



Experimental Evidence of A_{2A}–D₂ Receptor–Receptor Interactions in the Rat and Human Carotid Body

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Adenosine A_{2A} receptors (A_{2A}R) and dopamine D₂ receptors (D₂R) are known to be involved in the physiological response to hypoxia, and their expression/activity may be modulated by chronic sustained or intermittent hypoxia. To date, A_{2A}R and D₂R can form transient physical receptor–receptor interactions (RRIs) giving rise to a dynamic equilibrium able to influence ligand binding and signaling, as demonstrated in different native tissues and transfected mammalian cell systems. Given the presence of A_{2A}R and D₂R in type I cells, type II cells, and afferent nerve terminals of the carotid body (CB), the aim of this work was to demonstrate here, for the first time, the existence of A_{2A}R–D₂R heterodimers by *in situ* proximity ligation assay (PLA). Our data by PLA analysis and tyrosine hydroxylase/S100 colocalization indicated the formation of A_{2A}R–D₂R heterodimers in type I and II cells of the CB; the presence of A_{2A}R–D₂R heterodimers also in afferent terminals is also suggested by PLA signal distribution. RRIs could play a role in CB dynamic modifications and plasticity in response to development/aging and environmental stimuli, including chronic intermittent/sustained hypoxia. Exploring other RRIs will allow for a broad comprehension of the regulative mechanisms these interactions preside over, with also possible clinical implications.

Keywords: carotid body, type I cells, adenosine A_{2A} receptors, dopamine D₂ receptors, heterodimers, *in situ* PLA

INTRODUCTION

In mammals and humans, the carotid bodies (CBs) are chemosensory organs located at the bifurcations of the common carotid arteries with a critical role in maintaining homeostasis during both development/aging (Di Giulio, 2018; Sacramento et al., 2019) and environmental variations (e.g., levels of O₂, CO₂, and arterial blood pH) (Iturriaga and Alcayaga, 2004; Iturriaga et al., 2016; Prabhakar and Peng, 2017; Di Giulio, 2018; Iturriaga, 2018) with also a sensing function with respect to metabolic factors (Porzionato et al., 2011; Conde et al., 2018; Cunha-Guimaraes et al., 2020; Sacramento et al., 2020).

The CB regulatory function is strictly related to its specific organization. Morphologically, in the CB parenchyma two types of cells can be distinguished: “neuron-like” chemosensitive type I cells, positive for tyrosine hydroxylase (TH), and “glial-like” supportive type II cells, positive for glial fibrillary acidic protein (GFAP) (Pardal et al., 2007; Tse et al., 2012). Sensitive innervation of the CB

is mainly mediated by afferent terminals of the carotid sinus nerve, branch of the glossopharyngeal nerve, arising from neurons located in the petrosal ganglion (PG).

Neurotransmission in the CB involves a complex interplay of excitatory and inhibitory signals (Iturriaga and Alcayaga, 2004; Nurse, 2005; Fitzgerald et al., 2009; Porzionato et al., 2018; Stocco et al., 2020). Type I cells produce several neurotransmitters [e.g., dopamine, noradrenaline, adrenaline, acetylcholine, serotonin, adenosine, adenosine 5'-triphosphate (ATP)] and neuromodulators (e.g., enkephalins, neuropeptide Y, calcitonin gene-related peptide, galanin, endothelins, bombesin, adrenomedullin, kisspeptins, leptin) (Varas et al., 2003; Iturriaga and Alcayaga, 2004; Porzionato et al., 2008), in turn acting in an autocrine/paracrine manner on a broad spectrum of different ionotropic/metabotropic receptors located in afferent nerve fibers, type I cells, and type II cells, these latter also showing a role in the coordination of chemosensory transduction (Nurse, 2014; Porzionato et al., 2018; Stocco et al., 2020). Among these receptors, some metabotropic G protein-coupled receptors (GPCRs) (e.g., A_{2A}, D_{1/2}, H_{1/2/3}, M_{1/2}, 5-HT_{2A}, and others) are also involved; in particular, A_{2A} and D₂ have attracted the attention of many researchers, resulting among the most studied GPCRs (Aldossary et al., 2020).

The presence of A_{2A}R was verified in rat (Gauda, 2000; Kobayashi et al., 2000; Xu et al., 2006; Bairam et al., 2009) and human (Fagerlund et al., 2010) CB specimens, where it showed to be expressed in type I cells, colocalizing with tyrosine-hydroxylase (TH) (Gauda, 2000; Gauda et al., 2000; Kobayashi et al., 2000; Bairam et al., 2009) or β -III-tubulin (Fagerlund et al., 2010). Considering the methodological approaches, different techniques were adopted, including *in situ* hybridization analysis (Gauda, 2000), immunohistochemistry (Kobayashi et al., 2000; Fagerlund et al., 2010), Western blot analysis (Bairam et al., 2009), and Ca²⁺ imaging technique (Xu et al., 2006). Also, D₂R presence was reported in CB type I cells in rats (Czyzyk-Krzeska et al., 1992; Holgert et al., 1995; Gauda et al., 1996, 2001; Bairam and Khandjian, 1997; Gauda, 2000; Kinkead et al., 2005; Wakai et al., 2015), rabbits (Bairam et al., 1996b; Bairam and Khandjian, 1997; Bairam et al., 2003), cats (Bairam and Khandjian, 1997), and humans (Fagerlund et al., 2010). CB specimens were analyzed through *in situ* hybridization (Czyzyk-Krzeska et al., 1992; Holgert et al., 1995; Gauda, 2000), RT-PCR (Bairam et al., 1996b, 2003; Bairam and Khandjian, 1997; Kinkead et al., 2005), and immunofluorescence (Wakai et al., 2015).

Apart from type I cells, some data support the expression of A_{2A}R and D₂R also in type II cells (Kaelin-Lang et al., 1998; Leonard and Nurse, 2020). Additionally, A_{2A}R (Gauda, 2000; Gauda et al., 2000; Conde et al., 2006, 2017, 2012; Zhang et al., 2018; Sacramento et al., 2019) and D₂R (Czyzyk-Krzeska et al., 1992; Schamel and Verna, 1993; Bairam et al., 1996a,b) were also demonstrated in PG neurons and afferent fibers in the CB.

As demonstrated for transfected mammalian cell systems and different native tissues (i.e., central nervous system, mammary gland, liver, cancer tissues), A_{2A}R and D₂R can establish transient physical receptor–receptor interactions (RRIs) giving rise to a dynamic equilibrium between their specific monomeric form and homo/heterocomplexes (dimers or receptor mosaics)

(Ferré et al., 2014; Guidolin et al., 2015). Such RRIs, in turn, likely modulate ligand binding and signaling, thus affecting the physio-pathological features but also the pharmacology of the nervous system.

Despite that the presence of A_{2A}R and D₂R has been broadly recognized in the CB, the possible existence of A_{2A}R–D₂R heterodimers was never verified before, but only hypothesized in a previous work (Porzionato et al., 2018). Thus, in this study, rat and human CB specimens were investigated by proximity ligation assay (PLA) technique to assess the eventual interaction between A_{2A}R and D₂R, thus corroborating the above working hypothesis and possibly opening the doors to the analysis of further possible RRIs in the CB.

MATERIALS AND METHODS

Tissue Collection

The animal study was reviewed and approved by the ethical committee of Padua University, in agreement with the Italian Department of Health guidelines (Authorization No. 702/2016-PR of July 15, 2016). Human tissues were managed by the Body Donation Program of the Section of Human Anatomy, University of Padova (Macchi et al., 2011; Porzionato et al., 2012), according to European, Italian, and Regional guidelines (De Caro et al., 2009; Riederer et al., 2012). Excision was further authorized by the Italian law No. 10 of February 10, 2020, entitled “Rules regarding the disposition of one’s body and post-mortem tissues for study, training, and scientific research purposes” (Boscolo-Berto et al., 2020). Donors’ written informed consent was signed upon joining the Body Donation Program; here, Donor’s authorization expressly allowed to use Body and Body Parts also for research purposes, after donation.

Rat CBs were excised from 5 adult Sprague-Dawley rats; tissue isolation occurred immediately after euthanasia. Human CBs were obtained at autopsy from 5 adult subjects [3 males, 2 females; mean age 46 years, standard deviation (SD) \pm 3.6] with no clinical sign of chronic pulmonary and/or cardiovascular diseases. Eventual pharmacological therapies that could have influenced the CB plasticity constituted a further exclusion criterion. Autopsies occurred within 30 h after death, according to Italian Law. On the basis of our previous experience (Porzionato et al., 2005, 2006, 2011), the tissues are viable and adequate for immunohistochemistry/immunofluorescence studies after this death–autopsy interval.

According to routine protocols, once isolated, the CBs were promptly fixed in 10% phosphate-buffered formalin for 72 h, dehydrated through ascending alcohols and xylene, clarified through xylene, and paraffin embedded.

Immunohistochemical Analysis

Preliminarily, the primary antibodies used were tested by immunohistochemistry; this is an important step before PLA assay, whose performance critically depends on the antibodies’ quality as the GPCR antibodies are notoriously problematic (Michel et al., 2009; Trifilieff et al., 2011).

Longitudinal serial sections of the whole fixed carotid bifurcation (5 μm in thickness) were prepared, dewaxed according to routine protocols, and immunostained by anti-A_{2A}R antibody (monoclonal mouse antibody; ab79714, Abcam, United Kingdom) and anti-D₂R antibody (polyclonal rabbit antibody; ab150532, Abcam). The anti-A_{2A}R antibody and the anti-D₂R antibody were used with a dilution of 1:100 and 1:200, respectively; antigen retrieval occurred before both staining with high pH (EnVision™ FLEX, High pH, K8012) and low pH (EnVision™ FLEX, Low pH, K8005) buffer. The sections were incubated using the DAKO Autostainer Plus Staining System (EnVision™ FLEX, High pH). Immunostaining specificity was confirmed by sections incubated without primary antibody, which did not show immunoreactivity.

Proximity-Ligation Assay (PLA)

PLA technology allows easy visualization of endogenous protein–protein interactions at the single-molecule level. The method relies on the use of combinations of antibodies coupled to complementary oligonucleotides that are amplified and revealed with a fluorescent probe. Each single protein–protein interaction is visualized as a fluorescent spot.

In situ PLA was performed according to the manufacturer's instructions on 5-μm rat and human CB slices using the following: mouse anti-A_{2A} primary antibody (dilution: 1:100); rabbit anti-D₂R primary antibody (dilution: 1:200); Duolink® *in situ* PLA detection kit (DUO92014, Sigma-Aldrich, St Louis, MO, United States); Duolink® anti-rabbit PLUS probe (DUO92002, Sigma-Aldrich); and Duolink® anti-mouse MINUS probe (DUO82040, Sigma-Aldrich). Briefly, the slices were blocked with Duolink® blocking solution, in a humid chamber for 60 min at 37°C and then incubated with the primary antibodies (anti-A_{2A}R and anti-D₂R) solution prepared in the antibody diluent solution; incubation occurred in a humid chamber for 1 h at room temperature (RT). Thereafter, the primary antibody solution was tapped off and the slices were washed with wash buffer at RT, before incubation with the anti-rabbit and anti-mouse secondary antibody-conjugated PLA probes in a preheated humidity chamber, for 1 h at 37°C. After hybridization, ligation and amplification steps were performed. For TH and S100 colocalization analysis, after the amplification step, the slices were rinsed in wash buffer and (a) incubated with anti-TH (1:6,000) in Antibody Diluent solution (Dako) or (b) incubated with anti-S100 (1:7,000) in a humid chamber at 4°C, overnight. Subsequently, after a wash in PBS, incubation was performed using mouse Alexa Fluor-488 (1:100; 1 h at RT) for TH or rabbit Alexa Fluor-488 (1:500; 1 h at RT) for S100. Thereafter, the sections were rinsed in PBS and mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA, United States) for 15 min at RT.

Immunofluorescence and PLA signals were analyzed and acquired with Zeiss800 confocal microscope equipped with 63× oil objective (NA = 1.4). For each field of view, z-stacks were acquired for a total thickness of 10 μm. Images were acquired enabling the identification of the A_{2A}R–D₂R heterodimers at confocal microscopy as red dots.

In order to better detail the localization of the red dots with reference to nuclei and membranes of different cell types, images were analyzed with the help of z projections and 3D volume rendering through different perspectives. This permitted to better localize the red dots without bias due to plane overlapping. In particular, Z-stacks were acquired and exported with ZenBlue software. For image visualization in 2D, z projections were performed with FIJI software, while 3D volume rendering was reconstructed with IMARIS software.

Negative control experiments were performed avoiding the conjugation of the primary antibodies with the Duolink® Probes; in turn, no positive reaction occurred, and no red dots were visualized. The specificity of the double immunolabeling was verified by replacing the primary antibodies with PBS.

Quantification of the PLA signal was performed on z-stack images, and the number of red dots was manually counted with ImageJ, using the cell counter plugin. The average data referring to the density of positive PLA elements ± SD are related to images acquired from at least 3 randomly chosen fields from three slides of each animal/patient. The percentages of colocalization of red dots with TH and S100 immunostainings were also calculated.

RESULTS

A_{2A}R and D₂R by Immunohistochemistry

Adenosine A_{2A}R and dopamine D₂R were identified through immunohistochemistry in both rat and human CBs (**Figure 1**). Considering 3'-diaminobenzidine tetrahydrochloride (DAB) distribution, A_{2A}R- and D₂R-positive elements were mainly localized in correspondence of type I cells, being similarly arranged in clusters. However, partial immunostaining of A_{2A}R and D₂R also in type II cells and PG nerve terminals cannot be excluded.

Detection of the A_{2A}R–D₂R Heterodimers by PLA

PLA is an antibody-based method to detect biomolecules in physical proximity, and thus, it is recognized as an important experimental approach to demonstrate physical RRI when native molecules are localized within a radius of 0–16 nm, a distance considered crucial for heteromer formation. Only in case of physical closeness of proteins will a signal be produced. Here, possible heterodimerization between A_{2A}R and D₂R was verified through PLA. Under a confocal microscope, in both rat and human CBs, most red dots were recognized close to the DAPI-stained nuclei but not inside them, supporting location at the plasma membrane level; 3D visualization of the tissues allowed for a better topographical analysis (**Figures 2, 3**). As it regards the few red dots which were far away from the nuclei, we cannot exclude a localization in afferent terminals from the carotid sinus nerve. A homogeneous distribution of heterodimers was observed in all specimens with a mean density (± standard deviation) of $(3.5 \pm 0.67) \times 10^{-3}$ heterodimers/μm² and $(5.9 \pm 1.4) \times 10^{-3}$ heterodimers/μm² in rat and human samples, respectively.

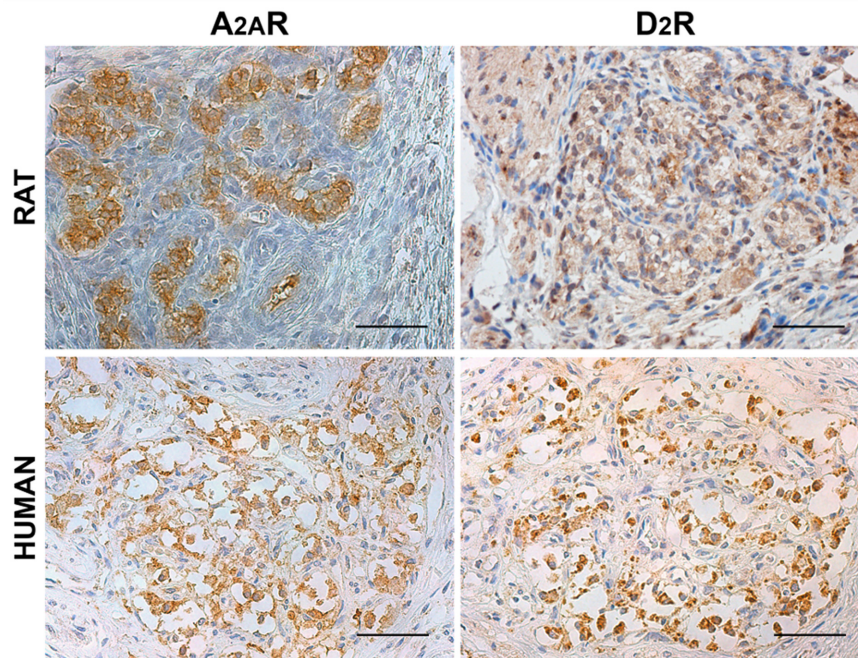


FIGURE 1 | Distribution of adenosine A_{2A}R and dopamine D₂R in rat and human CB. Positive immunostaining in clusters of roundish cells is consistent with type I cells, although some positive elongated cells may be considered as type II cells. Scale bar: 50 μ m.

After PLA, specific TH and S100 immunostaining was also performed to distinguish type I and type II cells. This methodological approach allowed to assess by confocal microscopy the localization of the A_{2A}R–D₂R heterodimers with respect to the CB constituent cells. In all stained specimens, S100-immunopositive cells, corresponding to type II cells, were specifically visualized as yellow elements; TH immunoreactivity was observed in the cytoplasm of CB type I cells and visualized in green (**Figure 4**). We cannot exclude possible visualization of TH-positive afferent nerve fibers, as also PG terminals may be TH immunoreactive (Katz et al., 1983; Katz and Black, 1986).

In rats, the percentages of TH and S100 colocalizations of red dots were 51.49 ± 5.97 and 55.84 ± 3.63 , respectively. In human samples, the percentages of TH and S100 colocalizations of red dots were 60.65 ± 8.49 and 50.75 ± 6.30 , respectively. Significant differences were not found between TH and S100 colocalizations in the two different species.

As previously stated, the presence of red dots far away from nuclei and possibly positive for TH may be interpreted as localization of A_{2A}R–D₂R heterodimers in PG terminals, as they may express A_{2A}R (Gauda, 2000; Gauda et al., 2000; Conde et al., 2012; Zhang et al., 2018; Sacramento et al., 2019) and D₂R (Czyzyk-Krzeska et al., 1992; Schamel and Verna, 1993; Bairam et al., 1996a,b).

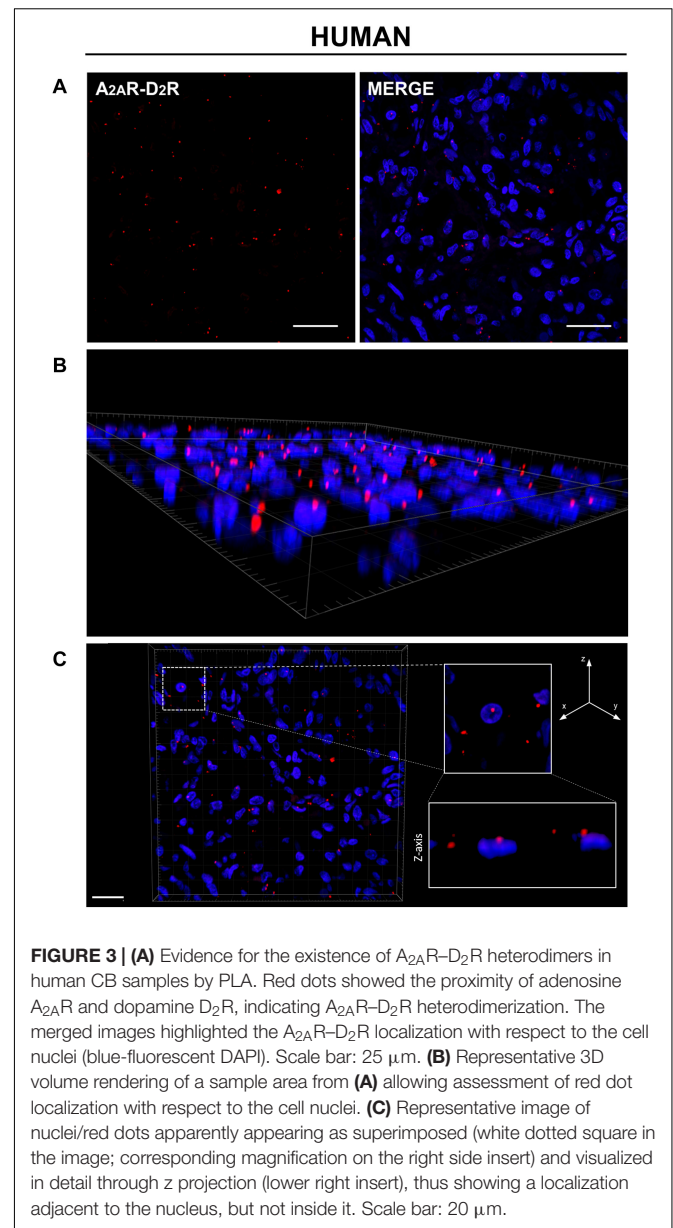
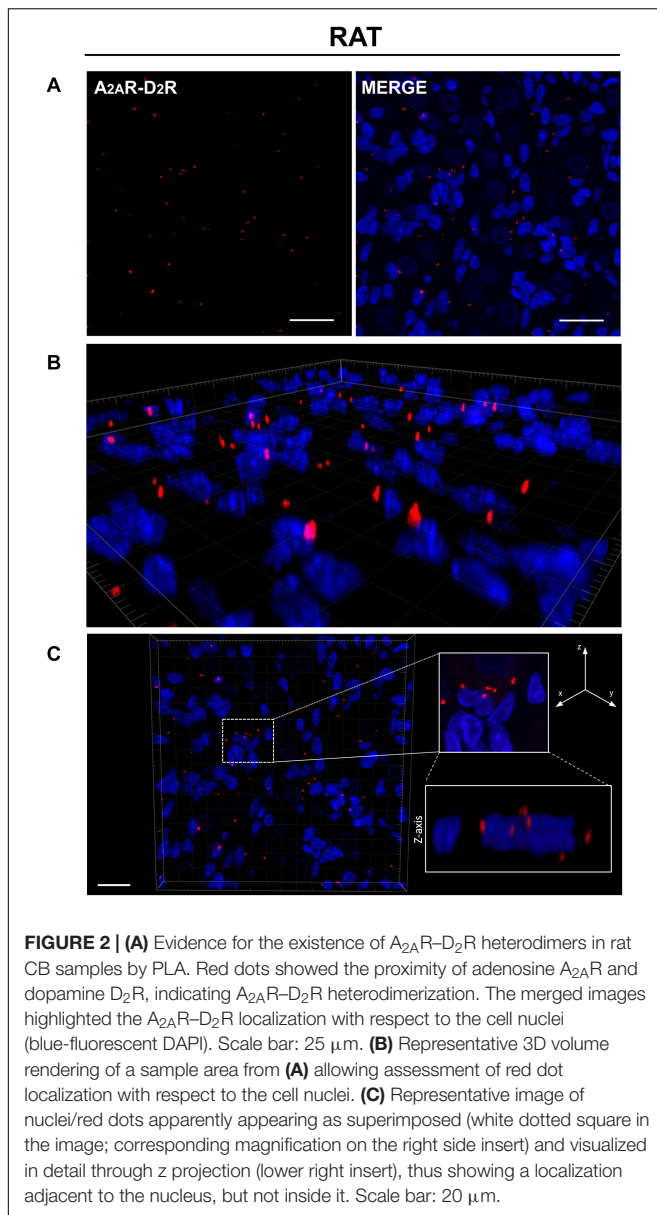
DISCUSSION

Type I CB cells release many different neurotransmitters (i.e., acetylcholine, adenosine, ATP, dopamine) with excitatory or

inhibitory effects (Iturriaga and Alcayaga, 2004). Among the main receptors mediating adenosine and dopamine function in the CB, A_{2A}R, and D₂R are included. Experimental data support the presence of A_{2A}R (Gauda, 2000; Gauda et al., 2000; Kobayashi et al., 2000; Xu et al., 2006; Bairam et al., 2009; Fagerlund et al., 2010) and D₂R (Czyzyk-Krzeska et al., 1992; Holgert et al., 1995; Gauda et al., 1996, 2001; Bairam et al., 1996b, 2003; Bairam and Khandjian, 1997; Gauda, 2000; Kinkead et al., 2005; Fagerlund et al., 2010; Wakai et al., 2015) in CB type I cells. Kaelin-Lang et al. (1998) also recognized, by *in situ* hybridization on rats, A_{2A}R-positive elements likely attributable to type II cells. Even referring to D₂R, a recent paper by Leonard and Nurse (2020), considering the possible inhibitory role of dopamine on type II cell function, suggests D₂R localization in type II cells. Our data indicated the presence of A_{2A}R–D₂R heterodimers both in type I and II cells of rats and humans.

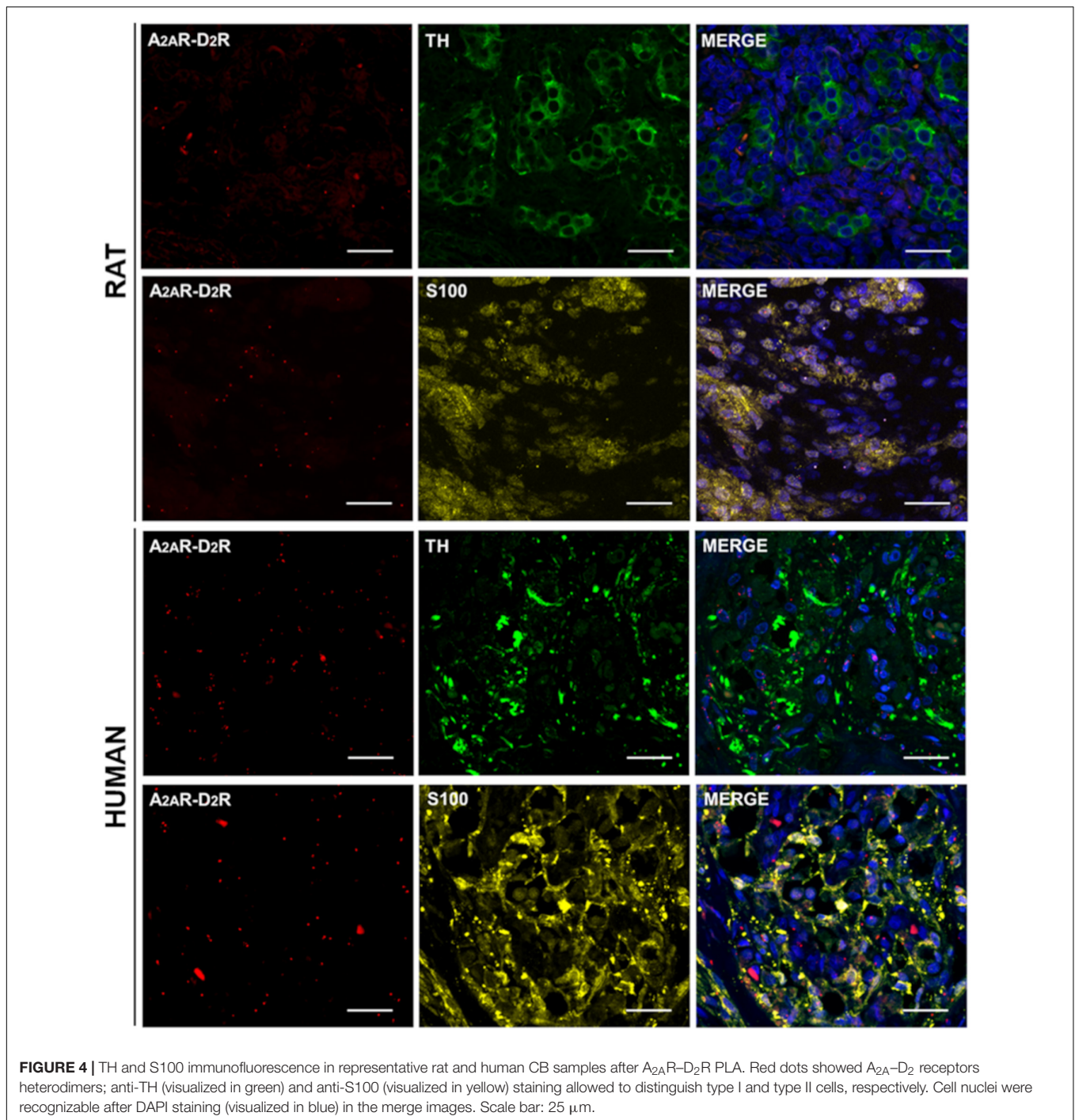
PG neurons and terminals are known to express A_{2A}R (Gauda, 2000; Gauda et al., 2000; Conde et al., 2012; Zhang et al., 2018; Sacramento et al., 2019) and D₂R (Czyzyk-Krzeska et al., 1992; Schamel and Verna, 1993; Bairam et al., 1996a,b). Moreover, afferent terminals in the CB may also express TH (Katz et al., 1983; Katz and Black, 1986). Thus, red dots far away from nuclei and colocalizing with faint fluorescent staining may be interpreted as nerve localization of A_{2A}R–D₂R heterodimers.

Typically, A_{2A}Rs are coupled to G_s protein, whose activation increases the cAMP levels, promoting an excitatory behavior (Weaver, 1993; Lahiri et al., 2007; De Caro et al., 2013). In the CB, the increase of adenosine levels determined by hypoxia induces the increase in intracellular cAMP through stimulation of A_{2A}R (Lahiri et al., 2007). Adenosine activation of A_{2A}R also triggers



Ca²⁺ rise during hypoxia (Kobayashi et al., 2000; Tse et al., 2012). Similarly, A_{2A}Rs also mediate the effect of hypercapnia (Sacramento et al., 2018). Conversely, D₂Rs are coupled to inhibitory G₁/G₀ proteins and their activation decreases cAMP levels with the onset of an autocrine/paracrine inhibitory signal (Zeng et al., 2007; Wakai et al., 2015; Zhang et al., 2018). Hypoxia is also responsible for dopamine release from CB type I cells and activation of postsynaptic D₂R (Prieto-Lloret et al., 2007) and D₂R activation exerting inhibitory effects (Gonzalez et al., 1994) on ventilation, both during rest (Zapata and Zuazo, 1980) and hypoxic exposure (Nishino and Lahiri, 1981), although direct activation of D₂R in PG terminals could also have a modulatory (Alcayaga et al., 1999; Alcayaga et al., 2003) or even excitatory effect (Alcayaga et al., 2006; Iturriaga et al., 2009), depending on species involved.

Another possible regulative mechanism exists for these receptors, based on direct reciprocal interactions. The formation of A_{2A}R–D₂R complexes was highlighted in transfected cells, including SH-SY5Y (Hillion et al., 2002; Xie et al., 2010) and HEK-293T cells (Navarro et al., 2014), neuronal primary cultures of rat striatum (Navarro et al., 2014) and enkephalin-containing GABAergic neurons from the mammal striatum (Fink et al., 1992; Fuxe et al., 1998; Trifilieff et al., 2011). A_{2A}R–D₂R heterodimers are key modulators of striatal neuronal function (Taura et al., 2018); here, heterodimerization showed to modulate GABAergic striato-pallidal neuronal activity. Reciprocal antagonistic interactions occur within the A_{2A}R–D₂R heterodimer (Fuxe et al., 2005). In particular, A_{2A}R ligands decrease both the affinity and the signal intensity of D₂R ligands (Ferré et al., 2016), determining the increased



excitatory activity of adenosine, while D₂R agonists decrease the binding of A_{2A}R ligands (Fernández-Dueñas et al., 2018), causing increased inhibitory activity of dopamine. For instance, after incubation of striatal membrane preparations with the A_{2A}R agonist CGS21680, the affinity of the high-affinity D₂R agonist-binding site decreases (Fuxe et al., 1998; Guidolin et al., 2018). A_{2A}R–D₂R interactions may be modulated by different drugs (some of which with well-known effects on the CB); for instance, the psychostimulant effects of caffeine are also

mediated by the blockage of the allosteric modulation within the A_{2A}R–D₂R heterodimer, by which adenosine decreases the affinity and intrinsic efficacy of dopamine at the D₂R (Bonaventura et al., 2015).

Existence of RRI (A_{2B}–D₂) in CB chemoreceptors was first postulated by Conde et al. (2008) to explain the possible mechanism involved in catecholamine release by the CB. Thereafter, due to the glomic expression of a huge amount of different G protein-coupled receptors, our group hypothesized

a possible heterodimerization between many other different receptors in the CB (Porzionato et al., 2018). Thus, the aim of this experimental work was to verify the existence of RRI in the CB, suggesting a possible experimental strategy for its future characterization but also a new interpretive key for a broad comprehension of the regulative mechanisms it presides over.

To date, many biochemical and/or biophysical methods are available to demonstrate receptor colocalization. Among them, the PLA technique allows easy visualization of endogenous protein–protein interactions at the single molecule level (Ristic et al., 2016). Our data by PLA analysis confirmed the existence of A_{2A}R–D₂R heterodimers in both type I and type II cells of the CB, indicating that RRI may have a role in the functional modulation of these cells.

The identification of A_{2A}R–D₂R RRI in type II cells further supports a role for these cells in chemosensory modulation, in accordance with other authors (Kaelin-Lang et al., 1998; Tse et al., 2012; Leonard and Nurse, 2020). This finding, to be further detailed, could be particularly intriguing as A_{2A}R–D₂R heterodimers have also been identified in astrocytes (Cervetto et al., 2017, 2018; Pelassa et al., 2019; Guidolin et al., 2020).

The confirmation of A_{2A}R–D₂R RRI across species strengthens the idea on their contributory role in physiological events mediated by the CB. The differences between rats and humans in terms of amount and distribution of A_{2A}R–D₂R RRI may conversely be ascribed to species-specific differences and/or to potential exposure to different stimuli. The CB is known to undergo plastic changes in response to development/aging and various environmental stimuli, including chronic intermittent/sustained hypoxia. Its function is strictly related to these dynamic modifications (Iturriaga et al., 2006; López-Barneo et al., 2009; Dmitrieff et al., 2011; Kumar and Prabhakar, 2012; Bavis et al., 2013; Del Rio et al., 2014; Pulgar-Sepúlveda et al., 2018; Bavis et al., 2019; Liu et al., 2019), which can be also ascribed to the specific receptor behavior.

Various environmental stimuli could potentially modulate A_{2A}R–D₂R RRI. For instance, A_{2A}R can be present intracellularly and migrate to the cell membrane upon stimulation (Arslan et al., 2002; Milojević et al., 2006; Yu et al., 2006; Sacramento et al., 2015). In this sense, hypoxic effects on RRI will be surely to be evaluated, as hypoxia exerts an increase in adenosine and dopamine release from CB chemoreceptors (Conde et al., 2012), likely inducing a receptor-level modulation, as shown in rabbits by Bairam et al. (2003). Chronic caffeine treatment induces an increase in both adenosine and dopamine (Conde et al., 2012), and neonatal caffeine treatment increases the mRNA levels encoding for A_{2A}R (Montandon et al., 2008; Bairam et al., 2009)

and D₂ in male rats CB (not in female) (Montandon et al., 2008). Moreover, A_{2A}R and D₂R expressions are also modulated by age. D₂R mRNA increases with maturation (Gauda et al., 2000, 2001; Gauda, 2000; Gauda and Lawson, 2000), whereas A_{2A}R mRNA decreases (Gauda, 2000; Gauda and Lawson, 2000). Thus, further analyses will also have to address possible changes in A_{2A}R–D₂R RRI in response to development/age, hypoxic stimuli, or possible effects by other factors (drugs, metabolism, and others), allowing for a broad comparative study in different pathophysiological conditions.

Moreover, future perspectives of the work will include the involvement of other methods to better detail RRI, such as biophysical (e.g., bioluminescence– and fluorescence–resonance energy transfer; specialized microscopic techniques; X-ray crystallography) and biochemical analyses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Body Donation Program of the Section of Human Anatomy, University of Padova, according to European, Italian and regional guidelines. Excision was furtherly authorized by the Italian law No. 10 of February 10, 2020, entitled “Rules regarding the disposition of one’s body and post-mortem tissues for study, training, and scientific research purposes.” The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethical Committee of Padua University, in accordance with the Italian Department of Health Guidelines.

AUTHOR CONTRIBUTIONS

AP, RD, VM, ES, and MS designed the study. MS and ES performed the experiments. MC and SB supported the experiments. GB and FR performed the confocal microscopy analyses. AP, RD, ES, MS, DG, and VM interpreted the data. ES and AP wrote the manuscript. RD and AP supervised the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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