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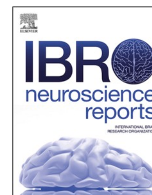

Research paper

Immunolocalization of kappa opioid receptors in the axon initial segment of a group of embryonic mesencephalic dopamine neurons

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ABSTRACT

The dopamine mesolimbic system is a major circuit involved in controlling goal-directed behaviors. Dopamine D2 receptors (D2R) and kappa opioid receptors (KOR) are abundant Gi protein-coupled receptors in the mesolimbic system. D2R and KOR share several functions in dopamine mesencephalic neurons, such as regulation of dopamine release and uptake, and firing of dopamine neurons. In addition, KOR and D2R modulate each other functioning. This evidence indicates that both receptors functionally interact, however, their colocalization in the mesostriatal system has not been addressed. Immunofluorescent assays were performed in cultured dopamine neurons and adult mice's brain tissue to answer this question. We observed that KOR and D2R are present in similar density in dendrites and soma of cultured dopamine neurons, but in a segregated manner. Interestingly, KOR immunolabelling was observed in the first part of the axon, colocalizing with Ankyrin in 20% of cultured dopamine neurons, indicative that KOR is present in the axon initial segment (AIS) of a group of dopaminergic neurons. In the adult brain, KOR and D2R are also segregated in striatal tissue. While the KOR label is in fiber tracts such as the striatal streaks, corpus callosum, and anterior commissure, D2R is located mainly within the striatum and nucleus accumbens, surrounding fiber tracts. D2R is also localized in some fibers that are mostly different from those positives for KOR. In conclusion, KOR and D2R are present in the soma and dendrites of mesencephalic dopaminergic neurons, but KOR is also found in the AIS of a subpopulation of these neurons.

Introduction

The dopaminergic mesolimbic system, composed of neurons that project from the ventral tegmental area (VTA) to the Nucleus Accumbens (NAc), is the primary circuit involved in the generation of goal-directed behaviors. Dopamine release modulates motivated behaviors by activating dopamine receptors located on GABAergic medium spiny neuron (MSN) in the NAc. Mechanisms regulating dopamine dynamics are essential for behavioral control. Indeed, dopamine unbalances within this circuit underlie several psychiatric diseases such as drug addiction (Volkow et al., 2017), schizophrenia (Brisch et al., 2014), and obsessive-compulsive disorder (Szechtman et al., 1999).

Dopamine D2 receptors (D2R) and kappa opioid receptors (KOR) are Gi-protein coupled receptors that control dopamine neurotransmission within the mesolimbic system. D2R are present both in the VTA and in the NAc. In the VTA, D2R are localized in tyrosine hydroxylase (TH) positive dendrites (Sesack and Pickel, 1994), where its activation decreases the firing of dopamine neurons (Beckstead et al., 2004). In the NAc, D2R are pre-synaptically localized on axon terminals and post-synaptically on dendrites of MSN GABA neurons (Pickel et al.,

Abbreviations: AIS, axon initial segment; AnkG, Ankyrin G; CPu, caudate putamen; DIV, days in vitro; D2R, dopamine D2 receptor; KOR, kappa opioid receptor; MAP2, microtubel associated protein 2; MSN, medium spiny neuron; NAc, nucleus accumbens; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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2006). Activation of D2R within the NAc decreases dopamine extracellular levels (Imperato and Di Chiara, 1988). Likewise, KORs are abundant in the mesolimbic system (Mansour et al., 1996) and negatively control dopamine neurotransmission. Like D2R, KOR activation in the VTA inhibits the firing of dopamine neurons (Margolis et al., 2003). Still, while D2R location has been established on TH-positive dendrites of the VTA (Sesack and Pickel, 1994), the KOR location remains to be firmly determined. In the NAc, KOR are localized in dopamine axon terminals (Svingos et al., 2001), and its acute activation decreases extracellular dopamine levels (Di Chiara and Imperato, 1988). Moreover, KOR knock-out mice have increased basal levels of dopamine in the NAc (Chefer et al., 2005), suggesting that KORs exert a tonic inhibition of dopamine release in the NAc.

Increasing literature indicates that KOR and D2R functionally interact to control dopamine neurotransmission and dopamine-related behaviors. For instance, KOR activation potentiates and accelerates locomotor sensitization and compulsive checking behavior induced by repeated D2R activation (Perreault et al., 2006, 2007). We recently found that KOR co-activation during repeated D2R treatment is associated with a sensitized D2R inhibition of tonic and phasic dopamine release in the NAc (Escobar et al., 2017). These data suggest that the enhancing effect of KOR over the locomotor activity and dopamine neurotransmission mediated by D2R arises both at presynaptic and postsynaptic levels within the NAc. Intriguingly, previous repeated activation of KOR diminishes D2R inhibition of dopamine release (Acri et al., 2001; Fuentealba et al., 2006) and decreases D2R binding in the striatum (Izenwasser et al., 1998). Also, it has been reported that KOR activation inhibits D2R-induced inhibitory postsynaptic currents in VTA neurons (Ford et al., 2007). The data indicate that KOR activation modifies D2R function, suggesting that these receptors may interact. However, their colocalization has not been addressed. To test whether KOR and D2R share similar locations within the dopamine system, we studied the localization of KOR and D2R in the meso-striatal pathway, mesencephalic primary cultured neurons, and the adult brain of mice. We show that KOR and D2R are present in dendrites and soma of cultured dopamine neurons but scarcely colocalize. Interestingly, in a group of mesencephalic dopamine neurons KOR is present in the axon initial segment (AIS), the structure responsible for the initiation of action potentials (Coombs et al., 1957; Kole and Stuart, 2008), and recently identified in dopaminergic neurons (González-Cabrera et al., 2017; Meza et al., 2018). In the adult brain, KOR and D2R also poorly colocalize in striatal tissue. While KOR was found on the axonal fibers of the striatum, corpus callosum, and anterior commissure, the D2R was found primarily within the striatum and NAc, surrounding the axonal fibers; and in fibers that are mostly different from those positive for KOR.

Experimental procedures

Animals

Female eighteen days (E18) pregnant Sprague Dawley rats (n = 3) were obtained from the animal care facility of the Faculty of Biological Sciences of Pontificia Universidad Católica de Chile. They were immediately euthanized for the removal of embryos.

Male C57BL/6 (n = 3) were maintained in the animal care facility of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile. Mice were group-housed (four-five mice/cage) under a 12/12 h inverted light/dark cycle (lights on at 10.00 pm), constant temperature (24 °C), with food and water available ad libitum. Mice were used for tissue immunofluorescence experiments.

All animal experiments were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Protocols were approved by the Bioethical Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile and the Bioethical Committee of the Comisión Nacional de Ciencia y Tecnología de Chile (CONICYT).

Primary mesencephalic cell culture and immunofluorescence

E18 pregnant rats were euthanized by decapitation, and the embryos were removed and placed in a petri dish containing ice-cold HANḰs buffered salt solution (HBSS). The mesencephalic culture was obtained as described previously (Slater et al., 2016; Pereira et al., 2017). Briefly, after decapitating embryos with scissors, the most ventral part of the mesencephalon was dissected and placed in a conic tube containing 10 ml of HBSS and centrifuged at 1000 RPM for 30 seg. The pellet was re-suspended by inversion with 4.5 ml of 0.075% papain solution in HBSS and disaggregated by incubation for 20 min at 37 °C. Then, the tube was centrifuged at 1000 RPM for 30 seg and papain removed. Four ml of warm Fetal Bovine Serum (FBS) was added to the pellet, mixed by inversion, and incubated for 2 min at room temperature. FBS was removed, and 5 ml of adhesion media (MEM, 10% FBS, 2 mM glutamine, 30 mM glucose, 1X penicillin/streptomycin) was added. 100.000–150.000 cells were seeded in coverslips pretreated with poli-L-lysine. Two hours after, the media was changed to complete Neurobasal media (2% B27, 1 mM glutamine, 1X Penicillin/Streptomycin). To reduce the number of glial cells in the cell culture, at DIV 1, half of the media was removed and replaced with Cytarabine (Ara-C) 1 μM. Cells were maintained for 9 days in vitro (DIV 9).

On DIV 9, cells were fixed with 3.7% formaldehyde, 1 μM $MgCl_2$ in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 and incubated with blocking solution (0.05% tween 20, 2 mg/mg BSA in PBS) for 1.5 hrs. Primary antibodies were incubated at 4 °C overnight. The following primary antibodies were used: guinea pig polyclonal anti-Tyrosine Hydroxylase antiserum (TH; 1/1000, 213004, Synaptic Systems), rabbit polyclonal anti-KOR antibody (1:500, Ab10566, Abcam), goat polyclonal anti-D2R antibody (1:250, sc-7522, Santa Cruz Biotechnology), goat polyclonal anti-Ankyrin G antibody (1:1000, sc-31778, Santa Cruz Biotechnology), mouse monoclonal anti-PAN Neurofascin antibody (1:1000, 73–172, Neuromab), rabbit anti-MAP 2 antibody (1:1000). After washing, cells were incubated with appropriate secondary antibodies: donkey anti-rabbit AlexaFluor 488, donkey anti-goat AlexaFluor 594, donkey anti-guinea pig AlexaFluor 647 (1/500; Invitrogen Life Technologies, Carlsbad, CA, US) for 1 hr at room temperature. Fluorescence imaging was acquired on a laser-scanning confocal microscope (Olympus®, Fluoview 1000) and Fluoview v6.0 software Olympus. Low magnification images were obtained with an appropriate 10X objective and high magnification with a 60X objective (NA 1.35 oil). High magnification images were acquired in z-stacks with 0.5 μm between images. Data presented was obtained from 4 to 6 pictures taken from different coverslips of three independent primary culture preparations, obtained from three pregnant rats with an average of 13 embryos each. We took pictures of 512 × 512 pixels and obtained were in average we visualized 8–10 neurons in the field.

Given that we could not validate primary antibodies in cell cultures obtained from knock-out since we did not have access to these KOR lines and because the D2R knock-out mice line available in our laboratory express a D2R truncated protein, recognized by our primary antibody (Sanchez et al., 2021), we validated them as mentioned in Escobar et al. (2017). D2R antibody (sc-7522, Santa Cruz Biotechnology) was validated in HEK293 cells transfected with a vector encoding D2R epitope-tagged with mcherry to its c-terminus Positive signal was observed only in transfected cells, which matched mcherry fluorescence (data not shown). KOR antibody Ab10566 (Abcam), recognizes the epitope formed by aminoacids 262–275 located in the third intracellular loop of the protein, this antibody was previously used in cell cultures of dorsal root ganglion, was validated by KOR silencing using KOR specific siRNA (Tsai et al., 2010).

We used Image J software for image analysis. We consider that the label for one protein collocated with the other when the marks

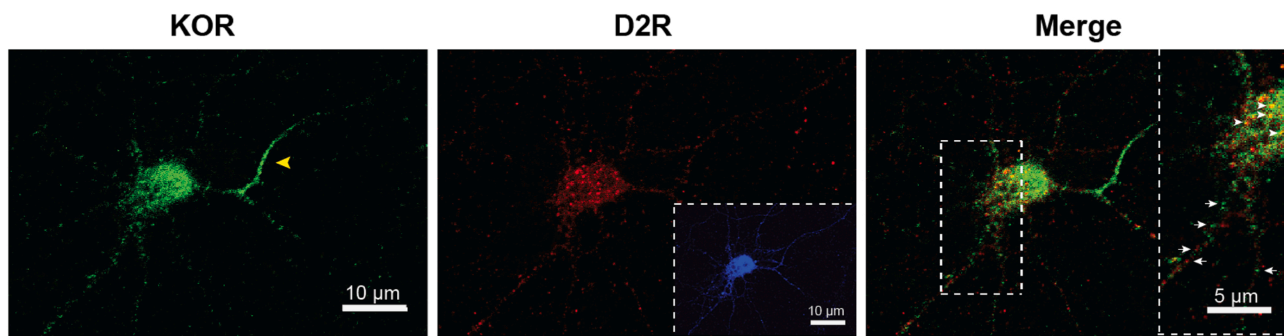

Fig. 1. KOR and D2R are located in somato-dendritic compartment and are segregated on axons of TH-positive mesencephalic neurons. Representative 100X image of a TH positive neuron of DIV 9 of E18 primary culture of rat mesencephalic neurons. Inset in the middle panel shows, in blue color, the positive immunostaining for TH. Yellow arrowhead depicts KOR exclusive localization. White arrowheads depicts KOR and D2R positive puncta. White arrows shows puncta exclusive for KOR or D2R.

overlapped, giving a mixture of colors in the merged image. To quantify KOR and AnkG colocalization, we manually quantified the number of TH-positive cells with both KOR and AnkG merging.

Immunofluorescence in mice tissue

C57BL/6 mice (n = 3) were anesthetized with a mixture of ketamine-xylazine (100–10 mg/kg, respectively) and perfused with 2% paraformaldehyde in PBS (pH 7.4). At the end of perfusion, brains were removed and post-fixed in 2% paraformaldehyde overnight, followed by

Fig. 2. Identification of axon initial segment in TH positive neurons. Representative 100X images of a TH positive neuron of rat mesencephalic neurons. **A)** Identification of AIS with AnkG (Ankyrin G) and reconfirmation with PAN Neuro (Neurofascin). Inset in the left panel shows positive immunostaining for TH. Inset in the right panel depicts higher magnification of AIS, arrowheads show AIS length. **B)** AIS localization in TH neurons, AnkG immunostaining respect to the somatodendritic compartment marker MAP2. Inset in the left panel shows, in blue color, the positive immunostaining for TH. Panel on the bottom shows higher magnification of AIS, arrowheads depicts AIS length.

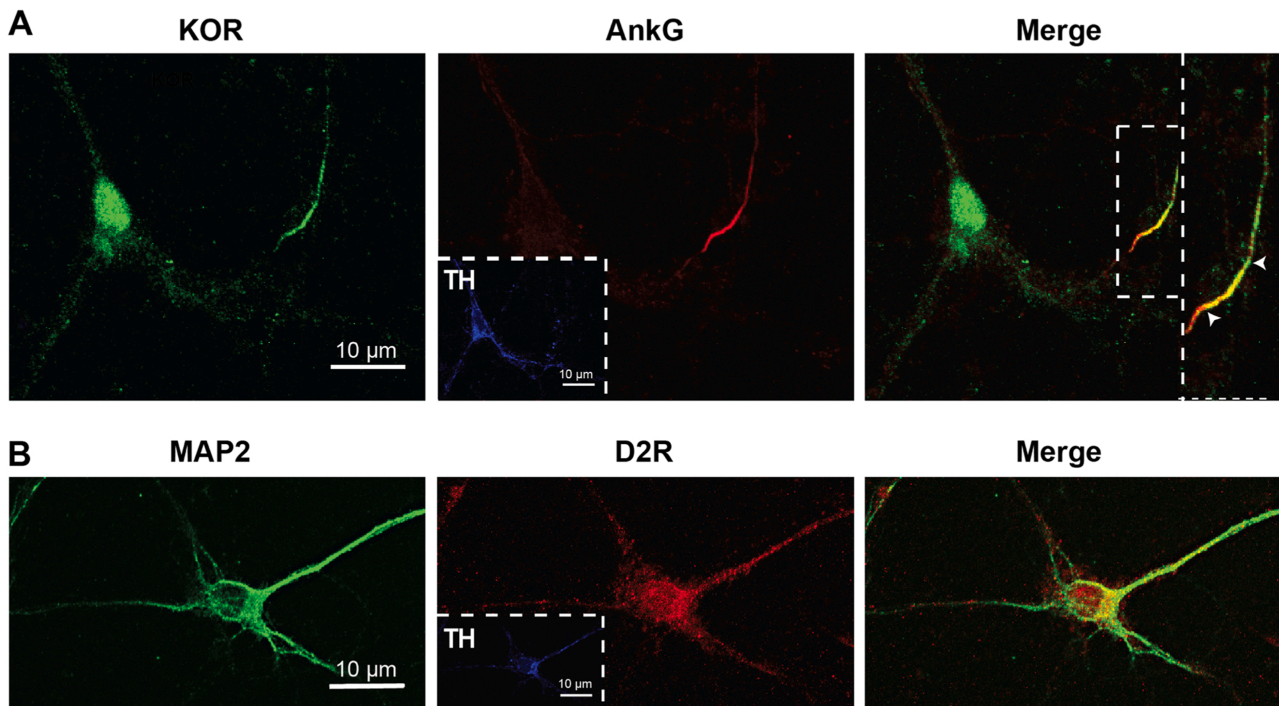

Fig. 3. KOR is located in the axon initial segment and D2R is located in somato-dendritic compartment of dopamine neurons. Representative 100X image of TH-positive neurons of DIV 9 of E18 primary culture of rat mesencephalic neurons. **A)** KOR co-localizes with AnkG, white arrowheads depicts the limits of higher co-localization. **B)** D2R co-localizes with somato-dendritic marker MAP2 Middle panel inset shows positive immunostaining for TH.

incubation in 30% sucrose for 48 hrs for cryopreservation. Brains were cut in 30 µm slices in the sagittal or coronal plane on a freezing-stage microtome (Reichert-Jung Mod. Hn 40). Sections containing the NAc and dorsal striatum (CPu) were selected. Slices were permeabilized using triton 0.05% in 3% normal donkey serum (NDS, vol/vol, Jackson Immunoresearch), for 1 h, and then blocked for 2 h with 3% NDS. Slices were incubated overnight with goat polyclonal anti-D2R antibody (1:250, sc-7522, Santa Cruz Biotechnology) and rabbit polyclonal anti-KOR antibody (1:1000, Ab10566, Abcam), followed by AlexaFluor488-conjugated donkey anti-goat IgG antibody (1:1000, Jackson Immunoresearch) and CY3-conjugated donkey anti-rabbit IgG antibody (1:1000, Jackson Immunoresearch). Slices were mounted in coverslips and covered with appropriate mounting media (Vectashield™, Vector Laboratories). Fluorescence imaging was acquired on a laser-scanning confocal microscope (Olympus®, Fluoview 1000) and Fluoview v6.0 software Olympus. Low magnification images were obtained with an appropriate 10X objective and high magnification with a 100X oil immersion objective. The primary and secondary antibodies used in mice tissue were validated in previously published studies from our laboratory (Escobar et al., 2017; Sánchez et al., 2021).

Results

Localization of KOR and D2R in cultured mesencephalic neurons of rat

To compare the localization of KOR and D2R, immunofluorescence assays were performed on DIV 9 of cultivated mesencephalic neurons obtained from E18 rat embryos. Immunolabeling shows that KOR and D2R signals spread all over the cell body and processes positive for TH (Fig. 1). Unexpectedly, co-localization of D2R and KOR is scarce, confined to a few dots in cell bodies (Fig. 1). Interestingly, in some neurons, an intense signal for KOR was observed in a single TH-positive process that resembled the axon initial segment (AIS) (Fig. 1). To test this hypothesis, we first assessed the localization of AIS in our primary cell culture by using Ankyrin G (AnkG) immunostaining as AIS marker (Kordeli et al., 1995; Kuba et al., 2006; Grubb and Burrone, 2010; González-Cabrera et al., 2017; Meza et al., 2018) and then reconfirming it by the immunostaining against Neurofascin, a cell adhesion molecule highly expressed in the AIS (Rasband, 2010) (Fig. 2**A**). AnkG immunolabel arises from a process positive for MAP2, a somatodendritic microtubule-associated protein (Fig. 2**B**), indicating that in the primary mesencephalic culture, the AIS occurs from a dendrite, as previously published (Meza et al., 2018).

Second, we analyzed the localization of KOR concerning AnkG, we found KOR immunolabeling was present in the cell body of every TH positive neuron, and that 20 ± 3.72% (mean ± SEM) of TH positive neurons colocalize KOR WITH AnkG, an example is depicted in Fig. 3**A**, indicating a group of dopamine neurons has KOR in the AIS, suggesting a role for KOR regulating dopamine neurons firing rate at this developmental stage. As expected, D2R signaling coincides with immunofluorescence for MAP2 (Fig. 3**B**). Together these data show that KOR and D2R are mostly segregated in the somatodendritic compartment of mesencephalic TH-positive neurons.

Robust post-synaptic localization of KOR in D2R positive and negative MSN in mice striatal structures

KOR-induced enhancement of D2R-mediated behaviors prompted us to evaluate postsynaptic colocalization of KOR with D2R in the striatum (CPu) and NAc. This set of experiments were carried out in mice tissue since the quality of the immunofluorescence for KOR and D2R was higher than that obtained with rat tissue. Low magnification images of sagittal slices show segregated signals for KOR and D2R in striatal tissue. While the KOR label is highly located in fibers of passage such as striatal streaks of the CPu, corpus callosum, and anterior commissure, D2R is located mainly within the CPu and NAc, surrounding the fibers of passage (Fig. 4). Higher magnification images of CPu and NAc show a clear localization of KOR in fibers (Fig. 5). As we previously showed (Escobar et al., 2017; Tejeda et al., 2017), a strong signal for KOR in some D2R positive and D2R negative MSN of the NAc and CPu was observed

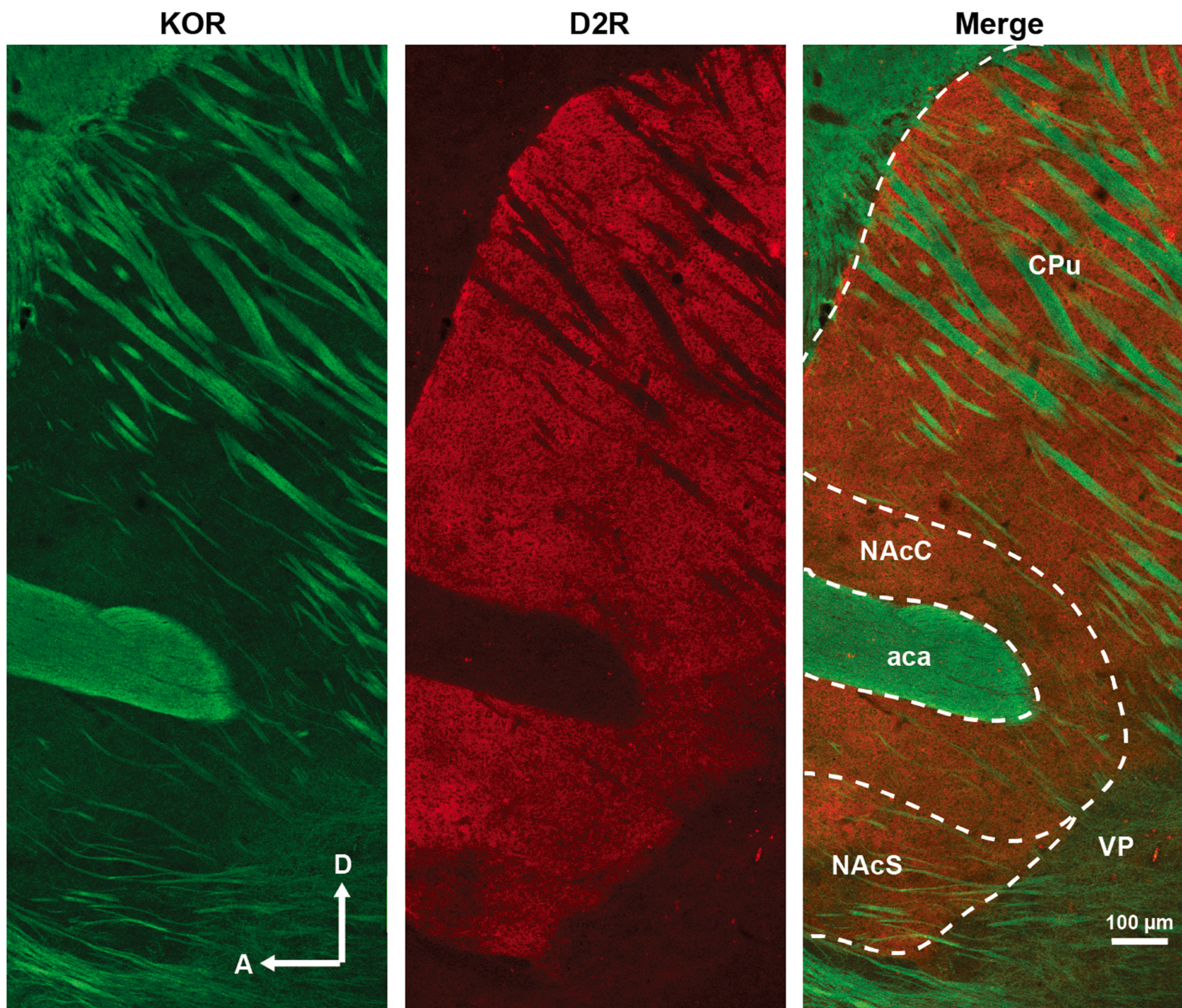

Fig. 4. KOR is localized in axonal tracts and D2R is localized within the striatum. Sagittal view of mouse striatum and NAc, 10X images reconstruction. CPu, caudate-putamen; NAcC, Nucleus accumbes core; NAcS, Nucleus accumbens shell; aca, anterior part of anterior commissure; VP ventral pallidum; D, dorsal; A, anterior; 1.08 L lateral, 1.08 mm lateral to middle line.

(Fig. 5), indicating that KOR is also located postsynaptically in these nuclei.

Discussion

In the present work, we studied the localization of KOR and D2R within the meso-striatal system of rodents. We show that both KOR and D2R are present in the somatodendritic compartment of mesencephalic TH-positive neurons but with intercalated positions. We found that a group of mesencephalic dopamine neurons localize KORs on the AIS. On the striatal tissue, we found that KOR and D2R co-localize in MSNs, but they are segregated in fibers such as in the anterior commissure, corpus callosum, and striatal streaks where only KOR were found.

The activation of either KOR or D2R inhibits dopamine neurons' firing within the VTA (Margolis et al., 2003; Beckstead et al., 2004), suggesting that both receptors are present in these neurons. Indeed, our data using the primary culture of mesencephalic neurons show KOR and D2R in the same TH-positive neurons. Both receptors are present in the somatodendritic compartment but with a segregated pattern, suggesting a lack of physical interaction. It is not possible for us to determine if the localization pattern of the D2R and KOR corresponds to a subpopulation of the dopaminergic neurons, since we did not use specific markers for each dopamine neuron subpopulation.

Margolis et al. (2006), in an electrophysiology-retrograde tracer combined-study, carried out in midbrain rat slices, showed that the direct activation of KORs in VTA inhibits the firing of neurons that project to the medial prefrontal cortex (mPFC) but not in those projecting to the NAc (Margolis et al., 2006). On the other hand, the activation of D2R inhibits the firing of neurons projecting from VTA to mPFC or NAc, but not those that project to the amygdala (Margolis et al., 2008). These data suggest that the coexistence of KOR and D2R occurs in a subpopulation of midbrain neurons in the adult brain, those that specifically project to the mPFC. In our cell culture, TH-positive neurons were positive for both KOR and D2R, suggesting that specialization of KOR- and D2R-containing subpopulations into VTA-projecting neurons occur at later stages of development.

Remarkably, we found that a group of TH positive neurons have KOR in axons, specifically on the AIS. We observed that the composition and position of the AIS in cultured dopaminergic neurons resemble that found in vivo, where the AIS preferentially emerges from a dendrite (Meza et al., 2018). Other G protein-coupled receptors have been reported to localize in this axonal compartment, as in the case of 5HT1A receptor in pyramidal neurons (Azmitia et al., 1996), muscarinic M1 receptor in granule cells of the hippocampus (Martinello et al., 2015),

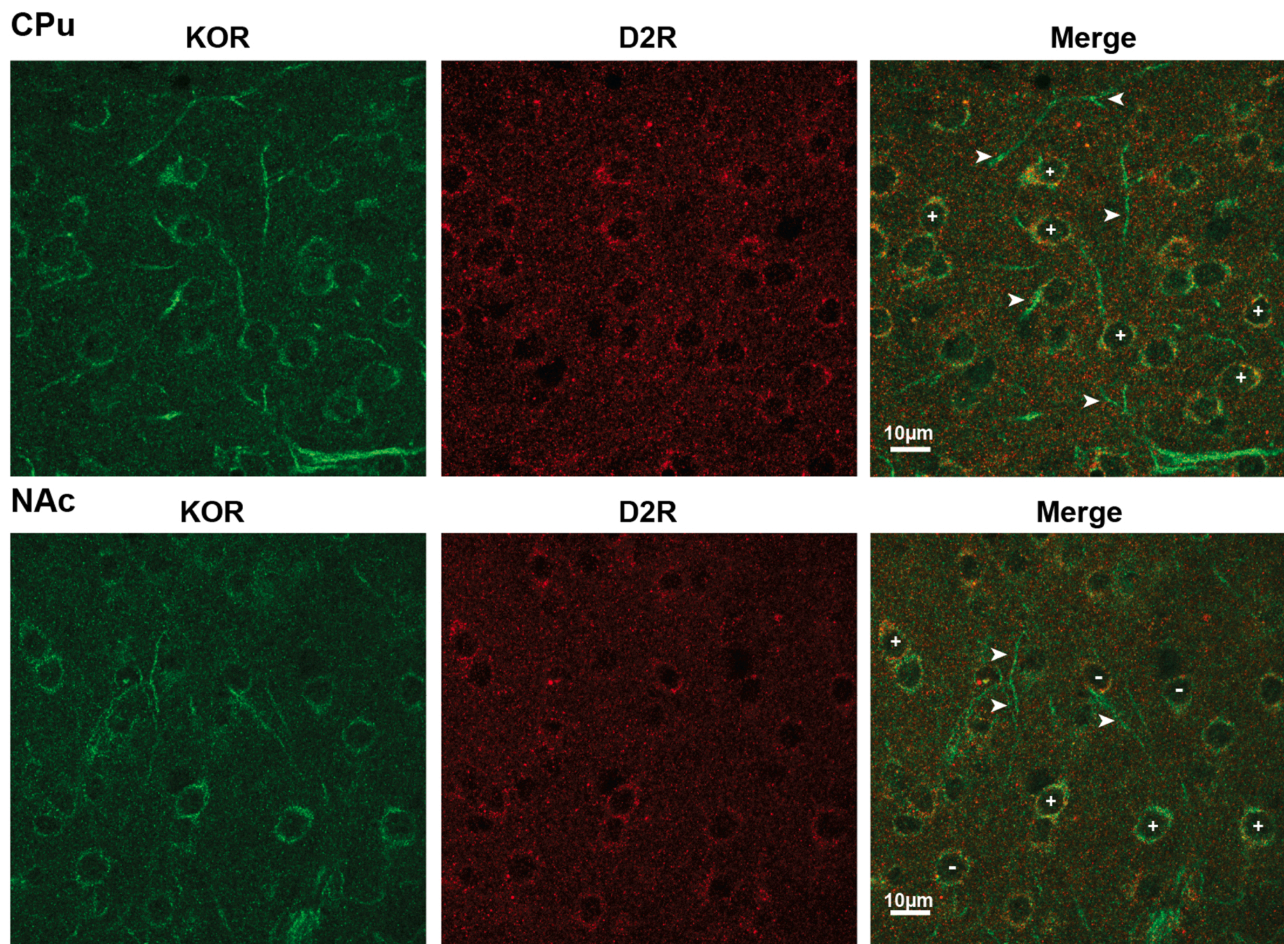

Fig. 5. KOR and D2R localization in cell bodies of mouse caudate-putamen and NAc. 100X images of striatal tissue. CPu: caudate-putamen; NAc: Nucleus Accumbens. Plus signs (+) indicate cell bodies positives for both KOR and D2R immunostaining. Minus signs (-) indicate cell bodies positives either for KOR or D2R, white arrowheads indicate fibers where only KOR immunostaining was found.

and dopamine D3 receptor in interneurons of the dorsal cochlear nucleus (Bender et al., 2010). The activation of these receptors in the AIS control neuron activity by directly modulating ion channels highly expressed in this compartment (Yamada and Kuba, 2016). A very recent study made in knock-in mice of D2R fused to SEP fluorescent protein showed, for the first time, that D2R localizes in the AIS of dopamine neurons of the SNc (Lebowitz et al., 2022), although quantification of the proportion of SNc dopamine neurons expressing D2R in the AIS was not addressed, this finding suggests a crucial role of D2R in regulating the firing of dopamine neurons during adulthood. In our primary cell culture, D2R immunostaining was confined to the somatodendritic compartment of TH neurons, indicating that contrary to the findings of Lebowitz et al. (2022) D2R does not localize in the AIS in the early developmental stage of the rat brain. Further studies are needed to decipher whether D2R locate in the AIS of dopamine neurons of the adult rat brain.

A recent in vivo study shows that the morphology of the AIS within dopamine neurons of the SNc determines their basal tonic activity, where a longer AIS defines a higher firing rate (Meza et al., 2018). Therefore, the localization of KOR in the AIS could have a crucial role in setting the tonic activity characteristic of dopamine neurons at this developmental stage.

The basal firing rate of dopaminergic neurons depends on the coupling between somatodendritic-AIS oscillations (Meza et al., 2018). The location of D2R and KOR found in our study suggests that D2R activation should inhibit the activity of the somatodendritic compartment. In contrast, KOR activation should hinder the activity of both somatodendritic and AIS compartments. Taken together, we suggest that at this developmental stage the independent inhibition of these compartments could affect the coupling between soma and AIS of dopamine neurons affecting their basal tonic activity and that consequently could trigger changes in dopamine levels in the target areas as has been previously found after the repeated D2R and KOR co-activation (Escobar et al., 2017).

We also compared the localization of D2R and KOR in target projections of midbrain dopamine neurons in adult mice. As expected, we found D2R highly expressed in the CPu and NAc, where it localizes in MSNs cells. This result is consistent with the ultrastructural analysis of D2R localization in the CPu showing that D2R is primarily located in dendrites, specifically in spines, and showing few co-localization with TH positives terminals (Sesack and Pickel, 1994). Accordingly, we observed few D2R label processes resembling axons. On the other hand, we observed that KOR was highly localized in axonal tracts such as the anterior commissure, corpus callosum, and striatal streaks. Autoradiography assays performed in the brains of rats (Atweh and Kuhar, 1977) and guinea pigs (Foote, 1987) also found this KOR pattern, indicating that KOR localization in fiber tracts is transversal to rodent species and suggesting a pivotal role of KOR on the control of neuron activity directly on axons. Svingos et al. 2001, showed that KOR was preferentially located in terminals in the NAc. Accordingly, we found high labeling of KOR in fibers similar to axons. As we previously reported (Escobar et al., 2017), we found that KOR was also in MSN colocalizing with D2R, giving an anatomical substrate for the potentiated locomotion found in rats after the repeated co-activation of KOR and D2R (Perreault

et al., 2006).

In conclusion, both KOR and D2R are highly expressed pre and postsynaptically in the mesolimbic system. Presinaptically, KOR, and D2R co-exist in mesencephalic dopaminergic neurons, but each in a specific and distinct location and segregated in striatal subdivisions. Postsinaptically, KOR, and D2R colocalize mainly in the cell bodies of CPu and NAc. Our data provide an anatomical framework for the functional interaction between KOR and D2R. Their inhibitory actions on dopamine neurons firing and dopamine release must arise from different mechanisms and neuronal compartments, highlighting a relevant role of KOR on the AIS of mesolimbic dopaminergic neurons.

Limitations of the study

Our study is based on immunofluorescent assays in primary cell culture of embryonic rats and adult mice tissue. Our study aims to give a characterization of D2R and KOR locations in the dopamine system at different developmental stages, the functionality of our findings should be evaluated in future electrophysiological works.

One of the main concerns of the technique is the specificity of antibodies, we did control for anti-D2R antibody in HEK-293 where immunolabeling was found to colocalize with the red fluorescence of transfected D2R coupled to mcherry protein. On the other hand, the KOR antibody was chosen based on its validation of dorsal root ganglion neurons, where no labeling was found when KOR was silenced with siRNA (Tsai et al., 2010), in this culture the KOR label was found along the axon, but not restricted to a particular region, as observed in our study in a fraction of dopamine neurons. Although this difference does not exclude the possibility of non-specific staining of the KOR antibody it might be also due to differences between cell types. The location of KOR in the AIS of dopamine neurons should be further characterized in future studies, using different antibodies and mesencephalic primary cultures obtained from KOR knockout embryos.

CRediT authorship contribution statement

A.P.E, R.C.M and M.E.A conceived and designed research; A.P.E, R.C.M, M.P.G performed experiments and interpreted results of experiments; A.P.E and R.C.M prepared figures; A.P.E drafted the manuscript; M.E.A and P.H edited and revised the manuscript and approved its final version.

Discontinued antibodies.

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R.C.M received ANID doctoral fellowship no 21120709.

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