectively. Blood glucose values were determined in whole venous blood using an automatic glucose monitor (Contour[®], Bayer). Glucose levels were determined in blood collected from the tail tip immediately before and 15, 30, and 60 minutes after the injection, with an additional value determined after 120 minutes for the glucose tolerance tests.

Analysis of body composition

Nuclear magnetic resonance (NMR) was employed to determine whole body composition of live animals on NCD at the age of 20 weeks using the NMR Analyzer minispec mq7.5 (Bruker Optik, Ettlingen, Germany).

Histology

Brains from male mice were removed, post-fixed in 4% paraformaldehyde (PFA) for the indicated time, and dehydrated in 20% sucrose in 0.1M phosphate-buffered saline (PBS) overnight. Brains were stored at -80°C until cutting.

Immunostaining

Brains were post-fixed for 6 h at 4 °C and cut (30 µm) with Leica CM3050 S Research Cryostat. Sections were incubated in 0.3% glycine for 10 min after washing in 0.1 M PBS for 2 × 10 min. After washing in PBS for another 10 min, sections were incubated in 0.03% SDS (in PBS) for 10 min before they got blocked with 3% donkey serum (in PBS, 0.25% Triton X-100) for 1 h at RT. Afterwards, they were incubated with primary antibodies, including goat anti-c-Fos (sc-526, Santa Cruz Biotechnology), chicken anti-GFP (ab13970, Abcam) and/or rabbit anti-Th (ab112, Abcam), for overnight (GFP) or 48 h (c-Fos and Th) at 4°C, washed in PBS for 3 × 10 min, and incubated with secondary antibodies, including Alexa Fluoro 594 donkey anti-goat, FITC donkey anti-chicken, Alexa Fluoro 488 donkey anti-rabbit (a11058, sa 1-7200 and a21206, Invitrogen), for 1 h at RT. After washing in PBS for 3 × 10 min, slices were mounted and covered with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories). Slices were stained together at one time for each experiment to have identical conditions for comparable signals. Slides were stored at 4 °C until imaging. Images were obtained with confocal laser scanning microscope Leica TCS SP8 and manually analyzed with Image J/FIJI (version 1.50d).

RNAscope fluorescent in situ hybridization

Brains from 12-wk-old male mice were post-fixed for 20 - 22 h at RT and cut (20 µm). Slides were stored at -80°C until staining. All reagents were purchased from Advanced Cell Diagnostics, and the staining was performed with the RNAscope Multiplex Fluorescent v2 kit (ACD, Advanced Cell Diagnostics) according to the user manual. Probes for Ox1R (*Hcrtr1*, 471561-C3, ACD) and Ox2R (*Hcrtr2*, 471551, ACD), DRD1 (*Drd1a*, 406491-C2, ACD), DRD2 (*Drd2*, 406501), and Th (*Th*, 317621-C4) were purchased from ACD. In brief, slides were briefly washed in diethyl-pyrocabonate (DEPC)-treated Millipore water, air dried, and then dried at 60°C overnight. On the second day, slides were treated with hydrogen peroxide (H₂O₂) for 10 min at RT, washed in water, and boiled in Target Retrieval solution (around 99.4 °C) for 8 -10 min. After a brief washing in water and dehydration in absolute ethanol, slides were incubated with protease IV for 30 min at RT. Slides were washed again in water and hybridized with the mixture of probes in different channels for 2 h in a humidified chamber at 40 °C. Afterwards, the hybridization was amplified with AMP 1 for 30 min, AMP 2 for 30 min and AMP 3 for 15 min. The signal was then developed for each channel. For example, for channel 1, slides were incubated with HRP-C1 for 15 min, the fluorophore for 30 min, and HRP blocker for 15 min. All amplification and development were performed at 40 °C, and 2 × 2 min of washing in ACD wash buffer was performed after each step. Finally, the slides were counterstained with DAPI for 1 min, mounted with ProlongTM Gold Antifade reagent with DAPI (Invitrogen), and covered with coverslips.

Slides were dried and stored at 4 °C. Imaging was performed with a confocal laser scanning microscope Leica TCS SP8. The images were manually analyzed with Image J/FIJI (version 1.50d).

Ca²⁺ imaging

Calcium dynamics were measured in dopaminergic substantia nigra neurons using the genetically encoded calcium indicator GCaMP6. Experiments were performed on brain slices from 18-19 week-old male control and Ox1R^{ADAT} mice. A minimum of 4 weeks separated the GCaMP6 virus injection and the imaging experiments. Animals were kept under standard laboratory conditions, with tap water and chow available ad libitum, on a 12h light/dark cycle. The animals were lightly anesthetized with isoflurane (B506; AbbVie Deutschland GmbH and Co KG, Ludwigshafen, Germany) and decapitated. Coronal slices (280 µm) containing SN were cut with a vibration microtome (VT1200 S; Leica, Germany) under cold (4°C), carbogenated (95% O₂ and 5% CO₂), glycerol-based modified artificial cerebrospinal fluid (GCaSF). GCaSF contained (in mM): 244 Glycerol, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 10 HEPES, 21 NaHCO₃, and 5 Glucose adjusted to pH 7.2 with NaOH. The brain slices were continuously superfused with carbogenated aCSF at a

flow rate of $\sim 2.5 \text{ ml} \cdot \text{min}^{-1}$. aCSF contained (in mM): 125 NaCl, 2.5 KCl, 2 MgCl_2 , 2 CaCl_2 , 1.2 NaH_2PO_4 , 21 NaHCO_3 , 10 HEPES, and 5 Glucose adjusted to pH 7.2 with NaOH. To reduce GABAergic and glutamatergic synaptic input the aCSF contained 10^{-4} M PTX (picrotoxin, P1675; Sigma-Aldrich), $5 \times 10^{-6} \text{ M}$ CGP (CGP-54626 hydrochloride, BN0597, Biotrend), $5 \times 10^{-5} \text{ M}$ DL-AP5 (DL-2-amino-5-phosphonopentanoic acid, BN0086, Biotrend), and 10^{-5} M CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, C127; Sigma-Aldrich).

The imaging setup consisted of a Zeiss AxioCam/MRm CCD camera with a 1388x1040 chip and a Polychromator V (Till Photonics, Gräfelfing, Germany) that was coupled via an optical fiber into the Zeiss AxioExaminer upright microscope (Objective W “Plan-Apochromat” 20x/1.0 DIC D=0.17 M27 75mm). The camera and polychromator were controlled by the software Zen pro, including the module ‘Physiology’ (2012 blue edition, Zeiss). The SN neurons were identified according to their anatomical location and expression of the GCaMP6. Calcium signals in GCaMP6 expressing cells were monitored by images acquired at 470 nm excitation wavelengths with 20 ms exposure time at $\sim 0.2 \text{ Hz}$. The emitted fluorescence was detected through a 500-550 nm bandpass filter (BP525/50), and data were acquired using 4x4 on-chip binning. Images were recorded in arbitrary units (AU) and analyzed as 16-bit grayscale images.

Orexin A (ab120212, Abcam) in the concentration of 100 nM was applied for 8 min. To analyze the orexin effect, we compared the fluorescence measured during 4 min intervals that were recorded immediately before and at the end of the peptide application. This protocol was followed by applying high K^+ concentration saline (40 mM KCl; osmolarity was adjusted by reducing the NaCl concentration accordingly) to elicit the maximal calcium response.

The image analysis was performed offline using Image J (version 1.53a), Igor Pro 6 (WaveMetrics, USA), and Prism 8 (GraphPad, California, USA). After the experiments, regions of interest (ROI) were defined based on the high K^+ saline responses. The mean AU values of the ROIs were calculated in Image J. Time series analysis was performed with Igor Pro 6. To correct for bleaching artifacts, the baseline fluorescence (without orexin application) was fit. The extended fit was subtracted from the raw data. The orexin-induced Ca^{2+} signals were normalized to the high (40 mM) K^+ response. On the single-cell level, a neuron was considered orexin-responsive if the change in fluorescence induced by orexin was 3 times larger than the standard deviation (SD) (3 sigma criterion) of the baseline fluorescence.

Food intake and spontaneous activity

Mice (20 weeks old) were analyzed for food intake, spontaneous activity, respiratory exchanging ratio and energy expenditure in a PhenoMaster System (TSE Systems) as previously described (Jordan et al., 2011 [link](#)).

Behavioral studies

Mice were moved into the behavioral test room, weighed, and handled 1 week before the measurement. On the day of the tests, mice (9-15 weeks old) were moved near to the equipment and handled 1 h before the tests. There was a 1-week break between different behavioral tests for anxiety, exploration or locomotion when using the same mice.

Open field test (OFT), dark/light (D/L) box and Hole-board tests

OFT and D/L box tests were performed with a Seamless Open Field Starter Package for Mouse plus the Light/Dark Insert or the hole-board floor (Med Associates, Inc.) between 10 am to 4 pm.

To start OFT, mice were put in the middle of a clear Plexiglas[®] chamber (27.3 cm $\times$ 27.3 cm $\times$ 20.3 cm) sitting in a sound-attenuating cubicle, and mouse movement was automatically traced for 30 min with 48 infrared beams divided into the X, Y and Z plane. For the D/L box test, a black

Plexiglas[®] box (half the size of the chamber) was inserted at the left side of the chamber, with an opening at the middle side. Mice were put in the middle of the light side and faced the openings. Mouse movement was traced for 5 min. For the hole-board test, a metal hole-board floor with 16 equally spaced holes for exploration was inserted in the chamber. Mice were put in the middle of the chambers and the nose pokes were monitored automatically for 10 min per session. There was 1 h break between each session. 4 sessions per day were analyzed for 2 consecutive days.

Elevated 0-maze test

An elevated 0-maze test was performed with TSE elevated 0-Maze in combination with the video tracking system TSE VideoMot 3D version 7.01. There are 2 closed and 2 open runways without a center position. The outer diameter is 48 cm, the arm width is 5 cm, the wall height is 10 cm and the base height is 55 cm. Mice were put in the middle of an open arm and faced to the closed arm to start the experiment. Mouse movement was monitored for 5 min.

Novel object recognition test

Novel object recognition test was modified from the publication before (Ruud et al., 2019¹). The mouse was acclimated and measured for 10 min in the 1st session and for 5 min in the 2nd session on the same day, in a polycarbonate box with non-transparent walls (50 cm $\times$ 50 cm $\times$ 30 cm). Two objects were present in the box. In the 1st session, two identical objects (glass beaker filled with blue marbles) were present and fixed with odor-free adhesive to the ground flooring at two sides of the box delineated diagonally. In each diagonal corner, the object is 11.5 cm and 14 cm from each wall respectively, and the distance between the two objects is 17.5 cm. Mice always started at the same corner which was between the two fixed objects and were allowed to explore and learn the experimental area and objects. In the 2nd session which started after a 1-h break in the home cage, one of the two identical objects was replaced with a novel object (Lego[®] Primo brick), and mice were allowed again to explore the environment and objects. The positions of the mouse nose, tail and center were monitored with a video tracking system TSE VideoMot 2. The interaction with a fixed object was identified when the nose was facing the object within a distance of 2 cm.

Conditioned place preference test for cocaine

The test was modified from previous publication (Ruud et al., 2019¹), and was performed in a polycarbonate box with non-transparent walls. The box was separated into 3 compartments with 2 independent partitions. It contains a left compartment with zebra stripes (white and black) painted on the wall and a rough ground (16.5 x 13.2 x 21.2 cm), a small middle area (7.0 x 13.2 x 21.2 cm), and a right compartment with a white wall and smooth ground. Mouse movement was monitored with TSE VideoMot 2 for 30 min per session, 1 session per day. Mice started from the middle area and had access to both left and right compartments through an opening (4.0 cm x 4.2 cm) in the partition during the habituation and pre-test days (d1-3) and on the test day (d10), and were put into and kept in either the left or right compartment without an opening in the partition during the conditioning phase (d4-9). The preference of the two compartments by nature was analyzed during the pre-test days. At the conditioning phase, on alternative days, mice received i.p. injection of cocaine (5 mg/kg BW) or saline immediately before entering the respective compartments. Cocaine and saline were always paired to the non-preferred and preferred compartments, which were determined by pre-tests, respectively. There was no injection on the habituation, pre-test or test days. The percentage of time spent in the reward-paired compartment was calculated: $100 \times \text{time spent in the compartment} / (\text{total time} - \text{time spent in the middle area})$. The CPP score was then analyzed using the calculated percentage of time: $100 \times (\text{time on the test day} - \text{time on pre-test days}) / \text{time on pre-test days}$.

Sucrose/sucralose preference test

Two-bottle tests were performed with 2 15-ml falcon tubes connected to sippers. Mice were single caged 1 week before the beginning of the experiment. 2 falcon tubes filled with water (side-by-side) replaced the normal water bottle. Water intake was monitored for a week to make sure mice adjusted to drinking from the sippers. When the test started, 2 new sipper tubes of water were used for 2 days of baseline measurement and were then replaced with one containing water and one containing sucrose/sucralose solution for 2 days of preference measurement. Animals received a 0.5%, 1% and 2% (w/v) sucrose solution and 1 mM sucralose solution in sequential. Between the preference tests of different solutions, 2 days of baseline measurement were performed with 2 new sipper tubes of water as a washout. To avoid side bias, the positions of 2 sipper tubes were switched every day, with the sucrose/sucralose tube always starting on the non-preferred position, which was determined by water intake on the day before the preference measurement. Sipper tubes were weighed every day.

ICV cannula implantation and injection

12-wk-old mice received 1 mg/ml of tramadol in drinking water 2 d before the surgeries. On the day of surgery, mice were anesthetized with isoflurane, and a bolus of buprenorphine (0.1 mg/kg BW) was given (i. p.) to reduce pain. Brain regions were identified according to the atlas (Franklin & Paxinos, 2007 [\[1\]](#)). After alignment of the brain in the stereotaxic surgery platform, one small hole was drilled in the skull and the ICV cannula (26 gauge, Plastics One) was immediately implanted (AP: -0.2 mm, ML: +1 mm, DV: -2.4 mm) and fixed to the skull with dental acrylic (Super-Bond C&B). The dummy cannula caps were inserted to close and protect the cannula. Mice received a bolus of meloxicam (5 mg/kg BW, s.c.), and tramadol in drinking water for 3 d after surgeries to relieve pain. BW was checked twice a day. Experiments started at least 1 week later. For injection, the catheter was attached to an injector (Plastics One), which was inserted through the cannula. orexin A (Phoenix Pharmaceuticals) was injected slowly at the dose of 1 nmol dissolved in 2 μ l of saline. Starting from 5 days before the injection, mice and the dummy cannula caps were handled for acclimation daily. The locomotion activity was measured in a PhenoMaster System (TSE Systems), and mice were acclimated to the system at least 5 days before the test.

PET imaging

PET imaging was performed using an Inveon preclinical PET/CT system (Siemens). Male mice were anesthetized with 2% isoflurane in 65%/35% nitrous oxide/oxygen gas, injected with saline or orexin A and positioned on a dedicated mouse carrier (MEDRES, Germany) carrying two mice. Body temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ by a thermostatically controlled water heating system. For injection of the radiotracer, a catheter consisting of a 30G cannula connected to a polythene tubing (ID = 0.28 mm) was inserted into the tail vein and fixated by a drop of glue. After starting the PET scan, 7- 8 MBq of [^{18}F]FDG in 50-100 μ l saline were injected per mouse. Emission data were acquired for 45 minutes. Thereafter, animals were automatically moved into the CT gantry, and a CT scan was performed (180 projections/360°, 200 ms, 80 kV, 500 μ A). The CT data were used for attenuation correction of the PET data, and the CT image of the skull was used for image co-registration. Plasma glucose levels were determined from a tail vein blood sample using a standard glucometer (Bayer) after removing the tail vein catheters. PET data were histogrammed in time frames of 12x30s, 3x60s, 3x120s, 7x240s, Fourier rebinned, and images were reconstructed using the MAP-SP algorithm provided by the manufacturer. For co-registration, the imaging analysis software Vinci was used (Cizek et al., 2004 [\[2\]](#)). Images were co-registered to a 3D mouse brain atlas constructed from the 2D mouse brain atlas published by Paxinos (Paxinos et al., 2013 [\[3\]](#)).

Kinetic modeling

An image-derived input function was extracted from the PET data of the aorta, which could be identified in the image of the first time frame of each animal. Input function data were corrected for partial volume effect by assuming a standardized volume fraction of 0.6 (Green et al., 1998 [↗](#)). Parametric images of the [18F]FDG kinetic constants K_1 , k_2 , k_3 , and k_4 were determined by a voxel-by-voxel (voxel size = 0.4 mm x 0.4 mm x 0.8 mm) fitting of data to a two-tissue-compartment kinetic model. K_1 is the constant for transport from blood to tissue, k_2 for transport from tissue to blood, k_3 the constant for phosphorylation of [18F]FDG to [18F]FDG-6-phosphate, and k_4 the constant for dephosphorylation. The ratio of tissue and plasma glucose concentrations (C_E/C_P) is a measure for glucose transport and is given by $C_E/C_P = K_1/(k_2 + k_3/0.26)$ (Backes et al., 2011 [↗](#); Jais et al., 2016 [↗](#)). Since neuronal activation is accompanied by increased glucose transport and this parameter is less sensitive to changes in plasma glucose level, we use alterations of glucose transport (C_E/C_P) as surrogate for alterations in neuronal activation.

Statistics

Statistical testing was performed by application of a voxel-wise t-test between groups. 3D maps of p-values allow for the identification of regions with significant differences in the parameters. From these regions, we defined volumes of interest (VOIs) and performed additional statistical testing for these VOIs. For presentation only, 3D maps of p-values were re-calculated on a 0.1 mm x 0.1 mm x 0.1 mm grid from the original dataset using trilinear interpolation.

Virus injection

Male mice received 1 mg/ml of tramadol in drinking water 2 d before the surgeries. On the day of surgery, mice were anesthetized with isoflurane and a bolus of buprenorphine (0.1 mg/kg BW) was given (i. p.) to reduce pain. Brain regions were identified according to the atlas (Franklin & Paxinos, 2007 [↗](#)). After alignment of the brain in the stereotaxic surgery platform, 1 or 4 small holes were drilled in the skull at specific coordinates. The virus, pAAV-Ef1a-DIO EYFP ($\sim 1 \times 10^{13}$ GC/ml, 300 nl, Addgene) was bilaterally injected into VTA and SN of 10 wk old mice, with micropipettes pulled in house with a heating system. The coordinates from Bregma were: anterior-posterior, AP: -3.4 mm; medial-lateral, ML: $\pm 0.5/1.25$ mm; dorsal-ventral, DV: -4.2 mm. The GCaMP6 virus (AAV1.Syn.Flex.GCaMP6s.WPRE.SV40, $\sim 4 \times 10^{12}$ GC/ml, 300 nl, Penn Vector Core) was injected to left SN (AP: -3.4 mm, ML: 1.1 mm, DV: -4.2 mm) of 12 wk old mice, to specifically express GCaMP6 in dopaminergic neurons. After virus injection, mice received a bolus of meloxicam (5 mg/kg BW, s.c.), and tramadol in drinking water for 3 d after surgeries to relieve pain. BW was checked twice a day. Experiments started at least 4 weeks later than virus injection to allow virus expression.

Statistical methods

All values are expressed as the mean $\pm$ SEM. Statistical analyses were conducted using GraphPad PRISM (version 8) unless stated otherwise. Datasets with only two independent groups were analyzed for statistical significance using an unpaired two-tailed Student's t-test. Datasets subjected to two independent factors were analyzed using two-way ANOVA followed by Sidak's post hoc test. All p-values < 0.05 were considered significant (*p < 0.05, **p < 0.01, and ***p < 0.001, ****p < 0.0001).

Acknowledgements

We acknowledge Hella Brönneke for outstanding support, Jens Alber, Pia Scholl, Christiane Schäfer, Nadine Spenrath, Ina Stünkel, Kerstin Marohl, Antonia Lorenz and Helmut Wratil for outstanding technical assistance. We received funding by the DFG within the framework of the

TRR 134 (A.C.H., P.K.), CRC 1451 (P.K.) and within the Excellence Initiative by German Federal and State Governments (CECAD). This work was funded (in part) by the Helmholtz Alliance ICEMED (Imaging and Curing Environmental Metabolic Diseases) through the Initiative and Networking Fund of the Helmholtz Association. X.X. received funding from CECAD Family Support. G.Y. gratefully acknowledges financial doctoral support from the DFG-233886668/GRK1960.

Author Contributions

X.X. and A.C.H. conceived the project, designed the experiments, and wrote the manuscript with input from the other authors. A.C.H. generated the Ox1R flox mouse line. X.X. performed all the experiments apart from calcium imaging experiments (G.Y. and P.K.) and PET imaging experiments (A.L.C. and H.B.). All authors discussed the data, commented on the manuscript before submission, and agreed with the final submitted manuscript.

Competing Interests

The authors declare no competing interests.

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Editors

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Reviewer #1 (Public Review):

Summary:

In this manuscript, the role of orexin receptors in dopamine neurons is studied. Considering the importance of both orexin and dopamine signalling in the brain, with critical roles in arousal and drug seeking, this study is important to understand the anatomical and functional interaction between these two neuromodulators. This work suggests that such interaction is direct and occurs at the level of SN and VTA, via the expression of OX1R-type orexin receptors by dopaminergic neurons.

Strengths:

The use of a transgenic line that lacks OX1R in dopamine-transporter-expressing neurons is a strong approach to dissecting the direct role of orexin in modulating dopamine signalling in the brain. The battery of behavioural assays to study this line provides a valuable source of information for researchers interested in the role of orexin-A in animal physiology.

Weaknesses:

The choice of methods to demonstrate the role of orexin in the activation of dopamine neurons is not justified and the quantification methods are not described with enough detail. The representation of results can be dramatically improved and the data can be statistically analysed with more appropriate methods.

Reviewer #2 (Public Review):

Summary:

This manuscript examines the expression of orexin receptors in the midbrain - with a focus on dopamine neurons - and uses several fairly sophisticated manipulation techniques to explore the role of this peptide neurotransmitter in reward-related behaviors. Specifically, *in situ* hybridization is used to show that dopamine neurons predominantly express the orexin receptor 1 subtype and then go on to delete this receptor in dopamine neurons using a transgenic strategy. *Ex vivo* calcium imaging of midbrain neurons is used to show that in the absence of this receptor orexin is no longer able to excite dopamine neurons of the substantia nigra.

The authors proceed to use this same model to study the effect of orexin receptor 1 deletion on a series of behavioral tests, namely, novelty-induced locomotion and exploration, anxiety-related behavior, preference for sweet solutions, cocaine-induced conditioned place preference, and energy metabolism. Of these, the most consistent effects are seen in the tests of novelty-induced locomotion and exploration in which the mice with orexin 1 receptor deletion are observed to show greater levels of exploration, relative to wild-type, when placed in a novel environment, an effect that is augmented after icv administration of orexin.

In the final part of the paper, the authors use PET imaging to compare brain-wide activity patterns in the mutant mice compared to wildtype. They find differences in several areas both under control conditions (i.e., after injection of saline) as well as after injection of orexin. They focus on changes in the dorsal bed nucleus of stria terminalis (dBNST) and the lateral paraventricular nucleus (LPGi) and perform analysis of the dopaminergic projections to these areas. They provide anatomical evidence that these regions are innervated by dopamine fibers from the midbrain, are activated by orexin in control, but not mutant mice, and that dopamine receptors are present. Thus, they argue these anatomical data support the hypothesis that behavioral effects of orexin receptor 1 deletion in dopamine neurons are due to changes in dopamine signaling in these areas.

Strengths:

Understanding how orexin interacts with the dopamine system is an important question and this paper contains several novel findings along these lines. Specifically:

- (1) The distribution of orexin receptor subtypes in VTA and SN is explored thoroughly.
- (2) Use of the genetic model that knocks out a specific orexin receptor subtype from only dopamine neurons is a useful model and helps to narrow down the behavioral significance of this interaction.
- (3) PET studies showing how central administration of orexin evokes dopamine release across the brain is intriguing, especially since two key areas are pursued - BNST and LPGi - where the dopamine projection is not as well described/understood.

Weaknesses:

The role of the orexin-dopamine interaction is not explored in enough detail. The manuscript presents several related findings, but the combination of anatomy and manipulation studies does not quite tell a cogent story. Ideally, one would like to see the authors focus on a specific behavioral parameter and show that one of their final target areas (dBNST or LPGi) was responsible or at least correlated with this behavioral readout. In addition, some more discussion on what the results tell us about orexin signaling to dopamine neurons under normal physiological conditions would be very useful. For example, what is the relevance of the orexin-dopamine interaction blunting novelty-induced locomotion under wildtype conditions?

In some places in the Results, insufficient explanation and reporting is provided. For example, when reporting the behavioral effects of the Ox1 deletion in two bottle preference, it is stated that "[mutant] mice showed significant changes..." without stating the direction in which preference was affected.

The cocaine CPP results are difficult to interpret because it is unclear whether any of the control mice developed a CPP preference. Therefore, it is difficult to conclude that the knockout animals were unaffected by drug reward learning. Similarly, the sucrose/sucralose preference scores are also difficult to interpret because no test of preference vs. water is performed (although the data appear to show that there is a preference at least at higher concentrations, it has not been tested).