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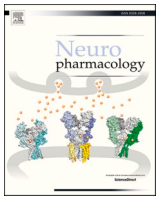

Striatal astrocytic A2A-D2 receptor-receptor interactions and their role in neuropsychiatric disorders

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ABSTRACT

It is now generally accepted that astrocytes are active players in synaptic transmission, so that a neurocentric perspective of the integrative signal communication in the central nervous system is shifting towards a neuro-astrocentric perspective. Astrocytes respond to synaptic activity, release chemical signals (gliotransmitters) and express neurotransmitter receptors (G protein-coupled and ionotropic receptors), thus behaving as co-actors with neurons in signal communication in the central nervous system. The ability of G protein-coupled receptors to physically interact through heteromerization, forming heteromers and receptor mosaics with new distinct signal recognition and transduction pathways, has been intensively studied at neuronal plasma membrane, and has changed the view of the integrative signal communication in the central nervous system. One of the best-known examples of receptor-receptor interaction through heteromerization, with relevant consequences for both the physiological and the pharmacological points of view, is given by adenosine A2A and dopamine D2 receptors on the plasma membrane of striatal neurons. Here we review evidence that native A2A and D2 receptors can interact through heteromerization at the plasma membrane of astrocytes as well. Astrocytic A2A-D2 heteromers were found able to control the release of glutamate from the striatal astrocyte processes. A2A-D2 heteromers on striatal astrocytes and astrocyte processes are discussed as far as their potential relevance in the control of glutamatergic transmission in striatum is concerned, including potential roles in glutamatergic transmission dysregulation in pathological conditions including schizophrenia or the Parkinson's disease.

1. Introduction

1.1. Astrocytes and signal communication in the central nervous system

Attention focused on neuron function in central nervous system (CNS) made the astrocyte function taking second place until recent years. Today astrocytes are recognized to be active players in synaptic transmission and co-actors with neurons in the brain function. As a matter of fact, a neurocentric view of the integrative signal communication in the CNS is moving towards an astrocentric perspective (see Robertson, 2002; Pereira and Furlan, 2010; Parpura and Verkhratsky, 2012; Zhang et al., 2021), or to a broad neuro-astrocentric view (Cervetto et al., 2017, 2021; Venturini et al., 2019). The neuro-astrocentric view of neuropsychiatric disorders is based on the idea that complex cellular networks (Agnati and Fuxe, 2000; Marcoli et al., 2023) and neuron-astrocyte crosstalk play crucial roles for brain integrative functions. Several pieces of evidence indicate a major involvement of astrocytes in the information processing in CNS and in

Abbreviations: CNS, central nervous system; GFAP, glial fibrillary acidic protein; GPCR, G protein-coupled receptor; Hcy, homocysteine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PLA, proximity ligation assay; RRI, receptor-receptor interaction.

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the pathophysiology of psychiatric disorders and neurodegenerative/neuroinflammatory pathological conditions. The astrocyte roles in healthy and diseased brain are excellently reviewed in recent papers (Sofroniew and Vinters, 2010; Oliveira et al., 2015; Habib et al., 2020; Meldolesi, 2020; Preman et al., 2021; Kruyer et al., 2023). The relevance of astrocytes to the brain function is not unexpected if one considers the abundance of astrocytes in particular in the human brain (Sherwood et al., 2006; Sofroniew and Vinters, 2010; von Bartheld et al., 2016; Sonninen et al., 2020), and the ability of perisynaptic astrocyte processes to regulate the function of large numbers of neurons at the tripartite synapses (Araque et al., 1999; Derouiche et al., 2002; Reichenbach et al., 2010; Verkhratsky and Nedergaard, 2014; Goenaga et al., 2023).

It is now generally accepted that glial cells are excitable: fast ionic signaling, in addition to slow signaling, occurs in glial cells in response to physiological stimuli (Verkhratsky et al., 2020). Notably, astrocytes have been proven to be equipped with transmitter-loading vesicles and to release gliotransmitters in a vesicular way (see Montana et al., 2004, 2006; Parpura and Verkhratsky, 2012; Araque et al., 2014; Goenaga et al., 2023). Activation of neuronal target receptors by gliotransmitters (e.g. glutamate, ATP, D-serine) then modulates synaptic transmission and plasticity (see Araque et al., 2014; Goenaga et al., 2023). Exocytotic vesicular release of the gliotransmitter glutamate has been confirmed by the effectiveness of vesicular glutamate transporter (VGLUT) blockers in astrocytes (see Bezzi et al., 2004; Montana et al., 2006 and references therein; Bergersen and Gundersen, 2009; Perea et al., 2009), as well as in isolated astrocyte processes (Stigliani et al., 2006; Cervetto et al., 2015, 2018, 2021). It has been repeatedly shown that functional astrocyte processes, retaining the typical ultrastructural features including transmitter-loading vesicles (Stigliani et al., 2006; Cervetto et al., 2015, 2018) and expressing ezrin (Cervetto et al., 2018), the selective marker of the perisynaptic processes (Derouiche and Frotscher, 2001; Derouiche, 2003), can be isolated from various CNS regions. The perisynaptic astrocyte processes are primarily involved in gliotransmitter release and astrocyte-neuron communication at synapses (see Reichenbach et al., 2010; Lavialle et al., 2011; Ghézali et al., 2016 and references therein). Consistently, the isolated processes were proven capable of Ca^{2+}-dependent exocytotic vesicular release of the gliotrasmitter glutamate (Stigliani et al., 2006; Cervetto et al., 2015, 2017) but also able to release signals transferred through exosomes (Venturini et al., 2019). Notably, the perisynaptic processes exhibit high plasticity in response to synapse activity and can rapidly modify the synapse coverage (Reichenbach et al., 2010; Bernardinelli et al., 2014a, 2014b; Kater et al., 2023) and the interstitial space volume (Xie et al., 2013). Signaling from the processes of astrocytes - source for both classical volume transmission through the release of gliotransmitters, and roamer-type volume transmission through the release of exosomes - might therefore play different roles depending on synapse activity and synaptic coverage.

The above evidence indicates that astrocytes, receiving messages from neurons and sending messages to neurons, are obligatory co-actors in brain signal communication and transmission control. The presence of astrocytic receptors that bind and respond to neurotransmitters, and of neuronal receptors that are acted upon by gliotransmitters is central to the bidirectional neuron-astrocyte signaling.

1.2. Neurotransmitter receptors on astrocytes

Astrocytes express heterogeneous receptors for neurotransmitters - mainly of the G Protein-Coupled Receptor (GPCR) superfamily (Ross, 1989; Vassilatis et al., 2003), but also ionotropic receptors (Höft et al., 2014)- expression of which varies between brain regions, being most probably regulated by the neurochemical environment (Verkhratsky et al., 1998; Verkhratsky and Nedergaard, 2018). It appears that GPCRs are not focally expressed in astrocytic plasma membrane rather they are expressed broadly, allowing detection and integration of neurotransmitter signals of multiple synaptic contacts in a single astrocyte (Charles et al., 2003). Evidence from various studies (Shigetomi et al., 2013; Poskanzer and Yuste, 2016; Ye et al., 2017; Stobart et al., 2018) showed a high level of Ca^{2+} activity in astrocytic processes. Calcium signals in astrocytes in discrete regions of the processes termed Ca^{2+} microdomains, dynamic Ca^{2+} changes spatially restricted to the fine perisynaptic astrocyte processes (Ahmadpour et al., 2021; Lia et al., 2021) are often uncorrelated with events in the soma (Otsu et al., 2015; Bindocci et al., 2017). Nevertheless, there is broad agreement that GPCR activation leads to an enhanced probability of Ca^{2+} signals in astrocytes in their microdomains and later, at higher levels of activation, these Ca^{2+} transients can be propagated to the soma (Araque et al., 2014; Volterra et al., 2014; Kofuji and Araque, 2021; Goenaga et al., 2023). Astrocytic Ca^{2+} signals evoked by GPCR activation in response to synapse activity, and their relationship with remodeling of astrocyte morphology and gliotransmitter release are reviewed in Goenaga et al. (2023). The Ca^{2+} transients in the processes seem to depend mainly on Ca^{2+} influx, while at the astrocyte soma they may depend on Ca^{2+} release from intracellular stores (Bazargani and Attwell, 2016; Lia et al., 2021). On this background it seems of interest to note that only in the processes of reactive astrocytes newly expressed GluA2-lacking AMPA receptors allowed Ca^{2+} influx coupled to vesicular release of glutamate (Marcoli et al., 2022), opening new investigation for a better understanding of Ca^{2+} microdomains in astrocyte processes in physiological and pathological conditions.

2. GPCR complexes and A2A-D2 receptor-receptor interaction

The idea that transmitters act upon their own receptors to contribute to brain information processes has changed dramatically with the finding that receptors for different transmitters can physically interact through receptor-receptor interactions (RRIs), forming receptor complexes. GPCR heteromers display unique pharmacology, changes in receptor agonist recognition, signaling and trafficking that add an unexpected complexity to the information handling at membrane level in CNS (Agnati et al., 2018; Marcoli et al., 2023). The existence of direct RRIs between different receptors was suggested in pioneering works by Agnati and Fuxe, based on the ability of neuropeptides to modulate the binding of monoamine receptors in membrane preparations (Agnati et al., 1980; Fuxe et al., 1983; Fuxe and Agnati, 1985). In the 1980s, evidence was provided that GPCRs could allosterically interact at the plasma membrane level forming macromolecular assemblies of two or more receptors (receptor mosaics, Agnati et al., 1980, 1982; Fuxe et al., 1983; see also Agnati et al., 2003a, 2004, 2005, 2010; Fuxe et al., 2008; Farran, 2017; Prasad et al., 2021). The identified GPCR complexes are continuously increasing (see Farran, 2017; Guidolin et al., 2018, 2022; Lazim et al., 2021; Dale et al., 2022 for recent reviews; see also the GPCR Interaction Network, http://www.gpcr-hetnet.com/). The GPCR complexes may be considered specialized plasma membrane micro-circuits acting as "intelligent interfaces" between the extracellular and the intracellular environment (Agnati et al., 2006a, 2007; Marcoli et al., 2022). The field of GPCR RRIs is now a major research area relevant to the understanding of signal integration in CNS, and to their use as targets for new drug development. In the groundbreaking field of GPCR complexes the attention was until recent years focused on neuronal GPCR RRIs in CNS.

One of the best-known examples of GPCR RRIs is given by adenosine A2A and dopamine D2 receptors interaction in A2A-D2 heteromers.

2.1. A2A and D2 receptors in the brain and their roles

The A2A receptor is a purinergic GPCR, in the mammal brain mainly expressed at striatal level (Svenningsson et al., 1999; Fredholm et al., 2011; Bartoli et al., 2020). The receptor is activated by adenosine, which can be released as a neurotransmitter (Liu et al., 2019) but is also produced extracellularly from ATP by the ecto-nucleotidase activity

(Cunha, 2005). As a matter of fact, the preferential localization of ecto-nucleotidases near the A2A receptor (Cunha, 2005; Augusto et al., 2013), may play a crucial role in the receptor activation. The A2A receptor is involved in the control of synaptic plasticity in healthy brain functioning while excessive receptor function seems related to neuron damage, and the receptor and has been considered a promising therapeutic target in different neurodegenerative/neuropsychiatric disorders (see Cunha, 2016; Domenici et al., 2019). A therapeutic use of A2A agonists was suggested in diseases including autism-spectrum disorders or schizophrenia, while A2A blockade, affording neuroprotection against brain damage, was suggested as an approach to neurodegenerative/neuropsychiatric conditions including Parkinson's disease (PD), Alzheimer's disease (AD), attention-deficit hyperactivity disorder, depression; (Cunha, 2016; Domenici et al., 2019). A2A antagonists are proposed as a novel strategy to improving deficits of goal-directed behavior and to enhance cognitive flexibility; approval of the A2A antagonist istradefylline for PD treatment (Chen and Cunha, 2020) opens a repurposing opportunity for pharmacological control of goal-directed behavior and cognition (Chen et al., 2023). In fact, changes of A2A levels in pathological conditions and their consequences in term of synaptic plasticity, neuro-glial communication and memory, support repurposing of adenosine drugs for the treatment of AD and related disorders (Orr et al., 2015, 2018; Merighi et al., 2022; Launay et al., 2023). Moreover, astrocytic A2A are suggested to mediate the release of cytokines involved in neuroinflammation, which is a high-risk factor for depression (Zhao et al., 2022).

The D2 receptor for dopamine is expressed in several mammal brain regions and enriched in the striatum (Missale et al., 1998). The D2 receptors are acted upon by dopamine mainly through non-synaptic volume transmission (see Agnati et al., 2006; Vizi et al., 2010; Fuxe et al., 2015a). Notably, decreased expression of dopaminergic receptors, especially of the D2 subtype, is observed in the aging brain, and D2 agonists displayed positive effects on cognitive deficiency in AD patients (see Kourosh-Arami et al., 2023). Down-regulation of D2 receptors in the aging brain has been also suggested to compromise the immune homeostasis, contributing to PD pathogenesis (Wang et al., 2015). It appears that glia-mediated neuroinflammation, a hallmark of neurodegenerative diseases contributing to neuron death and disease progression in PD or AD, might be triggered by impaired dopaminergic transmission (Possemato et al., 2023). D2 receptors were reported to inhibit inflammasome activation and neuroinflammation, to maintain immune homeostasis and to play neuroprotective roles in striatum or brain cortex against injury-induced neuroinflammation, neurodegeneration, and synaptic dysfunction (Zhang et al., 2015; Alam et al., 2021; Possemato et al., 2023). Inhibition of the inflammasome activity by D2 agonist could pave the way for the identification of new pharmacological targets for therapeutic intervention in early PD and AD (see Possemato et al., 2023 and references therein). Various disorders that are known to involve dysfunction of the dopamine system, including schizophrenia and PD, but also drug abuse disorder, dysregulation of reward-related mechanisms and food addiction, compulsive eating and obesity, as well as attention deficit/hyperactivity disorders, exhibit D2 alterations in striatum (Baik, 2013; Gallo, 2019; Wise and Robble, 2020). Notably, genetic variants in the *DRD2* gene have been suggested to potentially contribute to susceptibility for post-traumatic stress disorder and major depression (Zhang et al., 2023).

2.2. A2A-D2 receptor-receptor interaction

It is well established that when expressed on the same cells transfected A2A and D2 receptors can interact and heteromerize (Hillion et al., 2002; Canals et al., 2003; Kamiya et al., 2003; Vidi et al., 2008; Cabello et al., 2009; Borroto-Escuela et al., 2013). Functional and physical evidence proved that native A2A and D2 receptors as well can heteromerize on plasma membrane of striatal neurons, where the effects of heteromerization on receptor recognition and signaling have been widely investigated (Hillion et al., 2002; Azdad et al., 2009; Cabello et al., 2009; Trifilieff et al., 2011; Navarro et al., 2014; see also Agnati et al., 2003a,b; Fuxe et al., 2005; Gomes et al., 2016). Notably, A2A-D2 heteromerization provide an example of the interaction of volume transmission with synaptic transmission, and of their contribution to the integrative brain function, carried out through direct RRIs in receptor complexes (Fuxe et al., 2015a; see also Misganaw, 2021). It was reported that through A2A-D2 heteromerization at the striatopallidal GABA neuron plasma membrane, A2A receptor activation reduces the affinity of the D2 agonist binding site causing reduction of D2 receptor-G-protein coupling and D2 signaling (Hillion et al., 2002; Trifilieff et al., 2011). Accordingly, A2A-D2 heteromers regulate NMDA-mediated firing in striatopallidal GABA neurons, A2A receptor activation counteracting the inhibitory effect of D2 receptor on the NMDA-mediated firing (Azdad et al., 2009). Investigation on striatal A2A-D2 heteromers led to new perspectives on the molecular mechanisms of both schizophrenia or Parkinson's disease (PD) and on the pathophysiology of antiparkinsonian drug-induced dyskinesias, providing also new antipsychotic or anti-parkinson drug targets (Ferré, 1997; Agnati et al., 2003b; Soriano et al., 2009; Fuxe et al., 2014, 2015b; Guidolin et al., 2015; Borroto-Escuela et al., 2020; Prasad et al., 2021; Valle-León et al., 2021, 2023).

Despite the major attention to striatal A2A-D2 receptor heteromers as potential drug targets, and despite the recognized relevance of astrocytes in pathological conditions involving the striatum, including schizophrenia (see Matos et al., 2015; Tarasov et al., 2019; Chang et al., 2021) and PD (Villalba and Smith, 2011; Booth et al., 2017; Brandebura et al., 2022; Minchev et al., 2022), the presence and function of GPCR complexes on the astrocyte plasma membrane in the striatum was not investigated until recent years.

3. Striatal astrocytic A2A and D2 receptors

Adult striatal astrocytes were found to express adenosine A2A receptors (Matos et al., 2013). It is to note that in striatal astrocytes A2A activation could inhibit glutamate uptake, depending on a physical association of A2A with Na^+/K^+-ATPases, therefore linking neuronal activity to ion homeostasis and control of glutamatergic activity (Matos et al., 2013). Notably, ecto-nucleotidases, able to catabolize ATP to adenosine, are abundant at the astrocytic level in the vicinity of A2A receptors (Augusto et al., 2013). Blockade of striatal glial A2A receptors was suggested to be neuroprotective against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (Yu et al., 2008). Also, striatal astrocytic A2A receptor dysfunction, with disruption of striatal glutamate homeostasis, was suggested to be involved in schizophrenia (Rial et al., 2014; Matos et al., 2015).

Striatal astrocytes were also found to express dopaminergic D1, D2, D3, D4, and D5 receptors (Bal et al., 1994; Miyazaki et al., 2004; see also Miyazaki and Asanuma, 2020). *In vivo* evidence in rodents indicates that astrocytic striatal D2 receptors are involved in neuroinflammatory disorders: reduced astrocytic D2 function increases the vulnerability of dopaminergic neurons to MPTP, and astrocytic D2 activation suppresses neuroinflammation in PD models (Shao et al., 2012).

3.1. Receptor-receptor interaction between striatal astrocytic A2A and D2 receptors

The first demonstration of RRI between native astrocytic A2A and D2 receptors was obtained in astrocyte processes acutely prepared from the striatum of adult rats, which reflect the process behavior in striatal mature neuron-astrocyte networks (Cervetto et al., 2017). The purified preparation of the astrocytic processes was positive for the astrocytic marker glial fibrillary acidic protein (GFAP) and for ezrin (Cervetto et al., 2017, 2018), the selective marker of the perisynaptic processes involved in neuron-astrocyte crosstalk at the tripartite synapses (Derouiche and Frotscher, 2001; Derouiche et al., 2002; Lavialle et al.,

2011), and negative for neuronal, microglial or oligodendroglial markers (Cervetto et al., 2017, 2018). Ultrastructural analysis of the processes showed small transmitter-loading vesicles scattered within the cytoplasm (Cervetto et al., 2018, Fig. 1), resembling the synaptic-like vesicles carrying VGLUT1 in the perisynaptic and larger astrocyte processes in rat striatum *in situ* (Ormel et al., 2012). Notably the vesicles in the striatal nerve terminals obtained in parallel showed a clustered distribution (Cervetto et al., 2018, Fig. 1).

A2A and D2 receptors were found to be expressed on the same adult rat astrocytes (Cervetto et al., 2018; see Fig. 2) and on the same VGLUT1-positive and ezrin-positive astrocyte processes (Cervetto et al., 2018; see Fig. 3).

GFAP, glial fibrillary acidic protein.

VGLUT1, vesicular glutamate transporter 1.

Depolarization of the processes evoked a Ca^{2+}-dependent vesicular release of glutamate (Cervetto et al., 2017), consistent with the reported ability of striatal astrocytes to release glutamate in response to increased intracellular Ca^{2+} levels (Martín et al., 2015). Activation of D2 receptors inhibited the glutamate release from the processes; activation of A2A receptors, per se ineffective, abolished the D2-mediated inhibition (Cervetto et al., 2017, 2018; see Fig. 4). The finding indicates that A2A receptors may regulate the dopaminergic control of striatal glutamatergic transmission through RRI between native A2A and D2 receptors in striatal astrocyte processes.

3.2. Striatal astrocytic A2A-D2 heteromers

The synthetic peptide VLRRRRKRVN, corresponding to the region of the D2 receptor involved in the electrostatic interaction critical to A2A-D2 receptor heteromerization (Ciruela et al., 2004; Woods and Ferré, 2005) abolished the A2A receptor-mediated inhibition of the response to D2 receptor activation (Cervetto et al., 2017; see Fig. 4), suggesting that the interaction between native A2A and D2 receptors on striatal astrocyte processes was based on the formation of A2A-D2 heteromers.

Evidence for receptor heteromerization must rely on multiple biochemical and structural evidence (see Franco et al., 2016). Biochemical evidence for A2A-D2 receptor heteromerization on striatal astrocyte processes was obtained by co-immunoprecipitation (Pelassa et al., 2019, Fig. 5). The D2 receptor was found to co-immunoprecipitate with A2A, while the A2A only partially co-immunoprecipitated with the D2, consistent with its possible function as a hub in receptor complexes. Indeed, both the native A2A and D2 receptors sited on the striatal astrocytes were found able to heteromerize with oxytocin receptors (OTR, Amato et al., 2022; 2023). The relative importance of A2A-D2, A2A-OTR and D2-OTR heteromers and of receptor mosaics including A2A, D2 and OTR as well as their roles at the striatal astrocytes remain to be determined.

In situ proximity ligation assay confirmed the A2A-D2 heteromerization (Pelassa et al., 2019, Fig. 6).

Therefore, functional, biochemical and biophysical evidence converges to indicate that the RRI between native A2A and D2 receptors on the striatal astrocyte plasma membrane is based on receptor heteromerization (Cervetto et al., 2017; Pelassa et al., 2019). To the best of our knowledge, this was the first demonstration for the presence of functional native A2A-D2 heterodimers on adult astrocytes.

4. Striatal astrocytic A2A-D2 heteromers: potential relevance

The new astrocyte-related aspects of the regulation of striatal glutamatergic transmission suggest new perspectives on pathogenesis of pathological conditions such as schizophrenia or PD and on strategies for their pharmacological treatment. The striatal astrocytic A2A and D2

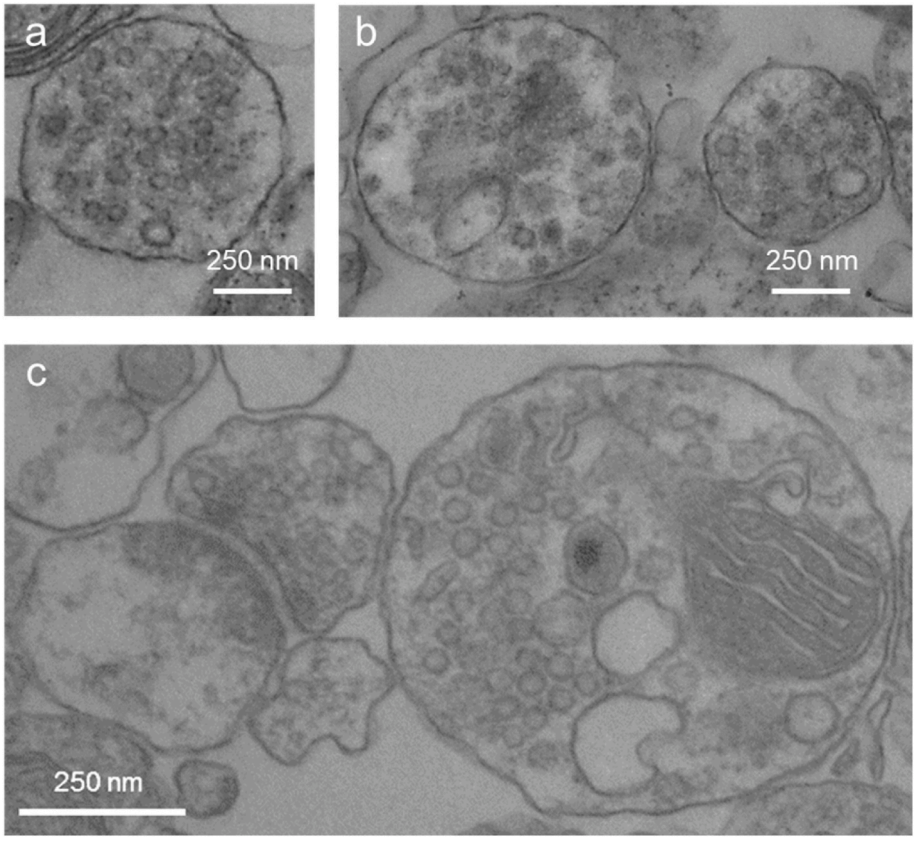

Fig. 1. Electron micrographs of isolated rat striatal astrocyte processes. Single astrocyte processes (gliosomes) are shown containing approximately 30-nm smooth and clathrin-coated vesicles scattered in the cytoplasm (a,b). For comparison, see single nerve terminals (synaptosomes) obtained in parallel from adult rat striatum: note the vesicle clusters in the active zones and postsynaptic densities and mitochondria (c). Modified from Cervetto et al. (2018).

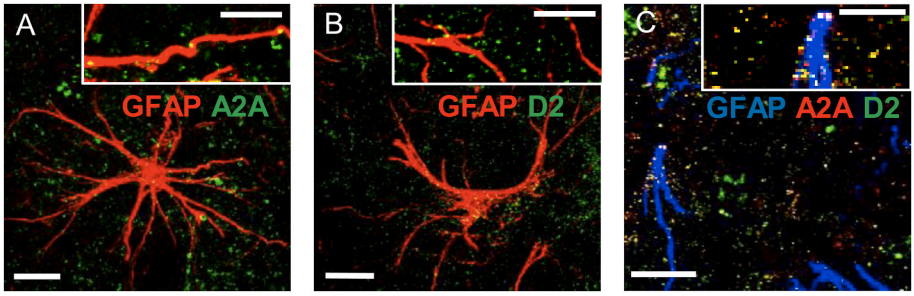

Fig. 2. A2A and D2 receptor co-localization on adult rat striatal astrocytes. Confocal images. Double immunofluorescence staining with antibody against the A2A (A, green) or the D2 receptor (B, green) and the astrocyte marker GFAP (A and B, red). In the figure two representative maximum intensity projections and their enlarged details are shown. Triple immunofluorescence labeling in striatal slices with antibody against the A2A (C, red), the D2 receptor (C, green) and GFAP (C, blue); in the figure an enlarged representative maximum intensity projection. Scale bar: 10 μm; 5 μm (inset). From Cervetto et al. (2018).

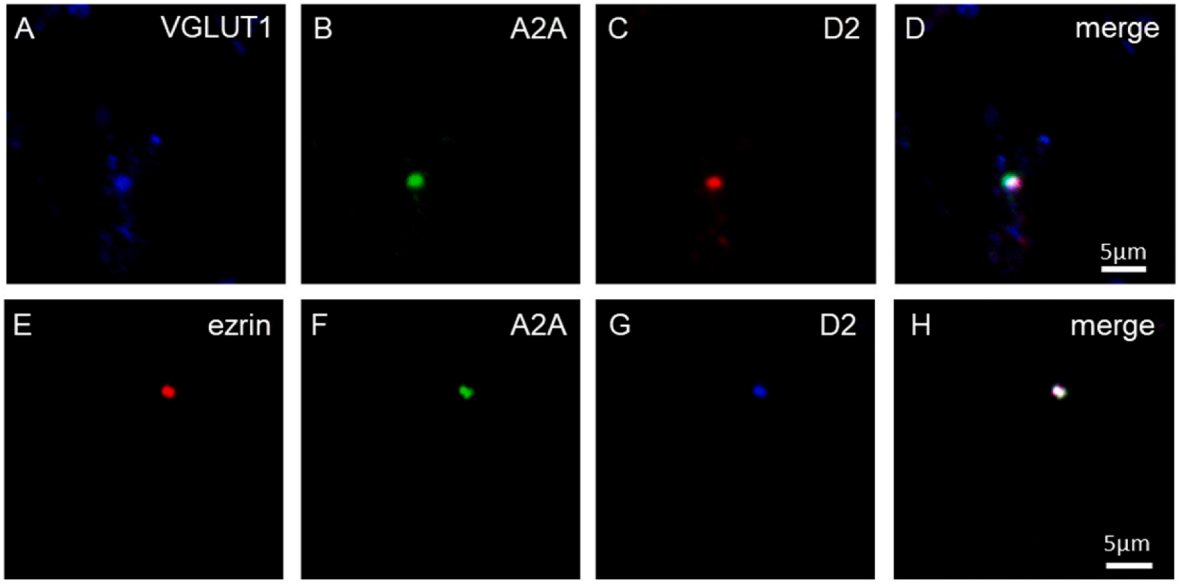

Fig. 3. Purified rat striatal astrocyte processes endowed with VGLUT1 and ezrin bear A2A and D2 receptors. Confocal images showing co-localization of A2A and D2 with VGLUT1 or ezrin in single isolated astrocyte processes. Immunofluorescence image showing co-expression of the markers VGLUT1 (A), ezrin (E), the A2A (B,F) and the D2 receptor (C,G). Merge images showing single VGLUT1 or ezrin-positive processes expressing both A2A and D2 receptors (D,H), and processes expressing VGLUT1 only (D). Modified from Cervetto et al. (2018).

receptors and A2A-D2 heteromers as possible targets for pharmacological approaches to these pathological conditions are discussed in the following sections.

4.1. Potential relevance of striatal astrocytic A2A and D2 receptors and A2A-D2 heteromers in schizophrenia

Hyperactivity of striatal and frontal cortex D2 receptor was proposed to be related to cognitive impairment and schizophrenia symptoms as well as to the antipsychotic drug actions (Brisch et al., 2014; Laruelle et al., 2005; Laruelle, 2014). The hypo-adenosinergic hypothesis for schizophrenia proposes that reduced adenosine levels could contribute to D2 receptor hyperactivity (Lara and Souza, 2000; Boison et al., 2012). Indeed, adenosine is recognized to be a crucial regulator of synaptic/non-synaptic transmission in basal ganglia (see Sperlagh and Sylvester Vizi, 2011). Reduced activity of striatal ecto-nucleotidases in schizophrenia patients (Aliagas et al., 2013) supports the hypo-adenosinergic hypothesis of schizophrenia, as the ecto-nucleotidase-mediated catabolism of extracellular ATP or AMP to adenosine near A2A receptors seems related to the receptor activation (Augusto et al., 2013).In schizophrenia patients, up-regulation of striatal A2A receptors was reported (Kurumaji and Toru, 1998; Deckert et al., 2003; see also Rial et al., 2014) possibly as a response to reduced adenosinergic activity (Lara and Souza, 2000; Lara et al., 2006; Boison et al., 2012). Despite the upregulation of A2A (and D2) receptors, however, a reduced density of A2A-D2 heteromers has been reported in postmortem caudate nucleus from schizophrenia patients, and the degree of A2A-D2 heteromer formation was proposed to constitute a hallmark of schizophrenia (Valle-León et al., 2021). It is of interest to note that in the phencyclidine animal model for schizophrenia, a reduced density of striatal A2A-D2 heteromers was reported as well; notably, administration of antipsychotic drugs counteracted the striatal A2A-D2 heteromer reduction in the animal model (Valle-León et al., 2021). Furthermore, in mammalian cells different antipsychotic drugs were found to differently affect A2A-D2 heteromerization, by destabilizing or enhancing the interaction of A2A with D2 in the heteromers (Valle-León et al., 2023). It was suggested that an increase in A2A-D2 heteromerization may be involved in the extrapyramidal side effects of antipsychotics such as haloperidol, while the clozapine destabilization of the A2A-D2 heteromerization can explain its low extrapyramidal side effects (Valle-León et al., 2023). These studies support the direct implication of A2A-D2 heteromers in schizophrenia, also indicating their modulation as a potential therapeutic approach to psychosis.

In addition, dysfunction of striatal glutamatergic transmission was proposed to be linked to early symptoms and cognitive impairment in schizophrenia (Simpson et al., 2010). The glutamatergic hypothesis for schizophrenia is consistent with the schizophrenia-like symptoms and cognitive impairment induced by blockade of glutamate NMDA receptors (see Bubeníková-Valešová et al., 2008; Moghaddam and Javitt, 2011).

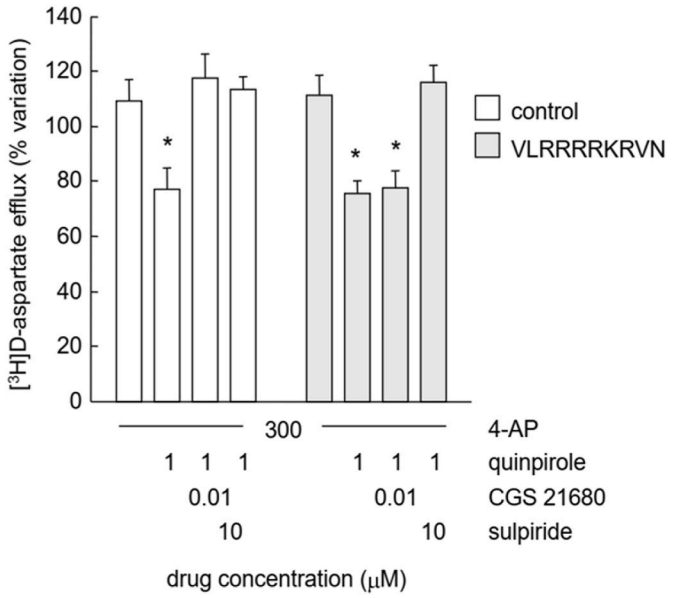

Fig. 4. A2A-D2 receptor-receptor interaction in striatal astrocyte processes: effect of the synthetic D2 peptide VLRRRRKRVN. Effects of A2A receptor activation (A2A agonist, CGS21680) on the D2-mediated (D2 agonist, quinpirole) inhibition of 4-aminopyridine (4-AP)-evoked efflux of the glutamate analogue [^{3}H]D-aspartate in control conditions (open bars) and in the presence of the D2 peptide VLRRRRKRVN (grey bars). Bars represent percent increase of [^{3}H]D-aspartate efflux in the presence of the drugs at the concentrations indicated. Gliosomes were prepared in the absence (control), or in the presence of VLRRRRKRVN (0.015 μM); release experiments were then run in parallel on the control preparation and on the preparation entrapping VLRRRRKRVN. 4-AP was added (2 min) during superfusion; quinpirole or CGS21680 were added together with 4-AP; the D2 antagonist sulpiride was added 8 min before the agonists. Other experimental details in Cervetto et al. (2017). Data are means ± SEM (bars) of 3–7 experiments performed in triplicate. *p < 0.05 compared with the effect of 4-AP. From Cervetto et al. (2017).

Evidence has accumulated for astrocyte involvement in schizophrenia (Matos et al., 2015; Tarasov et al., 2019; Chang et al., 2021; Kruyer et al., 2023), where extracellular matrix/glial abnormalities were proposed to contribute to dysfunction of glutamatergic and dopaminergic neurotransmission (Berretta, 2012). Notably, ecto-nucleotidases responsible for formation of adenosine in proximity of A2A receptors seem abundant in striatal gliosomes (Augusto et al., 2013). Indeed, in a mouse model of astrocytic A2A receptor knockout, enhanced behavioral sensitization to psychoactive drugs and reduced working memory - two behavioral schizophrenia symptoms - and impaired glutamate homeostasis were reported (Matos et al., 2015). A potential role of astrocytic A2A receptors in schizophrenia pathophysiology is consistent with the proposed involvement of astrocytes in the schizophrenia early phase (see Matos et al., 2015; Tarasov et al., 2019; Chang et al., 2021).

The astrocytic A2A-D2 heteromers involved in the control of glutamate transmission in striatum (Cervetto et al., 2017, 2018; Pelassa et al., 2019) can bring together three neurochemical pathways, namely the dopaminergic, adenosinergic, and glutamatergic ones, on which the major hypotheses for schizophrenia are based. In fact, a reduced adenosine activation of striatal astrocytic A2A receptors dependent on reduced adenosine production into the glial receptors (see Augusto et al., 2013) would result in hyperactivity of D2 receptors in astrocytic A2A-D2 heteromers and in dysregulation of glutamate transmission in striatal neuron-astrocyte networks. The striatal astrocytic A2A-D2 heteromers indeed may represent a druggable target for A2A agonist and/or antipsychotic drugs (see Wardas, 2008; Valle-León et al., 2023) to ameliorate the impaired glutamate homeostasis in schizophrenia.

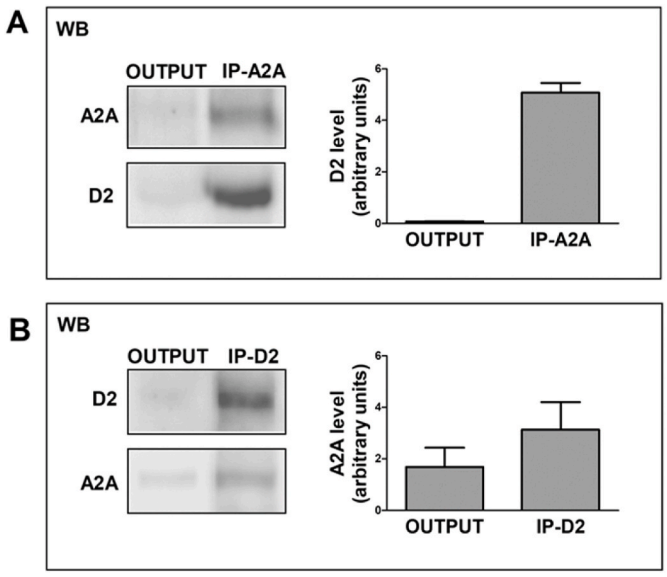

Fig. 5. A2A-D2 receptor-receptor interaction in striatal astrocyte processes: co-immunoprecipitation. Aliquots (300 μg) of Triton X-100-soluble proteins obtained from gliosomes were immunoprecipitated with 1 μg of anti-A2A antibody (A) or of anti-D2 antibody (B). Immunoprecipitated (IP) and not immunoprecipitated (OUTPUT) materials were analyzed by immunoblotting using the anti-A2A or the anti-D2 antibody. Other experimental details in Pelassa et al. (2019). A representative blot (of three) is shown; quantifications of D2 and A2A immunoreactive bands are also reported. Values are means ± SEM (n = 3). From Pelassa et al. (2019).

4.2. Potential relevance of striatal astrocytic A2A and D2 receptors and A2A-D2 heteromers in Parkinson's disease

Increased striatal ATP release and increased A2A activation by ATP-derived adenosine were identified as a key path involved in the onset of PD motor symptoms in animal models (Gonçalves et al., 2023), and adenosinergic transmission emerged as a crucial actor in PD pathology (see Zhao et al., 2023 and references therein). Notably, higher striatal A2A receptor expression was found in PD patients (Villar-Menéndez et al., 2014), and A2A-D2 heteromerization was reported to be increased in the PD patient caudate (Fernández-Dueñas et al., 2019). Indeed the A2A-D2 heteromers appear to be a target for new approaches to the PD, through the use of A2A antagonists such as istradephylline (Chen and Cunha, 2020; Jenner et al., 2021; Misganaw, 2021). The therapeutic efficacy of A2A antagonists, including caffeine and theophylline (Salamone et al., 2013; Podurgiel et al., 2015), is based on allosteric interactions within the heteromer, by which the blockade of the A2A can increase the effect of D2 agonists or L-dopa (Bonaventura et al., 2015; Ferré, 2016; Ferré et al., 2022).

Dysfunction of striatal astrocytes was suggested to have an initiating role in PD pathophysiology (Booth et al., 2017); the central role of astrocytes in PD is now generally accepted (Brandebura et al., 2022; Minchev et al., 2022). Reduced signaling of the astrocytic D2 receptors was found to increase the vulnerability of striatal dopaminergic neurons to MPTP, and activation of the astrocytic D2 receptors suppressed neuroinflammation (Shao et al., 2012). Notably, neuroinflammation is considered a major cause for dopaminergic neuron degeneration in PD (see Minchev et al., 2022; Zhao et al., 2023 and references therein). Interestingly, blockade of the striatal astroglial A2A receptors appeared to be neuroprotective, while blockade of neuronal A2A could control locomotion in the MPTP model of PD (Yu et al., 2008).

In PD models expansion of the astrocytic processes and altered astrocyte-neuron interactions at glutamatergic synapses (Villalba and Smith, 2011; Villalba et al., 2015) together with increase in extracellular glutamate levels (Dervan et al., 2004; see also Villalba and Smith, 2011;

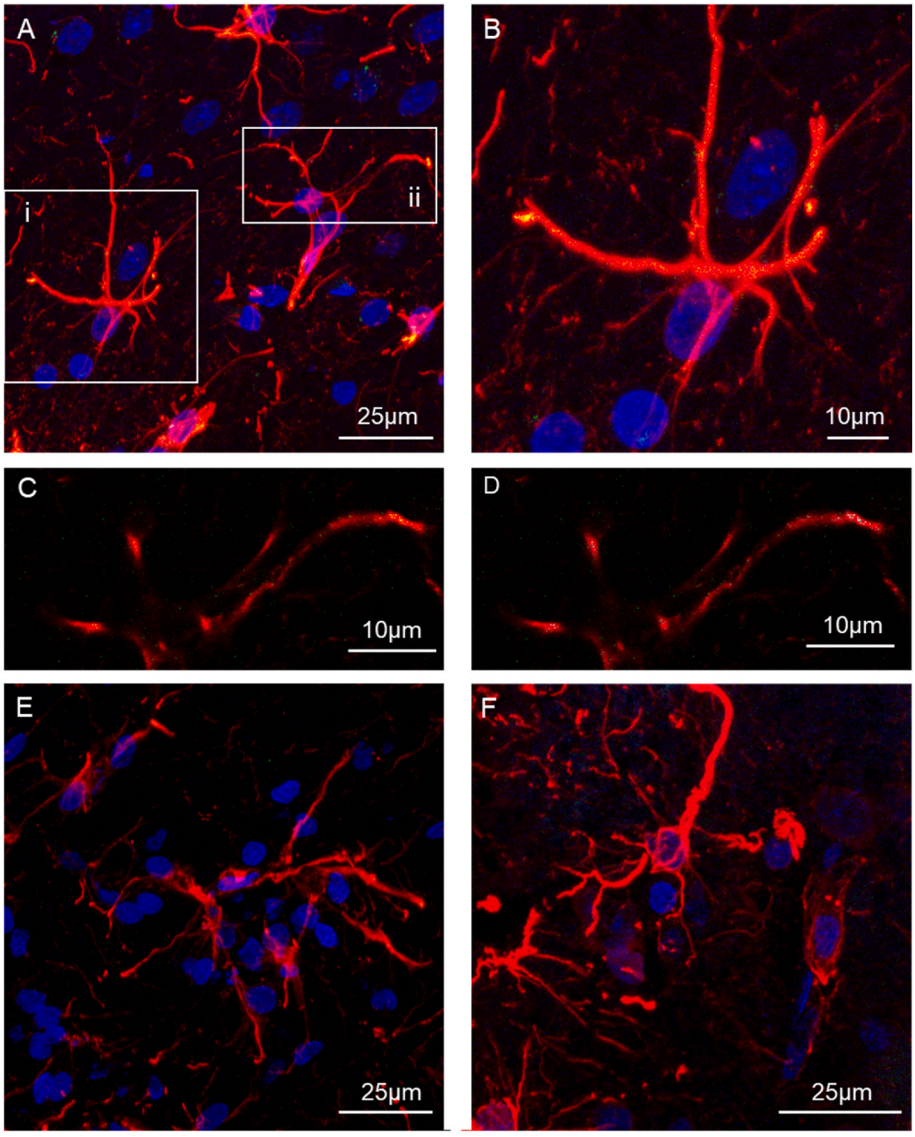

Fig. 6. A2A-D2 heterodimers on rat striatal astrocytes: proximity ligation assay. In adult rat striatal slices (10-μm thick), the *in situ* proximity ligation assay (PLA) for the A2A-D2 heteromers was carried out using two primary antibodies (mouse anti-A2A 1:200, Merck Millipore Corporation, 05–717, Burlington, MA, USA and rabbit anti-D2R 1:200, Alomone Labs, ADR-002, Jerusalem, Israel) and the Duolink *in situ* PLA Probes and detection kit (DUO92005, DUO92001, DUO92014 Sigma-Aldrich, St Louis, MO, USA). The astrocytes were labelled with goat polyclonal anti-GFAP (1:500, Santa Cruz Biotechnoloy Inc, Dallas, TX, USA, sc-6170), and Alexa Fluor 546-conjugated donkey anti-goat (1:500; Molecular Probes, Eugene, OR, USA). The nuclei were stained with DAPI. Other experimental details in Pelassa et al. (2019). (A) Merge of the maximum intensity projections of a representative field (240 × 240 μm; z = 10 μm) is shown; GFAP (red), DAPI (blue), PLA for A2A-D2 heteroreceptor complexes appears as green dots. The boxed region (i) is shown at a higher magnification in (B), while the confocal images of a single z stack of the boxed region (ii) is shown at higher magnification in (C). The colocalized map of (C) is shown in (D): the colocalized maps was created using ImageJ Fiji software and the plugin Colocalization Threshold, setting GFAP as region of interest (ROI). (E,F) A complete lack of stain for PLA was obtained in the negative control experiments, performed avoiding the conjugation of a primary antibody with the Duolink Probes. In the figure the merges of the maximum intensity projections of two representative fields are shown: PLA for A2A-D2 heteroreceptor complexes without the primary anti-A2A antibody (E) or the primary anti-D2 antibody (F). Scale bar 25 μm or 10 μm. From Pelassa et al. (2019).
GFAP, glial fibrillary acidic protein.

Chassain et al., 2016) have been reported. The relevance to PD pathology of the striatal extracellular glutamate level regulation by astrocytes is indicated by the finding that knocking down the striatal astrocytic glutamate transporter GLT-1 induces PD-like changes in rodents (Ren et al., 2022). The A2A-mediated inhibition of D2 signaling at striatal astrocytic A2A-D2 heteromers, by increasing the extracellular glutamate levels (see Cervetto et al., 2017, 2018), might contribute to striatal glutamatergic transmission dysfunction and circuit derangement in PD. Notably, modulation of the glutamate release from striatal astrocyte processes is expected to modulate the activation of NMDA and metabotropic glutamate receptors on specific medium spiny neurons, indicating selective signaling between particular astrocytes and neurons (see Martín et al., 2015).

Intracellular homocysteine (Hcy) was able to counteract the D2 inhibition of glutamate release from striatal astrocyte processes (Cervetto et al., 2018; see Fig. 7).

The finding was consistent with previous evidence in cell models, showing that the modulatory actions of A2A receptor and Hcy on the D2 receptor do not interfere (Agnati et al., 2006b, 2008). Intracellular Hcy could then work together with adenosine acting at A2A receptors to silence the astrocytic D2 signaling. The physio-pathological relevance of the finding emerges when considering that L-dopa can cause both synthesis of Hcy in astrocytes (inhibited by COMT inhibitors) and Hcy extrusion into the extracellular compartment (G. Huang et al., 2005). Increased Hcy plasma levels were reported in PD patients undergoing L-dopa treatment (Blandini et al., 2001; Mattson and Shea, 2003; Miller et al., 2003; Zoccolella et al., 2009; Paul and Borah, 2016) and extracellular Hcy-induced stimulation of NMDA receptors (Lipton et al., 1997) was suggested to be involved in dyskinesias during L-dopa treatment (Fahn, 2000; Hallett and Standaert, 2004; Tahar et al., 2004). Therefore, striatal astrocytes might actively participate in late side effects of L-dopa treatment both by synthesizing Hcy (therefore reducing the D2 inhibition of glutamate release from the astrocytes) and by releasing Hcy to directly activate the NMDA receptors. A2A receptor antagonists (see Schwarzschild et al., 2006; Yu et al., 2008; see also Zhao et al., 2023 and Gonçalves et al., 2023 and references therein) may be proposed to play synergistic roles with a reduction of Hcy production (e.g., by COMT inhibitors) in PD therapy.

These new astrocyte-related insights onto the regulation of striatal glutamate transmission through A2A-D2 heteromers could help to understand how altered astrocyte-neuron communication at striatal synapses and remodeling of the astrocyte processes can contribute to the PD pathophysiology, and to develop disease-modifying pharmacological strategies against PD (see Miyazaki and Asanuma, 2020). The striatal astrocytic A2A-D2 heteromers indeed may represent a new target for A2A antagonists (Schwarzschild et al., 2006; Yu et al., 2008; Dungo and

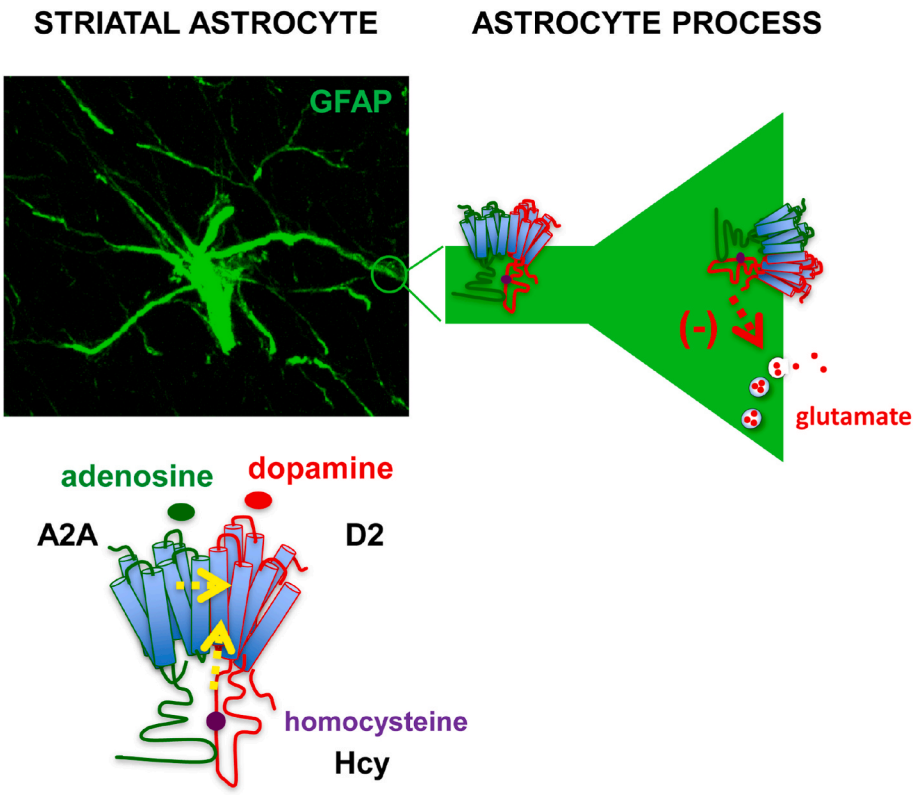

Fig. 7. Representation of astrocytic A2A-D2 receptor heteromers in the adult rat striatum. A confocal image showing immunofluorescence staining of an astrocyte with the astrocyte marker GFAP in a striatal slice from adult rat. The presence of A2A-D2 receptor heteromers was demonstrated in striatal astrocytes both on perisynaptic processes and astrocyte branchlets. The heteromers were involved in the control of glutamate release from the processes. Activation of the A2A receptor in the heteromer prevented the effect of D2 receptor activation; intracellular homocysteine (Hcy) behaved as a D2 allosteric antagonist. Yellow arrows: allosteric antagonism; red arrow: inhibition of vesicular glutamate release. From Marcoli et al. (2023).
GFAP, glial fibrillary acidic protein.

Deeks, 2013; Berger et al., 2020; see also Zhao et al., 2023; Gonçalves et al., 2023 and references therein) and bivalent compounds (A2A antagonist-D2 agonist, Soriano et al., 2009; Jörg et al., 2015; Pulido et al., 2018; Prasad et al., 2021) to control the striatal glutamate transmission derangement and to protect dopaminergic neurons in PD.

4.3. Potential relevance of A2A-D2 heteromers in other neuropsychiatric disorders

A2A-D2 RRI may have a potential role in other CNS diseases; the evidence, although currently limited, might help to provide a more comprehensive perspective on the importance of these heteromers in the context of neuropsychiatric disorders.

Dynamic regulation of A2A-D2 RRI has been proposed to have important implications for the pathophysiology and treatment of drug addiction, besides basal ganglia disorders or schizophrenia (Ferré et al., 2004, 2008). Indeed, drugs of abuse increase dopaminergic neurotransmission within the mesolimbic circuitry, and indirectly activate dopamine receptors; among the dopamine receptors, the D2 appears linked to drug abuse and addiction (Prasad et al., 2021). Subjects with substance use disorders have been observed to exhibit lower D2 receptor availability using positron emission tomography, allowing to hypothesize that upregulation of D2 receptors may provide a therapeutic effect (Prasad et al., 2021). On this background, D2 receptor heteromerization provides new avenues in the search for drug addiction therapies. An *in vitro* model suggested that changes in D2 and A2A-D2 trafficking induced by allosteric actions of cocaine may contribute to the alterations of D2 signaling in cocaine abusers (Genedani et al., 2010). Cocaine self-administration, extinction from cocaine use, and reinstatement of cocaine seeking differentially affected A2A-D2 heteromerization in the striatum (Pintsuk et al., 2016; Romero-Fernandez et al., 2022) leading to the idea that A2A-D2 heteromers are partly responsible for the psychomotor and reinforcing effects of psychostimulant drugs. In fact, targeting A2A receptors might offer innovative strategies for the treatment of cocaine use disorder and drug addiction (Filip et al., 2012; Ballesteros-Yáñez et al., 2018; Borroto-Escuela et al., 2018; Prasad et al., 2021). Notably, accumulating evidence support a direct involvement of striatal astrocytes in drug abuse disorder (Kruyer and Kalivas, 2021). Interestingly, a clinical trial is designed hypothesizing that the A2A antagonist istradefylline will increase striatal D2 binding and improve control of impulsivity (Okita et al., 2021). These considerations may be relevant also for PD patients. PD is now proposed as a complex neuropsychiatric disorder, and neuropsychiatric symptoms including impulse control disorders are recognized of similar relevance as the motor symptoms in some PD cases (Weintraub et al., 2022). Indeed, the search for safe efficacious treatment for the neuropsychiatric disorders is now a research priority in PD (Weintraub et al., 2022).

5. Conclusions and perspectives

Striatal astrocytic A2A-D2 RRI might help to get light on D2 signaling, adenosine transmission, and glutamate transmission in striatum. A2A-D2 heteromers at striatal astrocyte plasma membrane appear a research area for a better understanding of the striatal astrocyte-neuron communication and the astrocyte involvement in glutamatergic transmission derangement in neurodegenerative/neuropsychiatric conditions. Further studies are required to appreciate the relative contribution of astrocytic and neuronal A2A-D2 RRI in the derangement of glutamate transmission in such conditions.

Also, striatal astrocytic A2A-D2 heteromers might be exploited as druggable targets. In fact, novel molecules targeting the A2A-D2 heteromers might indeed target (also) astrocytic mechanisms. Distinct effects of A2A receptor activation in astrocyte processes and nerve terminals suggest that A2A-D2 RRIs may be diverse in striatal astrocytes and neurons, indicating that their characteristics and differences worth to be investigated. Indeed, striatal astrocytic A2A receptors do not appear coupled to the release of glutamate (only controlling the D2-inhibition of the gliotransmitter release; Cervetto et al., 2017), while presynaptic A2A receptors on striatal glutamatergic nerve terminals facilitated the neurotransmitter release (Rodrigues et al., 2005; Ciruela

et al., 2006, 2011; Cervetto et al., 2017). In corticostriatal glutamatergic nerve terminals activation of the D2 receptor was found to inhibit the A2A-mediated facilitation of glutamate release most probably through an interaction at the level of the transduction mechanism (Tanganelli et al., 2004; Higley and Sabatini, 2010; Ferré et al., 2011). No information is available indicating that presynaptic A2A and D2 receptors sited on the striatal glutamate-releasing nerve terminals can heteromerize. This point would be relevant to elaborate on the unique contributions of astrocytic *vs* neuronal A2A-D2 RRIs in the regulation of glutamate release and their potential as therapeutic targets. In fact, if the RRI between A2A and D2 receptors to control glutamate release from the astrocyte processes is based on receptor heteromerization, while the interaction at the presynaptic glutamatergic nerve terminals is at the level of adenylate cyclase 5 (Tanganelli et al., 2004; Higley and Sabatini, 2010; Ferré et al., 2011), this may help to design approaches selectively targeting the astrocytic heteromers (see below). As an example, heteromer-selective bivalent ligands, e.g. A2A antagonist/D2 agonist (Soriano et al., 2009; Jörg et al., 2015; Huang et al., 2021; Prasad et al., 2021) might control glutamate release by selectively targeting the astrocytic heteromers.

To a comprehensive view of the A2A-D2 RRIs involved in striatal glutamatergic transmission regulation, we should also consider the postsynaptic A2A-D2 heteromers on medium spiny neurons, where D2 inhibited the NMDA firing, and A2A receptor activation had no effect per se but reversed the effect of D2 receptor activation through heteromerization (Azdad et al., 2009). A predominant population of the postsynaptic A2A-D2 heteromers seem to possess tetrameric structure and form complexes with adenylyl cyclase subtype 5, which allows multiple allosteric interactions between different orthosteric ligands and Gs-Gi antagonistic interaction at the level of adenylate cyclase (Ferré et al., 2011, 2018). Further investigation is required to allow modelling of A2A-D2 heteromers at astrocyte level; selective targeting of striatal astrocytic *vs* neuronal A2A-D2 heteromers for selective pharmacological intervention would rely on this basis. Although the available knowledge does not allow to discuss about any progress in the development of such selective interventions, it is to be considered that GPCR heteromers, in which allosteric RRI occur and new allosteric sites may appear related to the heteromer structure, may offer a way to increase the selectivity of pharmacological treatments (Marcoli et al., 2023). Notably, several allosteric modulators are in clinical trials in CNS disorders, as allosteric ligands seem to show decreased side effects and provide greater receptor subtype and temporal selectivity (Nickols and Conn, 2014; Hauser et al., 2017). The assembly of the receptor protomers in heteromer complexes with allosteric RRI expands the possibilities of modulating the decoding processes, revealing attractive targets for drug development and showing the potential of allosteric modulators of GPCRs (see Marcoli et al., 2023). Knowledge of the distribution and functioning of astrocytic (and neuronal) A2A-D2 heteromers and of the receptor organization in receptor complexes (heterodimers and receptor mosaics, that may be different at neuronal and astrocyte level, as they may be composed of different receptor protomers) has the potential challenges for selective targeting of astrocytic *vs* neuronal mechanisms. In any case, by considering the colocalization of adenosine-producing ecto-nucleotidases with striatal astrocytic A2A receptors (Augusto et al., 2013), the expansion of striatal perisynaptic processes in PD (Villalba and Smith, 2011; Villalba et al., 2015) and the increased activation of astrocytes in schizophrenia (Tarasov et al., 2019), a great impact of blockade of the astrocytic A2A receptors on glutamate release regulation may be predicted in conditions of dysregulated striatal glutamate transmission.

As previously mentioned, in the complex scenario of the adenosine effects at striatal glutamatergic synapses, A2A receptor could play a crucial role in glutamate release facilitation by a double action, at presynaptic and at astrocytic level: direct stimulation of glutamate release from the nerve terminals, and indirect increase of the release from the astrocyte processes through interference with the D2 release-inhibitory mechanism; both the effects resulting in increased extracellular glutamate concentration. Therefore, based on the available information, A2A receptor blockers might play a dual role in the control of glutamate release at striatal glutamatergic synapses: by directly inhibiting the neuronal release of glutamate (also through effects at A1-A2A heteromers on cortico-striatal glutamatergic terminals; Ciruela et al., 2006; Orru et al., 2011), and by rescuing the D2-mediated inhibition of the glutamate release from the astrocyte processes. To a broader view of the impact of A2A receptor activation/blockade on striatal glutamatergic transmission, postsynaptic A2A-D2 heteromers controlling the glutamate receptor activation by the released glutamate should also be taken into account. It is therefore not surprising that in conditions of dysregulated striatal glutamatergic transmission the A2A receptor has been considered a suitable pharmacological target. In fact, A2A agonists were proposed as potential antipsychotic drugs, although effects on learning and memory and peripheral side effects limit their investigation for possible clinical use (Wardas, 2008). Interestingly, as already mentioned, a reduced density of A2A-D2 heteromers was proposed to be a hallmark of schizophrenia (Valle-León et al., 2021), and different antipsychotic drugs were reported to differently affect the A2A-D2 heteromerization in cell models, opening a new rationale for considering these heteromers as therapeutic targets in psychosis, with the possibility of changing their density and stability (Valle-León et al., 2023). Conversely, the A2A antagonist istradefylline has been approved as an add-on therapy in PD patients (Dungo and Deeks, 2013; Berger et al., 2020; Chen and Cunha, 2020). In fact, when proposing drug therapies targeting striatal A2A receptors, their effects at astrocyte levels should also be considered, as they could contribute to the drug potential usefulness.

In conclusion, this is an example of how the perspective is changing from a neurocentric to a neuro-astrocentric view of healthy and diseased brain functioning. As already stated, neuronal striatal A2A-D2 heteromers have been widely investigated, leading to new perspectives on molecular mechanisms involved in pathophysiology of schizophrenia and PD or of substance abuse disorder and providing novel targets for antipsychotic or antiparkinson drugs, but also possibly for pharmacological strategies against addiction. Consistent with recognized roles of astrocytes in brain function and neuropsychiatric disorders, striatal astrocytic A2A-D2 heteromers as well might represent a target worth investigation for subtle tuning of striatal glutamatergic transmission, of potential usefulness in neurodegenerative/neuropsychiatric diseases.

Author contribution

Chiara Cervetto: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Funding acquisition. **Guido Maura**: Conceptualization, Writing - Original Draft, Writing - Review & Editing. **Diego Guidolin**: Writing - Review & Editing, Funding acquisition. **Sarah Amato**: Writing - Review & Editing. **Cristina Ceccoli**: Writing - Review & Editing. **Luigi F. Agnati**: Conceptualization, Writing - Review & Editing. **Manuela Marcoli**: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Funding acquisition. All authors approved the submitted version of the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

In this review previously published results from our group are mentioned, and some figures are taken (with permission) from the previously published papers

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