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Astrocyte modulation of synaptic transmission in the reward circuitry of the Ventral Tegmental Area

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Abstract

The mesocorticolimbic dopaminergic (DA) system originating from the Ventral Tegmental Area (VTA) plays a prominent role in the cognitive processing of aversion, motivation, pleasure and reward, including the development of addiction. A central aspect for the function of the brain reward system is the switch in the pattern of action potential discharges of VTA DA neurons from a tonic, low firing frequency to a phasic, high frequency bursts which cause both an increased DA release in the VTA projecting structures and a local release of endocannabinoids (eCBs). Glutamatergic signalling to the VTA plays a central role in this transition from tonic to phasic-burst firing pattern and long-term changes of this signaling pathway can profoundly alter the output of DA neurons. Over the last years, astrocytes emerged as important regulatory elements of synaptic transmission in different brain circuits. They respond to neurotransmitters with Ca^{2+} elevations through a mechanism that involves the production of inositol-1,4,5-trisphosphate (IP3) and the release of Ca^{2+} from IP3-sensitive intracellular Ca^{2+} stores. In turn, these Ca^{2+} elevations in astrocytes evoke the release of gliotransmitters that modulates synaptic transmission. Whether a similar mechanism operates in VTA networks remains unexplored. In my thesis, by combining patch-clamp recording techniques and Ca^{2+} imaging experiments in horizontal VTA slices of juvenile mice (P14-17), we investigated whether astrocytes contribute to the modulation of synaptic transmission in the reward VTA circuitry. In a series of initial experiments, we found that VTA astrocytes show somatic Ca^{2+} increases in response to the cannabinoid receptor type 1 and 2 (CB1-CB2R) agonist WIN 55,212-2, suggesting their sensitivity to eCBs released by DA neurons. These Ca^{2+} responses were abolished in mice lacking the IP_3 receptor type 2 (IP_3R2KO mice). Patch-clamp recording experiments were then performed in order to investigate whether astrocytes can directly impact VTA circuitry by modulating glutamatergic inputs. We found that the burst firing of a single VTA DA neuron induces a heteroneuronal long-term potentiation (bLTP) of excitatory synaptic transmission onto an adjacent DA neuron in slices from female, but not male mice. This bLTP was blocked by CB1 and D2-type receptor antagonists and it depends on astrocyte Ca^{2+} signaling since it was absent in IP_3R2KO mice.

Furthermore, bLTP is dependent on activation of the metabotropic glutamate receptor 1 (mGluR1) and the concomitant production of nitric oxide (NO) by the burst firing cell. To validate the hypothesis that in the development of bLTP astrocytes are recruited to the DA neuron circuits, we performed a series of Ca^{2+} imaging experiments in slices from both female and male mice loaded with the Ca^{2+} indicator Fluo4 and the astrocytic marker SR101. We found that the D2-type receptor agonist quinpirole increases astrocyte somatic Ca^{2+} signals and this response is similar in slices from female and male mice. This latest result rules out the possibility that the lack of bLTP in male mice is due to a defective DA-mediated Ca^{2+} response in astrocytes from male mice. Finally, to investigate whether the results obtained in juvenile mice can be extended to later stages of development, we performed experiments in VTA slices from young adult mice (P30-70). We found that the burst firing of a single DA neuron evokes bLTP of excitatory transmission in both female and male mice. This result suggests that the gender difference observed in juvenile mice may depend on a developmentally regulated circuit mechanism in the VTA of male mice that confers a modulatory role to astrocytes. Whether the potentiation of synaptic transmission observed in young adult and juvenile mice shares the same mechanism remains to be investigated. In conclusion, the results described in the present thesis demonstrate that astrocyte signaling contributes to long-term plastic changes of glutamatergic transmission in VTA circuitry opening new perspectives for the understanding of the modulatory mechanisms in the brain reward system.

Riassunto

L' Area Tegmentale ventrale (VTA) è una piccola regione cerebrale, da cui ha origine il sistema mesolimbico-corticale che gioca un ruolo fondamentale in importanti processi cognitivi quali il piacere, la motivazione e la ricompensa. I neuroni dopaminergici, che rappresentano la popolazione cellulare maggiormente rappresentata in VTA, possiedono peculiari caratteristiche elettrofisiologiche. Essi, infatti possiedono un' attività tonica a livello basale che puo' improvvisamente tradursi in una di tipo fasico, o a burst, al presentarsi di situazioni comportamentali salienti, quali ad esempio, l'ottenimento di una ricompensa inaspettata e gratificante. Una delle conseguenze di tale cambio di attività nei neuroni dopaminergici è un maggiore rilascio di dopamina a livello delle strutture bersaglio e il rilascio di endocannabinoidi a livello locale. Gli inputs glutammatergici alla VTA giocano un ruolo fondamentale nel cambio di attività di questi neuroni. La plasticità della trasmissione glutammatergica è dunque importante nel determinare l'*output* dei neuroni dopaminergici stessi. Negli ultimi anni gli astrociti si sono rivelati essere importanti elementi regolatori in diverse funzioni cerebrali. Essi infatti rispondono ai neurotrasmettitori rilasciati dai neuroni con aumenti intracellulari dello ione Ca^{2+}, attraverso meccanismi che coinvolgono la produzione di inositolo-1,4,5-trisphosphate (IP_3) e rilascio di Ca^{2+} dagli stores intracellulari. Gli aumenti del Ca^{2+} degli astrociti regolano, a loro volta, il rilascio di gliotrasmettitori che possono modulare la trasmissione sinaptica. Tuttavia, non è noto se tali meccanismi siano operativi anche nella VTA. L'obbiettivo della mia tesi è quello di studiare una possibile modulazione della trasmissione sinaptica nella VTA da parte degli astrociti. Per caratterizzare questa interazione ho accoppiato registrazioni elettrofisiologiche a studi di imaging del Ca^{2+} in fettine di VTA. In una serie di risultati iniziali in fettine ottenute da animali giovani (P14-17) caricate con l'indicatore del Ca^{2+} OGB1 e con SR101 per marcare selettivamente gli astrociti, l'agonista per il recettore degli endocannabinoidi 1 e 2 (CB1-CB2R) WIN 55,212-2 induceva aumenti intracellulari dello ione Ca^{2+} a livello del soma in maniera dose dipendente. Ho effettuato in seguito esperimenti di patch-clamp recordings per valutare se l'interazione tra astrociti e neuroni potesse influire in maniera diretta sul circuito della VTA attraverso la modulazione degli afferenti glutammatergici. I risultati ottenuti suggeriscono che l'attività a burst di un singolo neurone

dopaminergico è in grado di indurre un potenziamento a lungo termine (bLTP) eteroneuronale, ovvero, in quei neuroni adiacenti alla cellula in cui il burst veniva indotto. Tale potenziamento era tuttavia presente solamente in fettine cerebrali ottenute da topi femmina, ma non in quelle ottenute da topi maschi. Abbiamo inoltre dimostrato che questo potenziamento dipende sia dal segnale Ca^{2+} all'interno degli astrociti - essendo assente in topi IP_3R2KO - sia dal reclutamento del recettore CB1 per gli endocannabinoidi e di quello di tipo D2 per la dopamina. Inoltre, questo bLTP dipende dall' attivazione del recettore metabotropico per il glutammato (mGluR1) e dalla produzione di ossido nitrico (NO) da parte della cellula dopaminergica in cui il burst veniva indotto. Ulteriori esperimenti di imaging del Ca^{2+} sono stati successivamente effettuati in fettine caricate con l'indicatore del Ca^{2+} Fluo-4 e la sulforodamina 101, un marcatore selettivo degli astrociti. In questi esperimenti, aumenti simili del Ca^{2+} intracellulare sono stati osservati dopo l'applicazione di quinpirolo (agonista del recettore di tipo D2 della dopamina) in fettine ottenute sia da topi maschi che da topi femmine, suggerendo quindi che la differenza nella durata del potenziamento tra maschi e femmine non dipende da una differenza nella risposta Ca^{2+} degli astrociti al rilascio di dopamina. Infine, per stabilire se i risultati ottenuti nei topi giovani potessero essere estesi anche ad animali più maturi, abbiamo ripetuto la stessa procedura sperimentale in topi di 30-70 giorni, considerati giovani adulti. In questi ultimi, abbiamo osservato che il potenziamento a lungo termine, presente nelle femmine giovani, era presente anche nei topi maschi suggerendo che questi ultimi abbiano dei tempi diversi nello sviluppo della modulazione astrocitaria dei circuiti della VTA. Rimane, tuttavia, da stabilire se il potenziamento osservato in topi giovani adulti condivida gli stessi meccanismi osservati nei topi più giovani. In conclusione, il contributo degli astrociti nella plasticità sinaptica in VTA, descritto nella presente tesi, apre nuove prospettive nella comprensione dei meccanismi modulatori presenti nei circuiti della ricompensa.

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1. Introduction

The Ventral Tegmental Area (VTA) is a region of the brain which plays a prominent role in the cognitive processing of aversion, motivation pleasure and reward [1]. Approximately 70% of VTA neurons are dopaminergic (DA) neurons that mainly project to the ventral part of the striatum, *i.e.* the nucleus accumbens (NAc), and the prefrontal cortex (PFC) [2, 3]. The release of DA in the NAc reinforces the addictive behaviors that are driven by a pleasurable reward or the anticipation of a reward [4-6]. Projections from the VTA to the PFC are also relevant for emotional and motivational processes [7]. VTA DA neurons show different activity states that have profound implications for their action in VTA circuits: i) a pacemaker, tonic-like activity which is characterized by a low frequency firing (2-4 Hz) and is maintained by a spontaneous, slow depolarizing membrane conductance [8, 9] and ii) a phasic multi-spike bursting activity pattern (firing rate, 15-30 Hz) which is activated by behaviorally salient stimuli, such as reward related events [9-11]. Glutamatergic afferents to the VTA plays a central role in the transition from tonic to phasic, burst-firing pattern [9, 12, 13] indicating that the modulation of the glutamatergic transmission onto DA neurons can profoundly alter DA neuron output and, thus, change DA release in the targeted regions with important consequences for motivated behaviour, learning and memory formation. While intensive investigations unveiled different forms of plasticity in glutamatergic innervation onto DA neurons [2, 14, 15], the potential role of neuron-astrocyte interplay in the modulation of VTA synaptic transmission remains unexplored. Astrocytes are the most abundant subclass of glial cells in the brain that provide mechanical, structural and metabolic support to neurons. Over the last three decades, however, accumulating evidence indicates that astrocytes contribute to the processing of information by mechanisms that are finely regulated in time and space [16, 17]. In the present study, we aim to address the following questions: i) Are astrocytes differently recruited by the tonic and the phasic DA neuron firing activity? ii) Can DA neuron-recruited astrocytes modulate excitatory synaptic transmission in the VTA circuitry? iii) What neurotransmitters and gliotransmitters are eventually involved in the modulatory action of astrocytes? iv) What functional relevance this modulation may eventually exert in brain

reward circuitry? The presence of a specific signaling between VTA DA neurons and astrocytes would open a new perspective to the understanding of the modulatory mechanisms in the VTA which may contribute to the brain reward processing. Most importantly, this would also hint at the possible involvement of neuron-astrocyte reciprocal signaling in the dysregulation of VTA circuitry induced by drugs of abuse suggesting the development of new targeted interventions in addiction.

1.1 The Ventral Tegmental Area

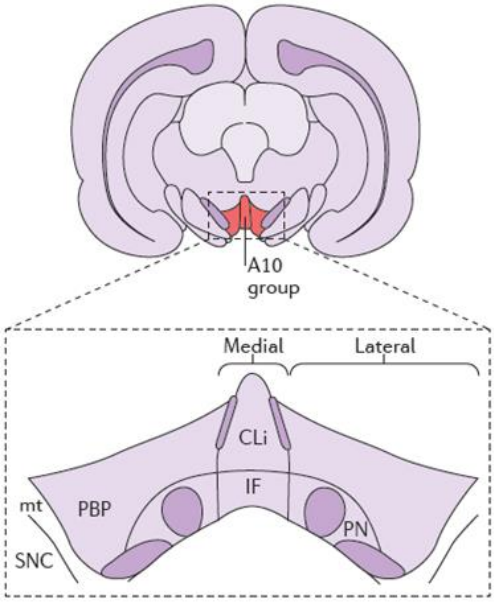

Figure 1. Ventral Tegmental Area location in the brain and regional composition. The A10 group (in red) is distributed within several subregions, including the parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), caudal linear nucleus (CLi), interfascicular nucleus (IF) and rostral linear nucleus of the raphe (RLi; not shown). From [3].

The Ventral Tegmental Area (VTA), i.e. the ventral tegmentum, was originally defined as a 'nucleus'. The term 'area' became then more appropriate to define such a heterogeneous cytoarchitectonic structure [3, 7]. The first description in the literature of the "nucleus tegmenti ventralis" (presently VTA) was reported by C. Tsai in 1925 [18] in Nissl and Golgi staining preparations of the opossum brain. This region has long been considered as part of the Substantia Nigra. However, Tsai suggested specific anatomical and functional features for the nucleus tegmenti ventralis. Some years later, Nauta's work confirmed Tsai conclusions by showing that some brain structures, such as the lateral hypothalamus, project to the VTA, but not to the Substantia Nigra. Finally, the Ventral Tegmental Area was mentioned for the first time by Nauta in 1958 [19]. In the early 1960s, the presence of three major groups of midbrain DA-releasing neurons (then termed the A8, A9 and A10 neurons) was revealed by the application of the Falck–Hillarp formaldehyde fluorescence technique [20].

Although there has not been an uniform agreement on the compartmentalization and nomenclature, the VTA is generally defined as a complex of 5 subregions containing the so-called A10 group of DA neurons: parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), caudal linear nucleus (CLi), interfascicular nucleus (IF) and rostral linear nucleus of the raphe (Fig 1). Anatomical, biochemical and electrophysiological studies show that there is heterogeneity among DA-releasing and non-DA-releasing neurons even within each of these subregions. Although some properties are organized in a medial-lateral or anterior-posterior gradient, others are highly intermixed [3]. Nowadays, the medial terminal nucleus of the accessory optic tract (MT) is used as an anatomic reference point to distinguish the medially located VTA neurons from the laterally located neurons of the substantia nigra that, together with the VTA, is considered one of the main dopaminergic areas in the brain [21]. These two brain areas are adjacent to each other and, although they do not show a clear anatomical separation, they differ in where most of their neurons project. Indeed, the large bundle of fibers leaving the substantia nigra, known as the nigrostriatal pathway, projects to the caudate and the putamen (together known as the striatum). On the other hand, two of the most prominent VTA outputs are the mesolimbic and mesocortical pathways, which travel to limbic and cortical areas, respectively [22]. Nowadays, circuities arising from the VTA are recognized to play a role in different adaptive brain functions related to reward, aversion and pleasure [1], as well as in more complex cognitive processes, such as motivation [23, 24] and memory formation [25-30].

1.1.1 Heterogeneity of DA neuronal population and connectivity. Roles in reward and aversion

Over the past decades, our view of the midbrain DA system changed from a simple organization of anatomically separated DA neurons in the substantia nigra and VTA to a more complex system of VTA DA neurons subtypes with different axonal projections and inputs, as well as distinct anatomical, molecular and electrophysiological features [31-35]. The ultrastructural findings on the mesocorticolimbic DA system are mainly based on immuno-labeling of DA

neurons with specific antibodies against the tyrosine hydroxylase (TH), a rate-limiting catecholamine-synthesizing enzyme (Fig 2).

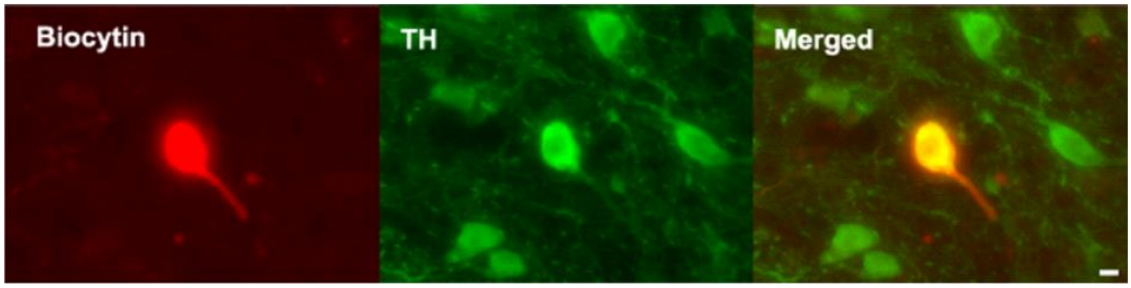

Figure 2: Dopaminergic VTA neuron immunolabeling. immunolabeling (tyrosine hydroxyilase TH, green) of a recorded DA neuron (filled with biocytin, red). Scale bar 10 µm). From [35].

Within VTA TH-positive neurons constitute approximately 55-65 % of the total numbers of cells [31]. However, the cellular composition of the VTA is highly heterogeneous and VTA DA neuron activity is differently regulated by inputs from a variety of brain regions as well as by interactions with local GABA- and glutamate-releasing neurons. Indeed, DA neurons in the VTA are intermingled with different subpopulation of GABAergic and glutamatergic neurons [3, 36, 37], representing approximately 25% and 5% of VTA neuronal population, respectively. Thanks to the heterogeneity of VTA DA neuron subtypes, it is not surprising that VTA DA neuron activity has been associated with a plethora of brain functions. They are activated in response to unpredicted reward, shift their activation to cues that predict reward following learning and transiently decrease their firing when an expected reward is omitted [38, 39]. In addition, VTA dopamine neurons can be inhibited, but also excited, by aversive stimuli or by cues that predict an aversive outcome [1, 40]. Given the participation of VTA DA neurons in different aspects of behavior, understanding the control of their activity and the resulting release of DA in the target areas is of great importance. DA neurons located within the VTA are clustered into specific anatomical niches and project to single target regions such as NAc, PFC, hypothalamus, basolateral amygdala (BLA), lateral habenula, pallidum and bed nucleus of the stria terminalis (Fig 3) [3]. The understanding of how these different circuitries contribute to the role of DA in reward and aversion is certainly a great challenge. To this aim, recent studies began to classify VTA DA neurons depending on their projection targets [31, 32, 34, 41, 42] and

powerful approaches have been used to disentangle the intricate anatomical and physiological connectivity of VTA circuitries. For example, the use of retrograde tracing allowed for a detailed functional identification of DA neurons residing within the VTA and projecting to NAc, PFC and BLA [33, 34]. Using these approaches, it was found that DA neurons in medial posterior VTA selectively project to the NAc medial shell and core, medial prefrontal cortex (mPFC) and BLA. In contrast, the DA neurons that are predominantly located in the lateral posterior (that are the object of study of the present thesis) and anterior VTA project to NAc lateral shell [1]. Recent findings showed that NAc activation by inputs from VTA dopamine neurons is sufficient to produce reward in rats [43] and that cue-evoked DA release in the NAc, that predicts reward delivery, rapidly modulates activity in medium spiny neurons (MSNs) expressing the DA receptor type 2 (D2-type receptors) [6]. In another study, optogenetic stimulation of dopamine transporter-expressing fibres from the VTA in dorsal hippocampus during learning improved recall in a complex spatial learning task [44]. The control of DA release in the target areas needs to be highly organized and finely regulated. To achieve this, VTA receives both excitatory and inhibitory inputs from a broad distribution of brain areas. Among the VTA inputs, the excitatory glutamatergic afferents are a key component in the regulation of DA neurons excitability (see 1.1.2 below). VTA DA neurons receive glutamate afferents mainly from the laterodorsal tegmentum (LDT) and the medial prefrontal cortex (mPFC) and these inputs seem to be important in DA neuron-related functions [3]. In particular, LDT neurons establish excitatory synaptic connections onto VTA DA neurons projecting to the lateral shell of NAc [45]. On the other hand, inhibitory GABAergic projections, that mostly originate from the rostromedial tegmental nucleus (RMTg), serve as a major brake for VTA DA neurons projecting to the lateral shell [13]. Recently, through the use of optogenetics, specific cell populations can be activated and this approach allowed to uncover the behavioral relevance of different afferent-specific inputs to VTA neurons and, ultimately, to exert an overall control of rewarding and aversive behaviors in mice. In particular, by expressing the light-activated channel channelrhodopsin-2 (ChR2) in the LDT, it was shown that optical stimulation of LDT terminals in the VTA activates mainly VTA DA neurons projecting to the lateral shell of NAc and induces conditioned place preference

(CPP) [45]. In contrast, light stimulation of axonal projections from the lateral habenula (LHb) in the VTA, that preferentially synapse with the VTA DA neurons projecting to the mPFC, and the GABAergic neurons in the RMTg - these latter inhibiting VTA DA neurons projecting to the NAc lateral shell - produced conditioned place aversion (CPA) [45]. These results suggest that specific inputs to the VTA differently influence DA neurons and VTA microcircuits and, as a consequence, they also differently influence behavior.

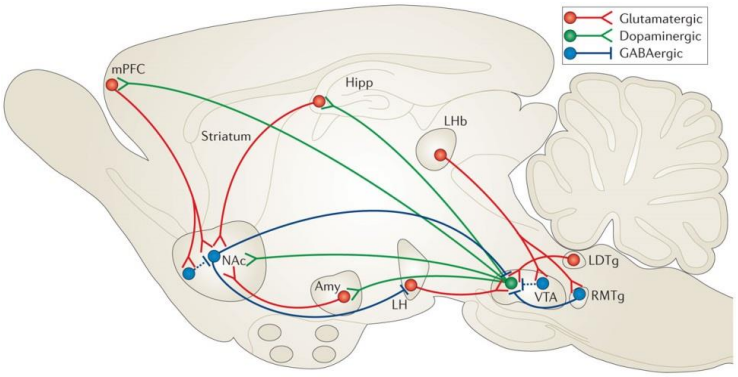

Figure 3. Input-output relationships in the VTA. A simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections to and from the VTA and NAc (green, dopaminergic; red, glutamatergic; blue, GABAergic projections) in the rodent brain. The primary reward circuit includes dopaminergic projections from the VTA to the NAc. The NAc receives dense innervation from glutamatergic monosynaptic circuits from the medial prefrontal cortex (mPFC), hippocampus (Hipp) and amygdala (Amy), among other regions. The VTA receives inputs from amygdala, lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH), among the others. The glutamatergic inputs control aspects of reward-related perception and memory. From [46].

1.1.2 Ventral Tegmental Area DA neuron activity

The behavioral heterogeneity of VTA DA neurons reflects not only the heterogeneity of the connections that these neurons receive from other brain areas and of microcircuit connectivity but also their unique electrophysiological properties. DA neurons that reside within the lateral portion of the VTA exhibit large I_h current [2], i.e. a hyperpolarization-activated cationic current, which represents an electrophysiological feature traditionally used to identify DA neurons in the VTA [13, 47]. Moreover, recent studies found that glutamatergic

synapses in these neurons display a low AMPAR/NMDAR ratio at resting state. On the other hand, DA neurons located in the medial part of the VTA do not show I_h currents. Moreover, in these neurons glutamatergic synapse configuration display a high AMPAR/NMDAR ratio at resting condition [2]. Although DA neurons display different electrophysiological properties, they all share common features in terms of their electrophysiological characterization. Indeed, as already mentioned, VTA DA neurons in slice preparations exhibit a basal, tonic-like activity, characterized by a low frequency firing rate (2-4 Hz) [8, 9], while *in vivo* local circuits and afferent GABAergic inputs change the pacemaker firing pattern into a slow, irregular firing pattern and in this way, they control the proportion of DA neurons that are firing spontaneously [9, 48]. Tonic, spontaneous discharge is important in determining the functional output of DA neurons because it sets the level of responsiveness of the system to rapid phasic stimuli [49]. Indeed, when exposed to behaviorally salient stimuli, such as reward related events or the anticipation of rewards, VTA DA neurons switch to a phasic multi-spike bursting activity pattern characterized by high frequency firing rate (15-30 Hz) [9-11, 39, 50]. DA neuron burst firing induces a large transient increase in synaptic DA release [51, 52] and this event is considered the functionally relevant signal sent to postsynaptic sites to encode reward prediction or to indicate incentive salience [53, 54] (Fig 4). Glutamatergic innervation to the VTA plays a central role in this transition from tonic to phasic burst firing pattern and is critical for reward-dependent learning [9, 12, 13, 55]. For example, electrical stimulation of the glutamatergic input LDT promotes burst firing of putative VTA DA neurons and increases DA release in the NAc [56, 57]. In particular, burst firing pattern seems to be initiated by the NMDA receptors activated by synaptic inputs [9, 50]. One important feature of this regulation is the presence of spontaneous activity in DA neurons while receiving excitatory signals. Indeed, in neurons that do not exhibit a basal active state, presumably due to GABA-mediated hyperpolarization, activation of glutamatergic afferents has little or no effect, likely attributable to Mg^{2+} blockade of the NMDA receptor–channel complex [48]. In contrast, in neurons that are spontaneously active, glutamatergic transmission can elicit a high frequency burst firing activity through which DA neurons can encode for reward prediction error signals. They fire constantly at a low rate, and speed up, firing a phasic

burst when reward exceeds prediction (for example an unexpected reward) or pause when an expected reward does not occur [10]. In this way, the firing activity of midbrain DA neurons codes for reward- and punishment-related information, and changes in firing rate may contribute to the pathophysiology of a number of disorders, including schizophrenia and addiction. Many studies have demonstrated how reward and reward expectation can trigger a burst firing activity pattern in VTA DA neurons. However, it is important to validate how this neuronal firing pattern induces behavioral changes in freely moving animals. Taking advantage of optogenetic tools that allow a selective control of specific neuronal populations with high temporal precision, it was demonstrated that DA neuronal activity can drive behavioral changes in freely-moving mammals. The first evidence that DA neurons phasic firing induction alone is sufficient to elicit reward-related behavior was provided by Tsai et al. in 2009 [52]. In this work the authors targeted ChR2 specifically to VTA DA neurons. They found that an optogenetic activation of phasic DA neuron firing activity induced behavioral conditioning in CPP. Moreover, they found that this optical stimulation protocol triggered a transient large increase of DA release in the NAc. In another study, phasic optogenetic activation of DA neurons in freely moving mice causally enhanced positive reinforcing actions in a food-seeking operant task [58].

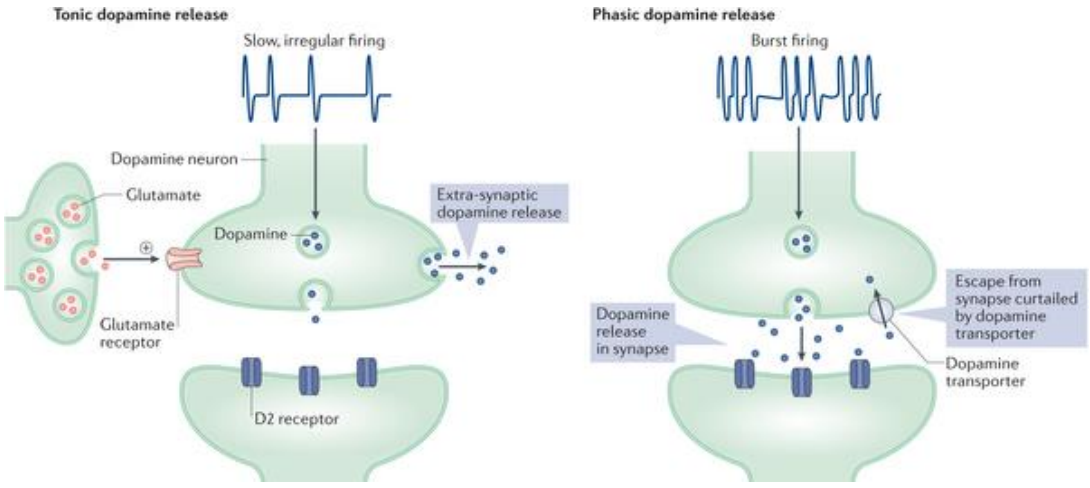

Figure 4. Different firing modes of DA neurons in the VTA. Tonic- like firing activity (characterized by low frequency firing rates) in basal conditions is related to tonic extra-synaptic DA levels. Burst firing activity (characterized by high frequency firing rates) is related to rapid, high amplitude, intra-synaptic phasic DA release in the projecting areas. From [48].

1.1.3 Endocannabinoid signaling in the VTA

Endocannabinoid (eCB) system comprises the G protein-coupled cannabinoid receptor type 1 and 2 (CB1R and CB2R) and small modulatory lipid ligands endocannabinoids (2-AG, AEA, 2-AGE and others) that are released on demand by high neuronal activity [59]. Burst firing activity of VTA DA neurons makes no exception to this rule and this pattern of neuronal activity elicits a local release of eCBs within the VTA [60, 61]. The CB1R is one of the most-abundant G protein-coupled receptors in the brain and it is highly expressed in regions that have a known involvement in reward, addiction, and cognitive function, including amygdala, PFC, and VTA [62, 63]. Activity-dependent eCB release generally occurs from post-synaptic neurons, while CB1Rs are often located on axon terminals [64]. Therefore, eCBs act in a retrograde way and activation of presynaptic CB1Rs leads to a decreased neurotransmitter release that results in short or long-term forms of synaptic depression [63, 64]. Similarly to other neurons, DA neurons use eCBs as retrograde neurotransmitters to finely tune their own activity. Within the VTA, CB1Rs are present in both excitatory and inhibitory terminals that signal to DA neurons whereby they exert widespread modulatory influences (Fig 5) [63]. In glutamatergic synapses, VTA DA neurons release eCBs on demand to decrease glutamate release [65-67]. In particular, among the different endogenous lipids molecules with eCB-like activity, 2AG seems to be the relevant eCB released after a brief burst of excitatory synaptic activity, through a mechanism that involves both mGluR1 activation and an increase in intracellular Ca^{2+} levels [66]. On the other hand, activation of CB1Rs on GABAergic synapses leads to a reduction of GABA release and subsequently to a disinhibition of DA neurons [68]. The precise regulation of DA neuronal activity by eCB system and the resulting changes in DA levels at the target regions have been proposed to play a central role in the control of DA-dependent behavior [63, 68, 69].

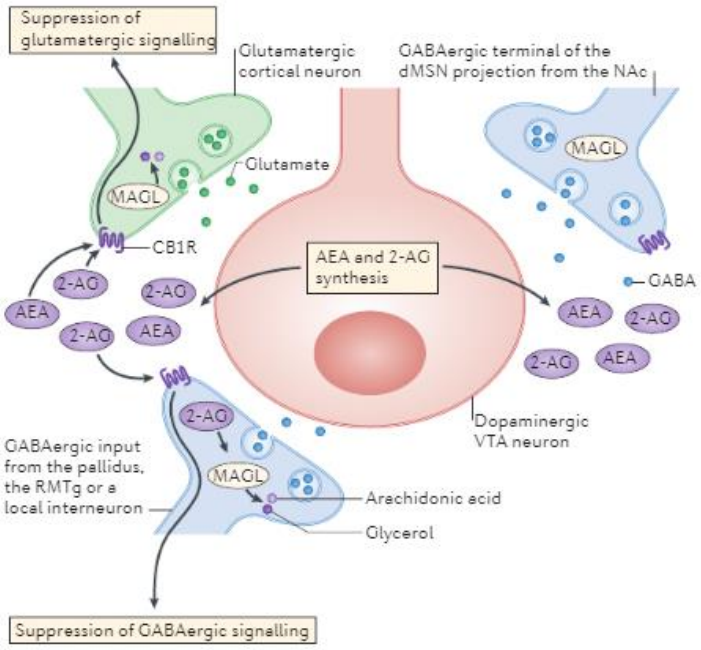

Figura 5. Endocannabinoid actions in the VTA. Endocannabinoids influence VTA synaptic signaling. 2-Arachidonoylglycerol (2-AG) produced by VTA DA neurons acts on CB1R of nearby glutamatergic and GABAergic terminals. Extensive evidence demonstrates eCB-mediated suppression of glutamate signaling in the VTA. Thus, eCBs have a prominent role in fine-tuning the activity of the mesolimbic DA projections through modulation of both excitatory and inhibitory signaling in the VTA. From [63].

1.1.4 Dopamine signaling in the VTA

It is well established that VTA DA neurons release DA through their axonal terminals in VTA projecting areas. In addition, release of DA occurs in the VTA through collaterals arising from DAergic axons as well as from soma and dendrites of DA neurons [70, 71]. The intra-VTA DA release, that is elicited under different behavioral and pharmacological conditions [72, 73], is involved in critical functions. The two types of receptors for DA (D1 and D2-type receptors) are G protein-coupled receptors, that trigger distinct intracellular signaling pathways [74]. In brief, Gα(s/olf)-coupled D1-type receptors (D1R and D5R), by activating the adenylyl cyclase (AC), boot the production of cyclic adenosine monophosphate (cAMP) and subsequently activate protein kinase A (PKA). In contrast, D2-type receptors (D2R, D3R and D4R), coupled to Gα(i/o) proteins, inhibit AC and suppress PKA signaling. Thus, DA displays different

physiological effects by binding to distinct DA receptors within the VTA. The first evidence that DA exerts an action in the VTA was provided by White et al. in 1984. In particular, they demonstrated that an exogenous DA application onto the VTA inhibited DA cell firing through activation of local D2-type receptors [75]. The D2-type autoreceptors are expressed mainly on the soma and dendrites of VTA DA neurons and the DA binding to these autoreceptors elicits inhibitory postsynaptic currents through activation of inwardly rectifying K^+ channels (GIRKs) [76, 77]. In addition, presynaptic D2-type receptors were reported to inhibit excitatory transmission onto VTA DA neurons [78]. In this way, D2-type receptor activation negatively regulates the firing pattern of VTA DA neurons, thus providing a feedback control of DA neuron activity [9, 47]. On the other hand, *in vivo* microdialysis with D1-type agonists increases the extracellular level of glutamate in the VTA [79] acting on D1R in excitatory presynaptic afferents [80]. In this sense, it has been suggested that activation of the presynaptic D1-type receptor increases glutamate release [81]. In contrast with these findings, in VTA slice preparations presynaptic D1-type receptor activation was reported to slightly decrease glutamate release [82]. The DA release in the VTA can, therefore, exert a variety of modulatory actions on DA neurons depending on the activation of different types of DA receptors and this, in turn, can have relevant implications in DA release also in target structures and in the functionality of the entire brain reward circuitry.

1.1.5 Synaptic plasticity in the VTA

DA in the brain is implicated in the promotion and the execution of goal-directed behaviors resulting in reward acquisition. In particular, DA neurons in the VTA play a central role in reinforcing rewarding behavior and attributing salience to important environmental cues [10, 23]. Fundamental goal-directed behavior, such as seeking food or water (that are considered natural reward), are not innate, but learned [83]. At a cellular level, the learning processes are associated to long-lasting changes in synaptic strength. This experience-dependent modulation of synaptic transmission is generally termed synaptic plasticity. In particular, the excitatory glutamatergic afferents onto DA neurons

exhibit a high degree of plasticity [2, 84-88] that contributes to profoundly alter DA neuron output, and, in turn, DA release in downstream regions. A better knowledge of the plasticity mechanisms at these synapses is, therefore, important given that glutamatergic afferents to VTA DA neurons drive the switch between tonic to burst firing [9, 12, 13]. Changes in the strength of excitatory synapses on DA neurons can, indeed, be elicited in vitro by high frequency stimulation protocols and spike-timing dependent protocols (STDP) [14]. Both protocols trigger a long-term potentiation of the excitatory transmission onto DA neurons that is mediated by NMDAR activation as well as by a subsequent increase in postsynaptic Ca^{2+}. Plasticity in VTA DA neuron synaptic activity can also be elicited in vivo during the acquisition phase of natural cue-reward learning, exposure to drugs of abuse and optogenetic stimulation of VTA DA neurons [84, 86-90]. In these cases, a long-term increase of the AMPA function is elicited. The general rule governing the modulation of synaptic strength in DA neurons seems to rely on an increase in the AMPA/NMDA ratio due to a switch in AMPAR subunit composition, from non Ca^{2+} permeable GluA2 subunit-containing, low conductance AMPARs to GluA2-lacking, Ca^{2+} permeable, high conductance AMPARs [90, 91]. Additionally, *in vivo* exposure to cocaine has been reported to reduce NMDAR function in VTA DA neurons which may amplify the increase in AMPA/NMDA ratio occurring under these conditions [92]. A requirement for eliciting this increase in the AMPA/NMDA ratio in VTA DA neurons is the activation of NMDARs [86, 89, 93]. In addition, D1-type receptors seem to be crucial for plastic changes evoked by cocaine exposure to isolated VTA slices and by optogenetic stimulation of burst firing in DA neurons, suggesting that an increase in DA within the VTA is a crucial event for the induction of synaptic plasticity [82, 90]. During natural reward, the increased AMPAR function associated to cue-reward learning is a transient event that is not required for the long-term maintenance of cue-reward associations [84]. In contrast, after drug-related experiences the potentiation of excitatory transmission onto VTA DA neurons became a long-persistent event that may drive pathological drug-seeking behavior [87]. In this sense, interfering with the persistent cocaine-evoked synaptic potentiation in VTA DA neurons can control in NAc early forms (depression of AMPA/NMDA ratio) and enduring forms (increased AMPA function due to insertion of GluR2-lacking AMPARs after

cocaine withdrawal) of cocaine-evoked synaptic plasticity and the subsequent cocaine-seeking behavior after cocaine withdrawal [94]. In fact, the initial synaptic changes triggered by drugs of abuse in VTA DA neurons represent the initial trace that will eventually lead to the establishment of addiction. As previously described, VTA DA neurons can be distinguished depending on their projecting targets. VTA DA neurons that project to NAc, and that are activated mainly by reward stimuli, display the potentiation of excitatory transmission in response to rewarding stimuli. In contrast, VTA DA neurons projecting to the mPFC, that are mainly activated by aversive stimuli, do not change their AMPA/NMDA ratio in response to cocaine. Instead, these cells display an increase AMPA/NMDA ratio in response to aversive experiences [34]. In this sense, the functional segregation of DA neurons in the VTA is not restricted to anatomical, electrophysiological and input/output characteristics and it is extended to the synaptic plasticity of these neurons in response to rewarding/aversive stimuli. While a number of different neuronal mechanisms have been identified in the regulation of synaptic plasticity in the VTA, a possible modulatory role in VTA circuits of the glial cells astrocytes remains unexplored.

1.2 Astrocytes

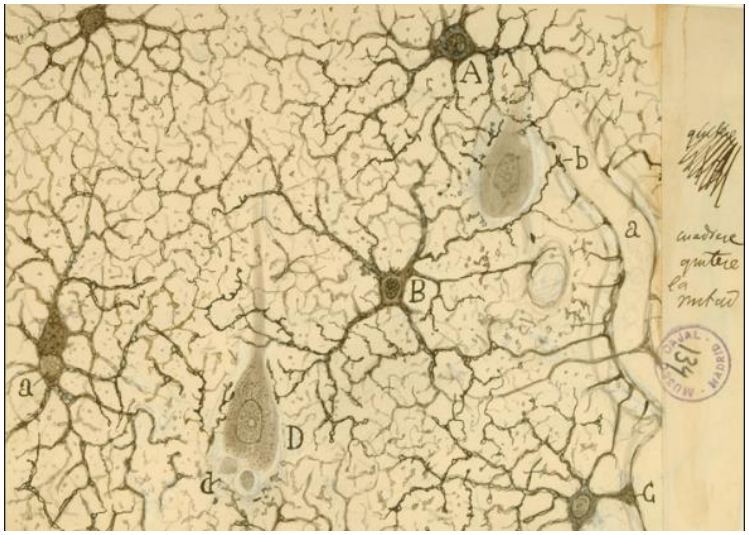

Figure 6. Santiago Ramon y Cajal drawing. Human hippocampal astrocytes based on brain tissue observation after Golgi method impregnation. The picture show the fine relationship existing between neurons (C, D), astrocytes (A, B) and microvessels (F).

In 1856 Virchow for the first time provided evidence that brain is composed not only of neurons, but also of glial cells. For the glial cells he used the term *nevernkitt*, i.e. nerve glue, that was then translated into *neuroglia*. He supposed that the main role of glial cells was indeed to provide structural support to neurons. Some years later, Camillo Golgi argued that glial cells serve as nutritive supporters for neurons and their association with blood vessels further supported his theory. Later on, Santiago Ramon y Cajal focused his attention on the study of the identification and of the classification of the glial cells on the basis of their morphology. Using metal impregnation methods developed by Camillo Golgi, he could study astrocytes from a structural point of view (Fig 6). Nowadays, astrocytes are considered the most abundant class of glial cells in the brain. They are star-shaped cells with long and thin processes through which they can interact with both neurons and blood vessels. Astrocytes play essential roles in brain homeostasis and metabolism. They exert a homeostatic control of the extracellular space regulating the pH and the extracellular concentrations of different neurotransmitters and ions. Thanks to their high resting K^+ conductance and the presence of gap junctions, one of the main function assigned to these cells is the clearance of extracellular K^+ following period of elevated neuronal activity [16]. Astrocytes processes also express high level of transporters for neurotransmitters, such as glutamate, GABA and glycine, that serve to clear neurotransmitters from the synaptic space [95]. Although astrocytes have long been known to be critical for providing structural and

nutritional support for neurons, their contribution to fast signal processing in the brain has been largely dismissed. However, numerous studies over the past three decades indicate that astrocytes are not merely passive supporters of brain function, but they rather actively participate in a dynamic and bidirectional communication with neurons. Astrocytes can respond with intracellular Ca^{2+} elevations to synaptic neurotransmitter release and signal back to neurons by releasing gliotransmitters which influence synaptic transmission, short and long-term plasticity and network operations [16, 17].

1.2.1 The tripartite synapse

From an anatomical point of view, astrocytes are characterized by a central soma and by a great number of processes through which they can sense incoming information and send signal to other cell types including neurons, other glial cells and blood vessels. They display an intricate morphological arborization composed by thin processes which cover non-overlapping territories in different brain regions and enwrapping tens of thousands of synapses [96, 97]. Electron microscope and immunohistochemical data reveal that astrocytes can interact with a great number of synapses in the brain thus introducing the concept of tripartite synapse (Fig 7). In the tripartite synapse, a single astrocytic process can enwrap both the presynaptic bouton and postsynaptic dendritic spine, the two neuronal elements of the synapse. Interactions between astrocytic processes with the pre- and post-synaptic elements form a structural and functional entity that is considered the information transfer unit of the brain. Consistent with this concept, in addition to the classical information flow between pre and post-synaptic elements, astrocytes exchange information with neurons, responding to synaptic activity and in turn, regulating synaptic transmission. The concept of tripartite synapse represents an integrative functional view of synaptic physiology, that consider astrocytes as active protagonists that in partnership with neurons govern information processing in the brain [16, 17]. Consequently, in contrast to the classical accepted paradigm that brain functions result from uniquely neuronal activity, tripartite synapse cause a paradigm shift in brain physiology in which brain functions arise from the coordinate activity of neurons and astrocytes.

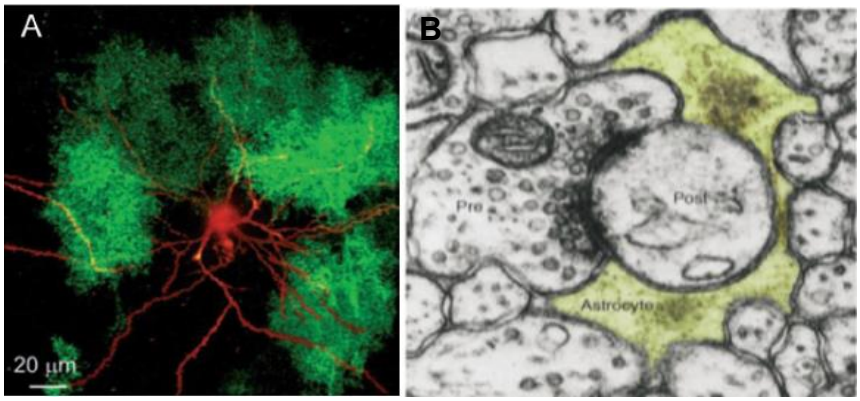

Figure 7. Astrocytes morphology. (A) Recostruction of a byoticine-filled neuron (red) in layer II/III from a dn SNARE animal. Astrocytes (green) cover the cortical volume with non-overlapping domains. **(B)** Electron microscopy image of an astrocyte process (yellow) at the axon-spine interfase revealing the presence of a tripartite synapse. From [97] and [98].

1.2.2 Astrocyte control of brain metabolism

Astrocytes play a central role in the modulation of brain circuits and also contribute to neurovascular coupling mechanism. Indeed, astrocytic processes are positioned like a hub between synapses and local vasculature and based on this anatomical organization, they sense synaptic activity and release vasoactive molecules, such as nitric oxide, adenosine trisphosphate (ATP) and prostaglandins [99, 100]. The contact of astrocytic end-feet with arterioles and capillaries was first described by Camillo Golgi and it has long been interpreted as an indicator that astrocytes can take up nutrients and metabolites from the blood and then distribute them to neurons. The glucose that astrocytes import from blood vessels has traditionally been believed to serve as the foremost substrate of energy directly supplied to and used by neurons. However, several studies have supported the hypothesis that, during physiologically condition of high energy demands, lactate is in fact preferentially utilized. A model proposed by Pellerin and Magistretti in 1994, known as astrocyte-neuron lactate shuttle (ANLS), suggests that the release of lactate by astrocytes is used as a fundamental energy substrate by glutamatergic neurons. The ANLS hypothesis originally derived from the finding that glutamate triggers the uptake of labeled glucose that is released as L-lactate by cultured astrocytes [101]. The model predicts that excitation, and hence glutamate release, stimulates the uptake of glutamate by astrocytes through two main subtypes of glutamate transporters,

i.e. the glutamate-aspartate transporter (GLAST or EAAT1) and the glutamate transporter type 1 (GLT1 or EAAT2). Glutamate into astrocytes is then rapidly converted into glutamine through glutamate-glutamine cycle, eventually sustaining the synaptic release of glutamate. This cycle requires energy from astrocytes, which would therefore activate glucose uptake from the blood and metabolize it into lactate. Lactate which is released by astrocytes via monocarboxylate transporter (MCTs), can enter other types of cells, including neurons, using similar transporters [102, 103] to be converted into pyruvate that through oxidative phosphorylation is subsequently metabolized into mitochondria to produce ATP. The lactate supplied by astrocytes to neurons help to understand how neurons handle the energy requirements during periods of intense activity. In line with this hypothesis, earlier work demonstrated that L-lactate was sufficient to maintain synaptic activity in rat hippocampal slices [104]. Additionally, *in vivo* studies from the barrel cortex demonstrated that young mice deficient in the glial glutamate transporter GLT1 or GLAST exhibit a reduced metabolic response to whisker stimulation [105]. Glycogen, the main form of glucose storage in the brain, plays also a central role in neurometabolic coupling and it is mostly exclusively localized in astrocytes [106, 107]. Under conditions of high energy demand, such as glucose deprivation or intense neuronal activity, glycogene is catabolized to rapidly deliver metabolic substrates (i.e. lactate, pyruvate) [106]. Several studies converged on the conclusion that glycogenolysis and aerobic glycolysis, resulting in the production of lactate, are critically linked to memory formation. In particular, glycogenolysis is a critical requirement for long-term memory storage. In line with this hypothesis, in a taste avoidance training in a day old chick intracranial injections of an inhibitor of glycogen phosphorylase, i.e. 1,4-dideoxy-1,4-imino-D-arabitol (DAB), impaired memory in a dose-dependent manner [108] and in mouse hippocampus lactate transported from astrocytes to neurons plays a critical role in long term memory consolidation [109]. Recent studies in the basolateral amygdala (BLA) of rats also indicate that the consolidation and reconsolidation of appetitive conditioning using drugs of abuse (i.e. cocaine-conditioned place preference or self-administration) are also dependent on glycogenolysis and on the directional transport of lactate from astrocytes to neurons via MTCs [110]. Astrocytes are thus important partners of neurons in

brain physiology. They receive inputs from nearby synapses and actively contribute both to the modulation of synaptic transmission in neuronal circuits and to the control of brain metabolism.

1.2.3 Astrocyte Ca^{2+} excitability

With their typical morphology of processes, astrocytes are in a privileged position to sense a large variety of signaling molecules and coherently respond to these signals. However, unlike neurons, astrocytes are electrically non-excitable cells and exhibit a form of excitability which relies on highly dynamic intracellular Ca^{2+} changes. The first clue of this Ca^{2+} mediated excitability was made in the early 1990s, when application of Ca^{2+} imaging techniques to cultured astrocytes led to the remarkable discovery that these cells respond to neurotransmitters by increasing the intracellular Ca^{2+} concentration [111, 112]. Moreover, astrocytes were observed not only to respond to external stimuli with an intracellular Ca^{2+} increases, but to transmit these Ca^{2+} signal to adjacent non-stimulated astrocytes, as intracellular Ca^{2+} waves. Since this pioneering study, in the following years these observations were confirmed and expanded in both cell cultures and in brain slices, revealing that astrocyte Ca^{2+} oscillations can occur in response to synaptic neurotransmitters as well as spontaneously as intrinsic Ca^{2+} oscillations independent of neuronal activity [113, 114]. This property of astrocytes was confirmed in different brain regions where astrocytes respond with Ca^{2+} elevations to the synaptic release of glutamate, GABA, ATP, norepinephrine and acetylcholine [115]. The expression in astrocytic membranes of a wide variety of neurotransmitter receptors suggests that astrocytes can sense and integrate different neuronal information coming from specific neuronal circuits. It is noteworthy that many of these receptors are high affinity, metabotropic G-protein coupled receptors that upon activation stimulate phospholipase C and the formation of inositol (1,4,5)-triphosphate (IP3) which increases the cytosolic Ca^{2+} concentration through the release of Ca^{2+} from IP3 sensitive Ca^{2+} stores [116]. More recently, it was also established that similar increases in intracellular astrocytic Ca^{2+} occur in vivo upon an increase in neuronal network activity thus providing evidence for the existence of active neuron-glia communication in the intact living brain [117]. Increase in astrocytic

Ca^{2+} signaling in the real *in vivo* context can also occur in response to sensory stimuli such as whisker stimulation [118] or visual stimuli to which astrocytes respond with distinct spatial receptive fields and sharp tuning to visual stimulus features such as orientation and spatial frequency [119]. Moreover, intracellular astrocytic Ca^{2+} signal activity can also raise during locomotor activity [120] as well as autonomic function [121]. The synaptically evoked, as well as the spontaneous Ca^{2+} signal, originate in spatially restricted area of astrocytic processes, called microdomains. The local activation of astrocyte Ca^{2+} signal can then propagate intracellularly to other astrocytic regions. As a single astrocyte might contact tens of thousands synapses [96], the control of the spatial extension of Ca^{2+} signals could have relevant functional consequences. Moreover, neurons can generate in astrocytes a wide range of Ca^{2+} signals with different patterns and kinetics depending on both the synaptic circuit involved [115], the pattern and frequency of afferent input activity [122]. Therefore, astrocytes can encode different neuronal information [123]. These properties underlie the level of complexity in the astrocyte response to neuronal activity.

1.2.4 Neuron-astrocyte signaling in the brain

The Ca^{2+} activity evoked in astrocytes by neuronal activity has important physiological consequences. One of these is the release of gliotransmitters that can target both pre- or post-synaptic neuronal receptors controlling neuronal excitability and modulating synaptic transmission (Fig 8) [16, 17, 124]. The main types of gliotransmitters released by astrocytes are glutamate, D-serine and ATP. This latter can be rapidly converted by membrane ectonucleosidases into adenosine. A number of evidence demonstrate that astrocytic transmitters are released in a Ca^{2+} dependent manner through vesicle exocytosis [125-128]. The presence in astrocytes of synaptic-like vesicles located at close proximity to synapses, was also confirmed by ultrastructural studies. Through gliotransmitters astrocytes can established a bidirectional communication with neurons.

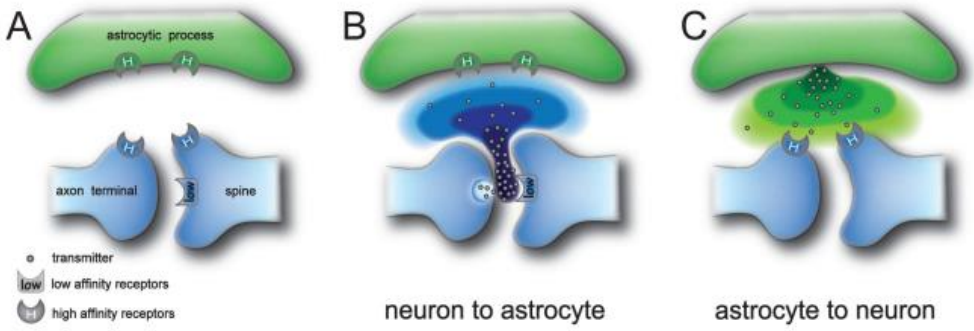

Figura 8. Bidirectional Neuron-astrocyte Communication Granted by High Affinity, Slowly Desensitizing Receptors. (A) Schematic drawing of the tripartite synapse illustrating the location of low and high affinity ligand receptors. **(B)** Neurotransmitters rapidly activate low affinity receptors at the postsynaptic neuronal membrane and diffuse outside the synaptic cleft to activate high affinity receptors at the astrocytic membrane. **(C)** Gliotransmitters activate high affinity receptors at perisynaptic locations in the neuronal membrane. Neurotransmitter **(B)** or gliotransmitter **(C)** decreasing concentrations over distance from release sites is illustrated by different color intensity. From [17].

Single gliotransmitters can have multiple effects depending on the type of circuit and targeted neurons, the pre- or post-synaptic location of receptors, and the receptor subtype activated (Fig 9). For instance, astrocytic glutamate potentiates or depresses excitatory transmission in the hippocampal dentate gyrus or neocortex, respectively, by acting on presynaptic NMDARs [129, 130], and it can also favor neuronal synchrony by acting on postsynaptic NMDARs in CA1 hippocampal region [131, 132]. On the other hand, at hippocampal CA3-CA1 synapses, astrocytic glutamate can activate presynaptic mGluRs to favour excitatory transmission [133-137]. In the CA1 hippocampal region, astrocytic glutamate has also been reported to potentiate inhibitory transmission by acting on presynaptic kainate receptors [138]. Moreover, astrocytes can release multiple gliotransmitters. For example, in addition to glutamate, astrocytes in CA1 can release the NMDA receptor co-agonist D-serine [139], a gliotransmitter involved in LTP induction [140]. Finally, ATP can also be released by astrocytes. This gliotransmitter can act on P2X and P2Y receptors to differently modulate synaptic transmission [141-146]. In addition, after conversion of ATP to adenosine, this latter gliotransmitter acts on either A1 or A2A receptors to depress or enhance excitatory synaptic transmission, respectively [141, 146-149].

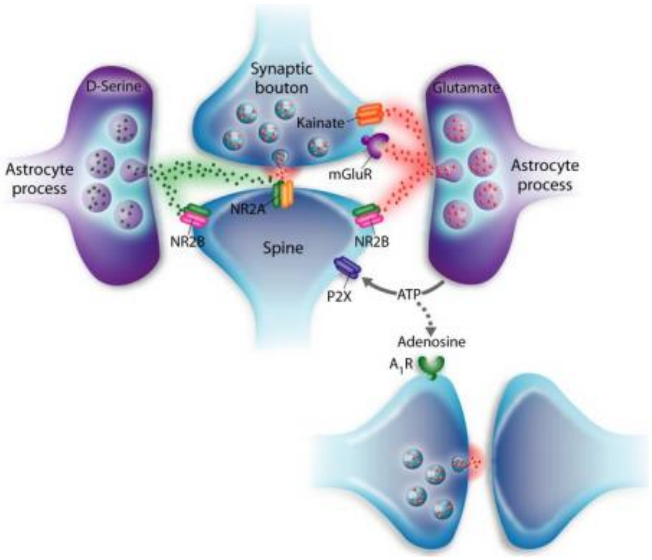

Figure 9. Astrocyte-derived signals act both presynaptically and postsynaptically to regulate synaptic transmission. The release of glutamate, D-serine, and ATP from astrocytes has a diversity of synaptic actions: presynaptically, glutamate can access metabotropic glutamate receptors to enhance synaptic transmission; postsynaptically, glutamate can act on extrasynaptic NMDA receptors to depolarize the neuronal membrane and promotes neuronal synchrony. D-serine acts on the glycine-binding site of NMDA receptors and can regulate synaptic plasticity. ATP may also act postsynaptically on P2X receptors to depolarize the neuronal membrane and regulate the insertion of postsynaptic AMPA receptors. After hydrolysis by ectonucleotidases to adenosine, ATP can have distant action on presynaptic A1 receptors to cause heterosynaptic depression of excitatory synaptic transmission. From [16].

Moreover, gliotransmission can coordinate networks of neurons and synapses. Astrocytic Ca^{2+} signals evoked locally by active synapses can eventually expand intracellularly from their initial source towards different cell locations. This intracellular Ca^{2+} propagation can trigger gliotransmitter release at relatively distant sites, affecting other synapses and circuits. Consistent with this view, it was found that endocannabinoids released by neurons can activate astrocytes through the stimulation of CB1Rs [150]. This activation eventually led to astrocytic glutamate release and a consequent activation of presynaptic mGluRs on synaptic sites relatively distant from the endocannabinoid source, that leads to enhanced glutamate release [134]. Through this mechanism, eCBs indirectly potentiate synaptic transmission, an action that is rather different of the direct activation of presynaptic CB1Rs which causes homosynaptic depression of neurotransmission [134]. As previously described, in hippocampal

CA1 region highly active synapses recruit astrocytes to release ATP. This gliotransmitter is then rapidly converted to adenosine and by acting on presynaptic A1 receptors it depresses other synapses [147, 151]. All together, these observations suggest that astrocytes operate as functional links to coordinate multiple synapses.

Finally, the involvement of astrocyte signaling in synaptic plasticity has been recently observed also *in vivo*. In particular, in hippocampus and in somatosensory cortex astrocyte activation by muscarinic receptors leads to a Ca^{2+}-dependent glutamate release that elicit synaptic plasticity [135, 152].

1.3 Endocannabinoid and dopamine signaling to astrocytes

eCBs and DA released by DA neuron in the VTA are known to exert widespread modulatory influences in the activity of the brain reward circuitry. Given the important physiological role of eCBs and DA in the VTA and the emerging role of astrocytes in the modulation of synaptic activity, in the following subheading, we provide a summary of the current knowledge of the reciprocal interactions between astrocytes and both eCBs and DA. A bulk of studies provide evidence that both eCBs and DA can induce intracellular Ca^{2+} elevations in astrocytes. Moreover, eCBs-mediated astrocytic modulation of synaptic transmission has been extensively investigated in different brain areas [130, 134, 136, 153].

1.3.1 Endocannabinoid-activated astrocytes in the modulation of synaptic transmission

eCBs signaling between neurons and astrocytes has multiple consequences for the function of the tripartite synapse and contributes to the richness of the mechanisms through which astrocytes interact with neurons to modulate neuronal excitability and synaptic transmission. eCB signaling in the brain is well recognized to be mainly mediated by CB1R. CB1R is highly expressed in different brain regions where it mediates several effects on neuronal transmission, plasticity and function [64, 154]. Over the last years the existence and the role of CB1Rs in astrocytes have been the subject of controversy.

Earlier studies showed discrepancies in the expression of CB1Rs by astrocytes in culture [155] and the extremely low levels of expression in these cells questioned their functional relevance in eCB signaling. However, even if they are expressed at low levels, the functional relevance of astrocytic CB1Rs is now well established. The first evidence that astrocytes can be activated by exogenous cannabinoid ligands, as well as by eCBs released by neurons, was provided by Navarrete and Araque in hippocampal slices. These authors found that eCBs elicited astrocytic Ca^{2+} elevations through the mobilization of Ca^{2+} from internal stores [150]. They also characterized the intracellular signaling pathway underlying this effect, that in contrast to the one observed in neurons [154], seems to be mediated by CB1Rs coupled to Gq/11 proteins that activate phospholipase C and produce inositol triphosphate (IP3). Moreover, they found that Ca^{2+} elevations evoked by eCBs released from pyramidal neurons stimulated the release of glutamate from astrocytes. This gliotransmitter activated NMDA receptors (NMDARs), evoking slow inward currents (SICs) in adjacent CA1 pyramidal neurons. These results indicate that neurons and astrocytes can communicate via eCB signaling and as a result of this reciprocal signaling there is a change in neuronal excitability. eCB signaling in astrocytes can also modulate synaptic transmission in different brain regions [130, 134, 136, 153]. The release of eCBs induced by neuronal depolarization evokes a transient depression of synaptic transmission in synapses that impinge onto that neuron by directly activating pre-synaptic CB1Rs. In pioneer studies, Navarrate and Araque demonstrated that, concomitantly, eCBs released by the stimulated neuron led to a transient potentiation of synaptic transmission in relatively distant synapses of adjacent neurons through activation of CB1Rs in astrocytes [134]. Interestingly, the dual effect of eCBs signaling is spatially controlled depending on the intracellular signaling pathway activated. While eCBs are known to exert their effects at short distances, eCB signals may spread through astrocyte branches reaching synapses distant from the original site of eCB release. Through activation of gliotransmitter release in astrocytes, eCBs may, therefore, indirectly lead to lateral potentiation of synapses faraway from eCBs sources. Similar actions were also found in striatum, where the functional relationship between neurons and astrocytes exhibit also cell and synapse specificity [153]. Activation of CB1Rs in astrocytes can also control long-lasting

synaptic changes in different brain areas. In barrel cortex astrocytes are crucially involved in synaptic transmission modulation [130]. In this cortical area, eCB-activated astrocytes release glutamate that interacts with presynaptic NMDA receptor to control spike-time dependent long-term depression (t-LTD) at neocortical synapses [130]. In hippocampus the coincidence of eCB signalling and post-synaptic activity, which leads to a eCB-induced long-term enhancement of transmitter release at single CA3–CA1 hippocampal synapses, was mediated by astrocytes [136]. This eCB-induced astrocyte-mediated long-term potentiation (LTP) occurs at single synapses in neurons relatively distant from the eCB source, corroborating the idea that eCBs, classically considered to act as retrograde signals that evoke synaptic depression, may also serve through activation of astrocytes as a lateral signal inducing LTP of synaptic transmission (Fig 10).

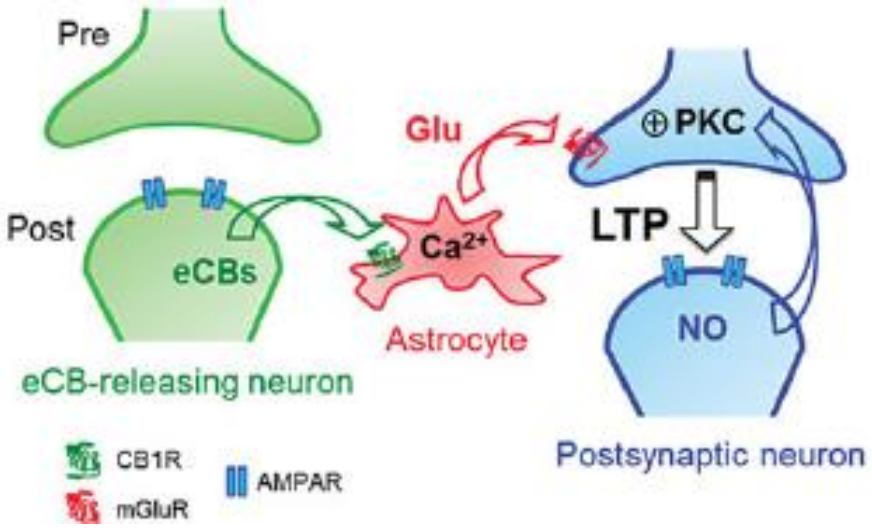

Figure 10. Endocannabinoid-induced LTP in the hippocampus. Schematic drawing representing the signaling pathways involved in the endocannabinoid-induced hippocampal lateral LTP. Endocannabinoid activated astrocytes release glutamate acting on presynaptic mGluR1 thus increasing the probability of neurotransmitter release at the excitatory glutamatergic afferents. Concomitantly NO production by postsynaptic neuron makes this modulation to became a long term change. From [136].

Finally, eCB signaling to astrocytes can have potential relevant roles in complex cognitive functions. CB1Rs have been indeed implicated in many specific

behaviors including the control of food intake, of emotion- and motivation-related responses as well as the expression of cognitive processes [156-159]. On the other hand, the use of a genetic mouse model in which CB1R is specifically deleted in astrocytes has revealed the involvement of CB-activated astrocytes in mediating the impairment of a specific behavioral task during marijuana intoxication [160]. In this study, Han al colleges demonstrated that activation of astrocytic CB1R is involved in the impairment of spatial working memory and the triggering of an in vivo LTD at hippocampal CA3-CA1 synapses induced by exogenous cannabinoids exposure. However, the endogenous roles of CB1R in astrocytes in learning and memory as well as in other specific behavioral paradigms needs to be further investigated. The involvement of eCB signaling to astrocyte in a plethora of brain function, from the modulation of synaptic transmission up to behavior, may open new insight in the understanding of the role of astrocytes in complex cognitive brain processes.

1.3.2 Dopamine signaling to astrocytes

Given the involvement of astrocytes in a plethora of brain functions, it is not surprisingly that a number of works are now investigating the involvement of these cells in dopaminergic systems. Indeed, the monoamine DA is a major modulatory neurotransmitter in the CNS, implicated in reward processing, decision-making and action initiation and termination [161]. Astrocytes are reported to express DA receptors and transporters [162-164]. Activation of astrocytic DA receptors (both D1- and D2-type receptors) has been linked to different intracellular pathway that control the levels of intracellular cyclic AMP (cAMP) and free Ca^{2+} [164-166]. Since intracellular Ca^{2+} elevations in astrocytes are of great importance in information processing, DA induced astrocytic Ca^{2+} elevations can have relevant implication for neuronal functions. However, the precise nature of these signals, their molecular pathways and possible functions are still unclear. It has been found that astrocytes in culture possess atypical D1-type receptors which couple to PLC through Gαq, and that a selective stimulation of this receptor triggers astrocytic Ca^{2+} transients [167].

On the other hand, D2-type receptors activate PLC via Gβγ, increasing intracellular IP3 [168], and application of a D2-type agonist to cultured cortical astrocytes marginally increases Ca^{2+} levels in these cells [162]. Moreover, DA induces Ca^{2+} elevations in cultured astrocytes through the production of reactive oxygen species in a DA receptor independent manner [169]. Recent findings in hippocampal slices showed that DA induces a dose-dependent bidirectional Ca^{2+} response in stratum radiatum astrocytes with Ca^{2+} elevations followed by below-baseline decreases. While D1/D2-type receptors engaged intracellular Ca^{2+} storage and removal to elicit Ca^{2+} elevations, the DA-induced Ca^{2+} decrease involved D2-type receptors only and it was sensitive to Ca^{2+} channel blockade. In contrast, in astrocytes from stratum lacunosum moleculare, DA generated higher-threshold Ca^{2+} responses which did not depend on DA receptors. These findings suggest that depending on the type of activated receptors DA elevates or decreases astrocyte Ca^{2+} signals [164]. The results reported here illustrate our limited knowledge about astrocyte Ca^{2+} signaling modulation by DA. Whether VTA astrocytes are recruited by DA signaling is, indeed, still unexplored.

2. Open questions

Given the critical role of VTA circuits in goal-motivated behaviors and the growing evidence that the output of a specific neuronal circuit depends on the integrated activity of both neurons and astrocytes, a great number of questions needs to be addressed to disentangle the astrocyte roles in VTA functions. In the present work, we aim to provide answers to the following questions: i) Are VTA astrocytes differently recruited by the tonic and the phasic DA neuron firing activity? ii) Can DA neuron-recruited astrocytes modulate excitatory synaptic transmission in the VTA circuitry? iii) What neurotransmitters and gliotransmitters are eventually involved in the modulatory action of astrocytes? iv) What functional relevance this modulation may exert in brain reward circutry? To provide answers to these questions, I studied the glutamatergic synaptic transmission onto VTA DA neurons, i.e. a key component in the regulation of DA neuron excitability, using Ca^{2+} imaging and patch-clamp recording techniques. The overall hypothesis at the basis of my PhD project is that astrocytes in the VTA are recruited by DA neuronal activity and that their activation results in the release of gliotransmitters that contribute to modulate VTA circuits. The clarification of the functional role of neuron-astrocyte reciprocal signaling in the excitatory circuitry of the VTA can, ultimately, provide a better understanding of the complex cognitive processes in mesocorticolimbic DA system. Most interestingly, the limited efficacy of available treatments for addiction asks for a better understand of the underlying cellular and molecular mechanisms that could help the development of new targeted interventions. Understanding the role played by astrocytes in the neuronal circuits of the brain reward systems could, therefore, indicate astrocytes as new targets to control drug addiction and other pathological reinforcement-dependent behaviors.

3. Results

3.1 Ventral Tegmental Area astrocytes express functional CB1 receptors

Beside their predominant neuronal localization, low but functionally relevant levels of CB1Rs are present in astrocytes from different brain regions [155]. Moreover, in different brain regions, including the hippocampus, the somatosensory cortex and the dorsal striatum, it has been found that through activation of astroglial CB1Rs, eCBs released by neurons can modulate neuron-astrocyte communication and trigger the release of astrocytic glutamate that induces short- and long-term changes of synaptic transmission [130, 134, 136, 153]. To investigate whether functional CB1Rs are also expressed in VTA astrocytes, we firstly tested in VTA slice preparations whether these cells respond with Ca^{2+} oscillations to an exogenous application of WIN 55,212-2, a specific CB1R agonist. To this aim, we loaded horizontal brain slices from juvenile mice (P14-P17) with the fluorescent Ca^{2+} indicator Oregon Green 488 BAPTA-1 (OGB1) and the selective astrocyte marker sulforhodamine 101 (SR101) (Fig. 11 A, B). We then recorded somatic fluorescence variations in astrocytes at basal conditions and after bath perfusion with WIN 55,212-2, at different concentrations (in μM: 1, 10, 25, 50). Similarly to what has been described in the hippocampus [150], WIN evoked in a significant group of VTA astrocytes intracellular Ca^{2+} elevations (Fig. 11 C, D). This response increases in a dose dependent manner to subsequent WIN applications at increasingly higher concentrations, in terms of both percentage of responsive astrocytes and frequency of Ca^{2+} peaks. To dissect out the intracellular signaling cascade involved in the CB1R-mediated Ca^{2+} elevations, we performed the same challenge with WIN 55,212-2 to astrocytes in slices obtained from mice lacking the IP_3 receptor type 2 (IP_3R2 KO mice). IP_3R2 represents the main IP_3 receptor involved in Ca^{2+} release from intracellular Ca^{2+} stores in astrocytes [170]. We found that WIN 55,212-2 failed to induce astrocyte Ca^{2+} increases in slices from IP_3R2 KO mice, in contrast to the Ca^{2+} elevations evoked in slices from wild type animals (Fig. 11 D). Taken together, these data demonstrate that i) astrocytes possess functional CB1R; ii) astrocytic CB1Rs can be activated in a

dose dependent manner and iii) CB1R-mediated Ca^{2+} responses in astrocytes are dependent on IP_3R2 signaling pathway.

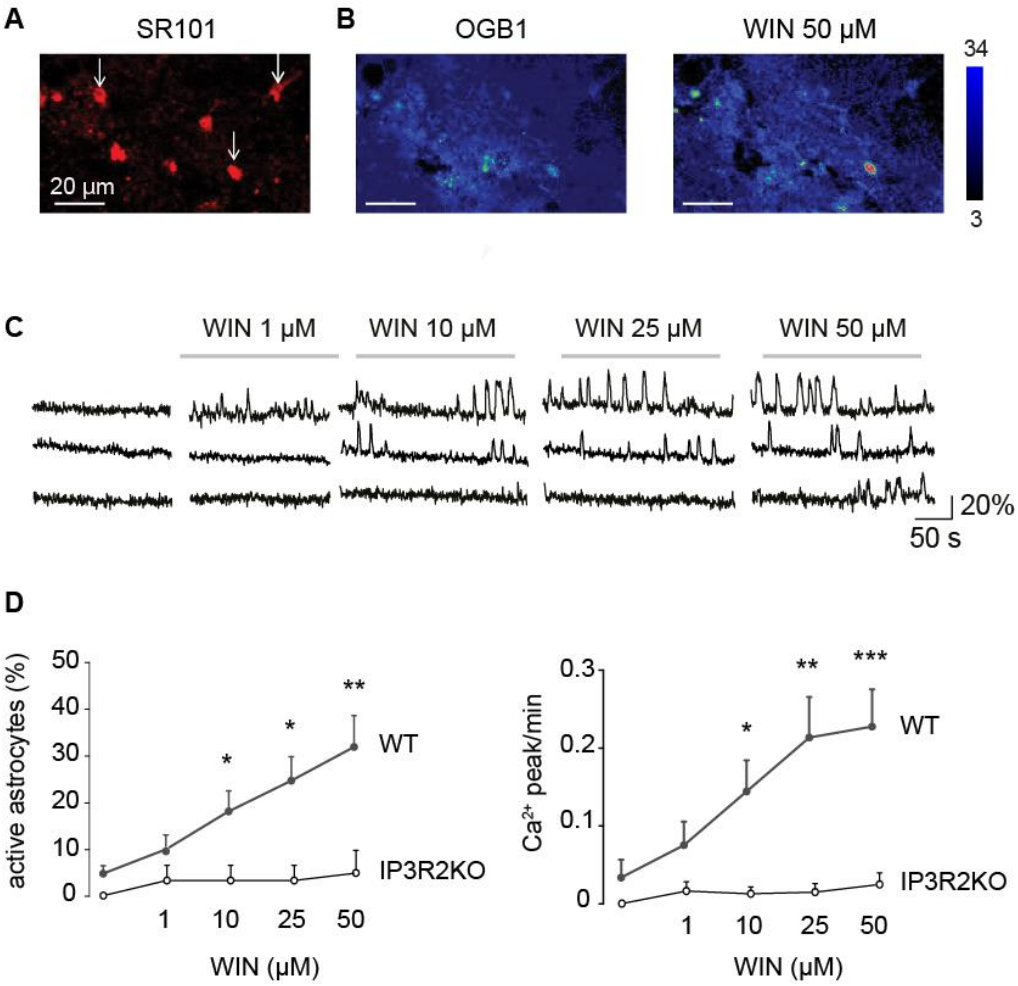

Figure 11: WIN induces dose dependent, somatic Ca^{2+} elevations in VTA astrocytes

(A) Red-fluorescent SR-101 selectively labels VTA astrocytes. **(B)** Pseudo-colored images of OGB1-loaded astrocytes before (left) and after (right) WIN 55,212-2 application (50 µM). **(C)** Fluorescence signal dynamics ($\Delta F/F_0$) from three responsive astrocytes (arrows in A) in basal conditions and after WIN applications at different concentrations (1µM, 10µM, 25µM, 50µM). Even if Ca^{2+} traces show significant bleaching this effect did not prevent our interpretation of the data. Indeed we could clearly discriminates Ca^{2+} peaks that even increased upon increasing concentration of the agonist. **(D)** Histograms showing the percentage of active astrocytes and the Ca^{2+} events frequency in different experimental conditions: WT (79 astrocytes, 4 slices, 4 animals); WIN 1 µM, for percentage p = 0.189, for frequency p= 0.234; WIN 10 µM, for percentage p = 0.030, for frequency p = 0.010; WIN 25 µM, for percentage p = 0.010, for frequency p = 0.002; WIN 50 µM, for percentage p = 0.008, for frequency p= 0.000008. IP_3R2KO (61 astrocytes, 4 slices, 2 mice) WIN 1 µM, for percentage p = 0.391, for frequency p = 0.181; WIN 10 µM, for percentage p = 0.391, for frequency p= 0.159; WIN 25 µM, for percentage p = 0.391, for frequency p= 0.181; WIN 50 µM, for percentage p = 0.391, for frequency p = 0.102.*p<0.05, **p<0.01, ***p<0.001; p values were calculated between basal and all the other conditions.

3.2 Heteroneuronal long-term potentiation of excitatory transmission in female mice and a heteroneuronal short-term potentation in male mice

To investigate whether astrocytes, possibly recruited by neuronal activity, exert an active role in the modulation of VTA synaptic transmission, we focused on the excitatory glutamatergic afferents to the VTA DA neurons. Among VTA inputs, glutamatergic afferents are a key component in the regulation of VTA DA neuron excitability and they play a central role in the transition from the tonic to the phasic, burst-firing activity [9]. To directly investigate whether neuron-astrocyte interplay impacts the VTA circuitry by modulating excitatory afferents, in horizontal slices from juvenile mice (P14-17) we performed whole cell patch-clamp recordings from pairs of VTA DA neurons located 70-120 μm apart (Fig. 12 A). In the *first* of the DA neurons we monitored the amplitude of the excitatory post-synaptic currents (EPSCs) evoked by stimulating the rostral glutamatergic afferents in the presence of the $GABA_A$ receptor antagonist picrotoxin (50 μM). Then, through injections of intracellular current pulses, we imposed to the *second* DA neuron a burst firing activity for 5 minutes (with an intraburst frequency of 20 Hz and an interburst frequency of 2 Hz) and evaluated whether following the imposed firing of this *second* DA neuron the EPSCs in the *first* DA neuron (i.e. the heteroneuronal synaptic activity) were modified or not (Fig. 12 A). We found that the DA neuron burst firing induces in the adjacent DA neuron a heteroneuronal potentiation of the excitatory synaptic transmission (Fig 12. B, C, D; female n = 11 , male n = 12). The plastic change in excitatory synapses occurs both in male and in female mice but the duration of the potentiation exhibited a remarkable gender difference. In male mice the potentiation was transient, lasting no more than three minutes after the burst firing induction, and was therefore defined as a heteroneuronal short term potentiation (bSTP). In contrast, in female mice this potentiation was higher in magnitude and long lasting, since it was still observed forty-five minutes after the burst firing (Fig. 12 D). We defined this change in EPSCs as burst-induced heteroneuronal long-term potentiation (bLTP). To understand whether the bLTP and the bSTP originate at pre or postsynaptic levels, we examined the paired-pulses ratio (PPR) before and after burst firing protocol.

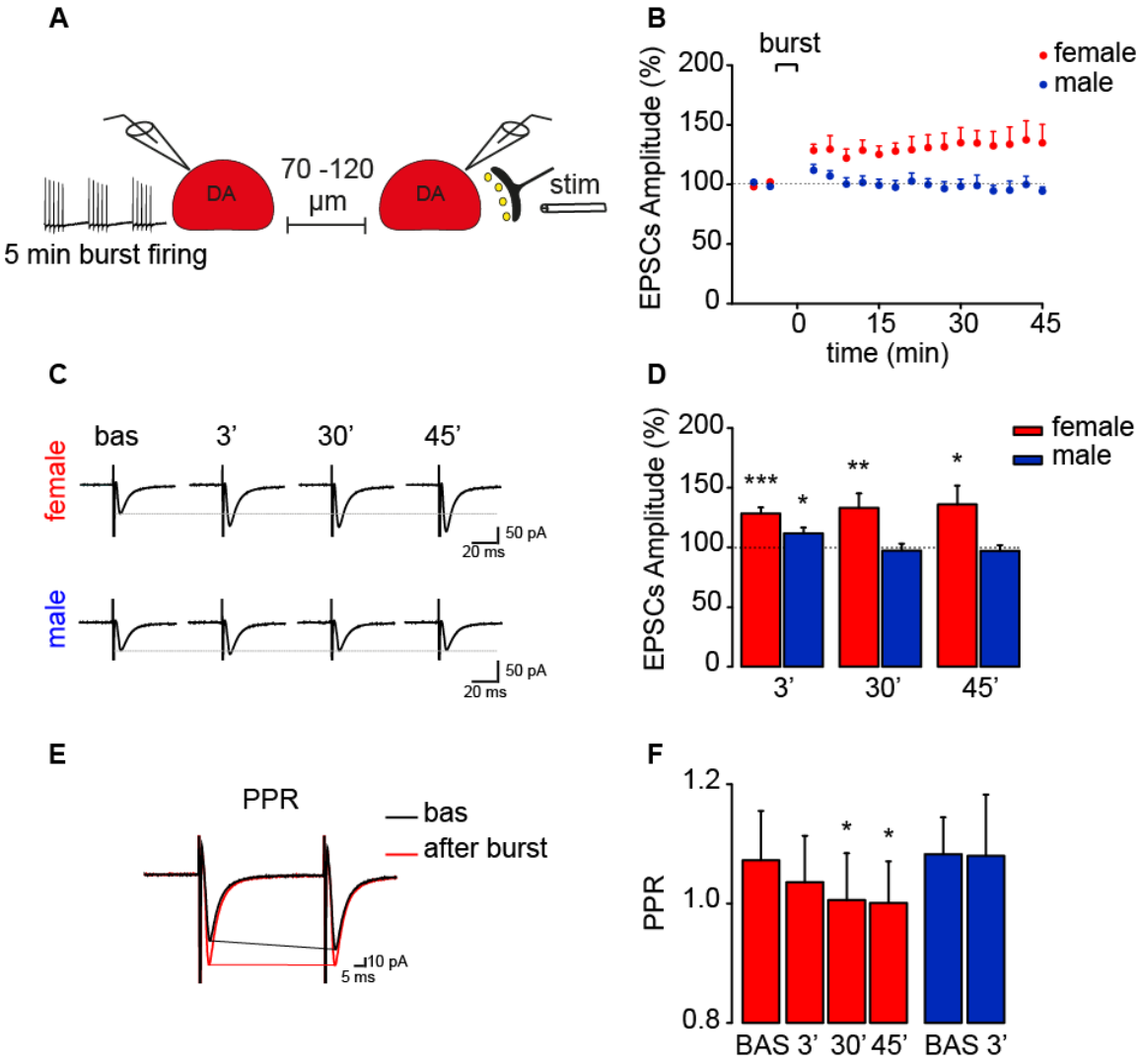

Figure 12: Burst firing induces bLTP of glutamatergic synaptic transmission onto DA neurons in the VTA of female mice

(A) Schematic representation of a pair recording experiment with the burst firing protocol, the two patch pipettes and the stimulating electrode. **(B)** Time course of the mean normalized EPSC amplitude (%) of female (red) and male (blue) mice before and after burst firing induction. In this and next figures, zero in the time course of the EPSC amplitude correspond to the end of burst firing. **(C)** Averaged EPSC amplitude (60 stimulations) of representative traces before (basal) and after three, thirty and forty-five minutes from the burst firing induction in female (upper traces) and male (lower traces) mice. **(D)** Quantification of the mean normalized EPSC amplitude after three, thirty and forty-five minutes from the burst firing induction in female (red) and male mice (blue) (female mice, three min: p= 0.0002, thirty min: p= 0.005, forty-five min: p= 0.044; male mice, three min: p= 0.037, thirty min: p= 0.664, forty-five min: p= 0.573). **(E)** Representative traces of paired-pulses ratio before (black trace) and after (red trace) the burst firing induction. **(F)** Mean paired-pulses ratio in basal condition and after three (p = 0.287), thirty (p = 0.037) and forty-five (p = 0.037) minutes from the burst firing induction in female mice (left) and in basal condition and after three (p = 0.96) minutes from the burst firing in male mice (right). *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and after burst firing induction.

Figure 12 E illustrates representative paired-pulse ratio traces of EPSCs recorded before (black line) and after (red line) the burst firing. In slices from male mice the PPR was not significantly reduced three minutes after burst firing, although at this time point the synaptic transmission was potentiated. However, in slices from female mice we observed a significant reduction of the PPR thirty and forty-five minutes after burst firing suggesting that bLTP is mediated by a presynaptic mechanism (i.e. an increase in the probability of release of glutamate from the excitatory terminals; Fig. 12 F). Taken together, these results suggest that the high frequency burst firing induced in a DA neuron translates into a potentiation of the excitatory synaptic transmission in adjacent DA neurons. While in male mice this potentiation is short-lasting (bSTP), in female mice the increase in the synaptic transmission efficacy is long-lasting (bLTP) and appears to be mediated by a presynaptic mechanism.

3.3 bLTP requires a switch from tonic to substained burst firing in DA neurons

VTA DA neurons are known to exibit a pacemaker conductance, i.e. a spontaneous, slow depolarizing membrane current that maintains their basal firing activity state [9]. Therefore, while during reward related events DA neurons exibit a phasic burst firing activity, in basal conditions they fire in a highly regular, slow, pacemaker or tonic-like pattern. To verify whether the potentiation of synaptic transmission that we observed in VTA was strictly dependent on a burst firing pattern, and not on tonic like activity, we imposed in a DA neuron of female mice VTA a tonic-like firing pattern (with a frequency of 2 Hz, for 5 minutes) (Fig. 13 A) instead of a burst firing pattern. We found that after the induction of a tonic-like activity in a DA neuron, the evoked EPSCs were not modified in adjacent DA neurons at any time point tested (Fig. 13 B, C; n = 8). This result suggests that the potentiation of excitatory synaptic transmission in VTA requires the switch from a tonic to a burst firing pattern in DA neurons. The bLTP is triggered by imposing a sustained burst firing lasting five minutes to a DA neuron.

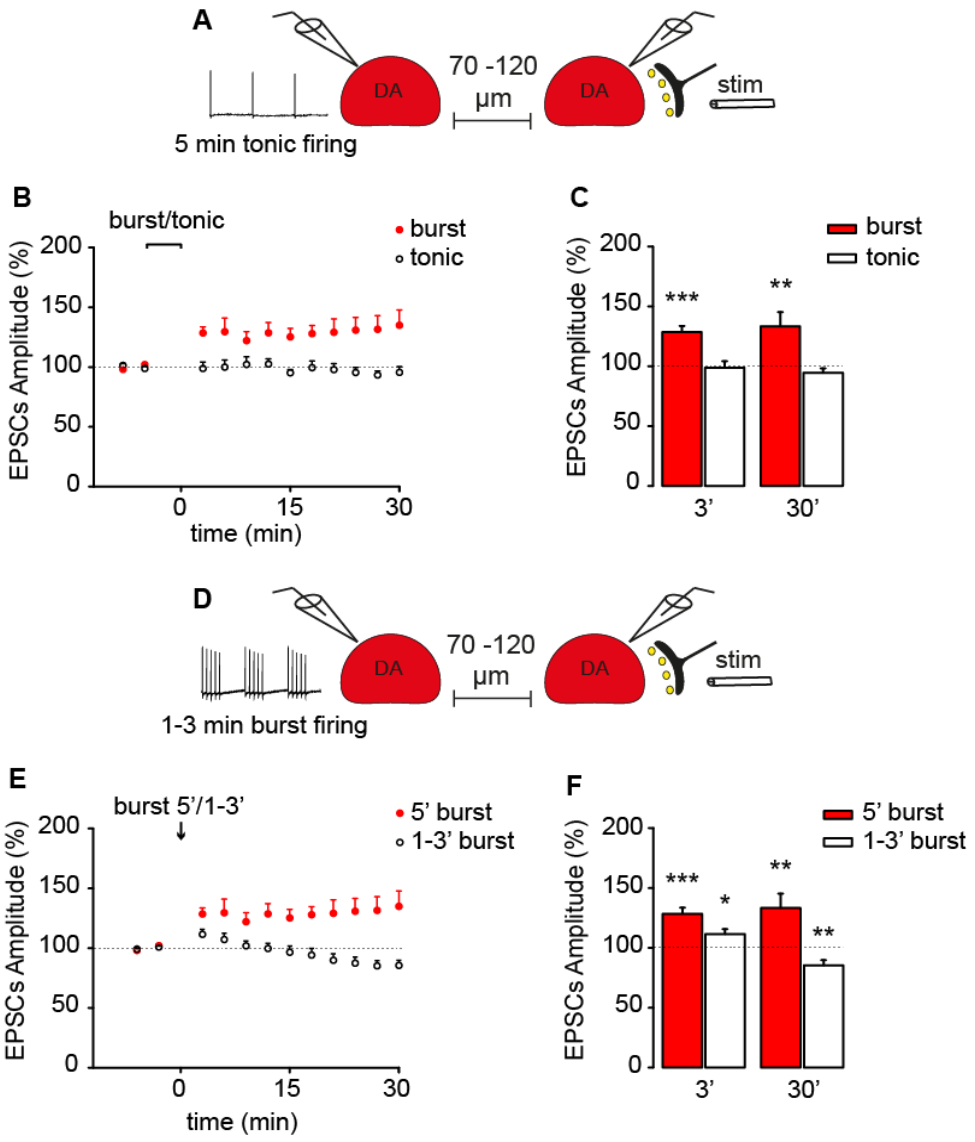

Figure 13: bLTP requires the switch from a tonic to a substained burst activity in dopaminergic neurons

(A) Schematic of the experimental approach with tonic firing. **(B)** Mean EPSCs amplitude (%) of female mice before and after burst (red) or tonic (white) firing mode. **(C)** Quantification of the mean EPSCs amplitude after three and thirty minutes from tonic (white) or burst (red) firing induction (tonic firing, three min: p = 0.826, thirthy min: p = 0.169). In this and other figures the p value for the potentiation of the EPSCs at the time point indicated after the five minute burst firing in control condition in female mice are those reported in fig. 12. **(D)** Schematic of the experimental approach with 1-3 minutes of burst firing. **(E)** Mean EPSCs amplitude (%) of female mice recorded after five (red) or one/three (white) minute lasting burst firing. **(F)** Quantification of the mean EPSCs amplitude after three and thirty minutes from 1-3 minutes (white) or 5 minutes (red) burst firing (1'-3' minute burst firing, three min: p = 0.0209, thirthy min: p = 0.0063). *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and after tonic or burst firing induction.

We then asked whether a shorter duration of the burst firing activity could be sufficient to induce changes in VTA synaptic transmission. To verify whether a shorter burst firing pattern is sufficient to induce changes in VTA synaptic transmission we reduced the burst firing from five to one/three minutes (Fig. 13 D). We found that after a short burst firing of a DA neuron in slices from female mice (n = 11) only a bSTP was observed (Fig. 13 E, F). This result suggests that bLTP is triggered only when a DA neuron displays a sustained burst firing of at least five minutes. On the other hand, bSTP can be induced with shorter burst firing patterns lasting 1 or 3 minutes. Furthermore, thirty minutes after this stimulation protocol, we observed a significant decrease, rather than an increase of the excitatory synaptic transmission. The mechanism of this decrease remains undefined. (Fig. 13 F).

3.4 Ca^{2+} elevations in astrocytes are crucial for bLTP generation

To investigate whether intracellular Ca^{2+} signaling in astrocytes plays a role in bLTP, we performed experiments on VTA slices from female mice lacking the IP_3 receptor type 2 (IP_3R2 KO mice). In these mice we found that the burst firing imposed to a DA neuron evokes in the other DA neuron the transient but not the long-lasting potentiation of synaptic transmission (Fig. 14 A, B, C; n = 12). These data suggest that bLTP, but not bSTP, depends on astrocytic intracellular Ca^{2+} elevations (Fig. 14 C). This set of experiments provides indirect evidence that astrocytic Ca^{2+} signaling is necessary for bLTP induction in female mice. To fully validate this hypothesis, direct evidence for burst-mediated Ca^{2+} elevations in astrocytes needs to be obtained.

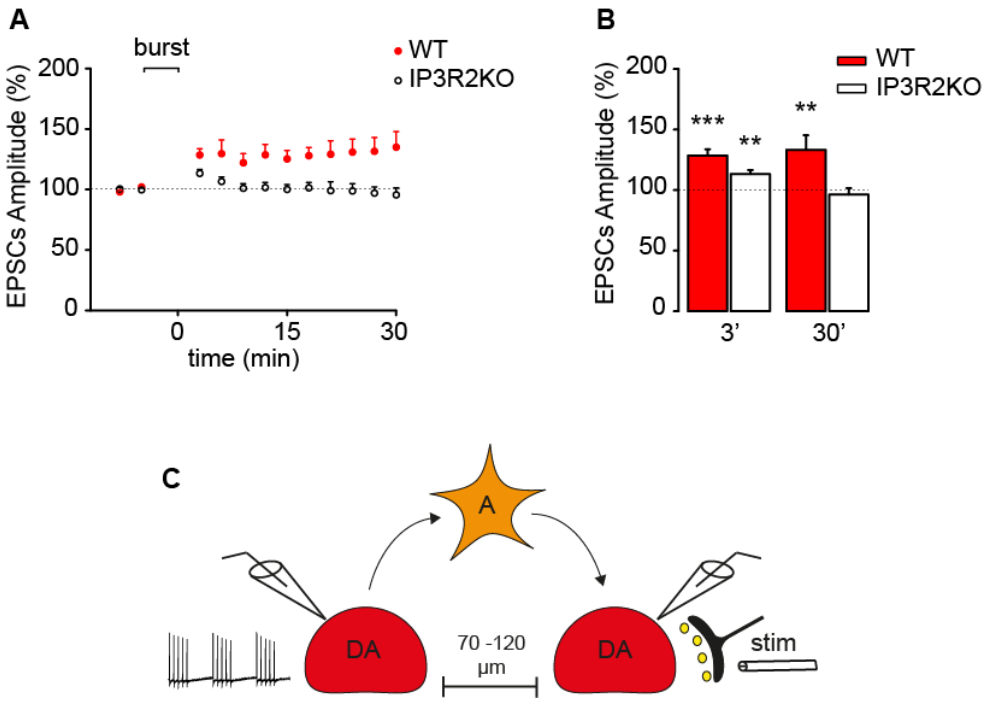

Figure 14: bLTP requires intracellular Ca^{2+} signals in astrocytes

(A) Mean EPSCs amplitude (%) of WT (red) and IP_3R2KO (white) female mice before (basal) and after burst firing induction. **(B)** Quantification of mean EPSCs amplitude (%) after three and thirty minutes from burst firing in WT (red) and IP_3R2KO (white) female mice (IP3R2, three min: $p = 0.002$, thirthy min: $p = 0.505$). **(C)** Schematic drawing showing that bLTP requires astrocytic Ca^{2+} signaling. *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and after burst firing induction.

3.5 bLTP requires CB1 and D2-type receptors signaling

Burst firing activity of DA neurons induces a somatodendritic release of both eCBs [61] and DA [171, 172]. These modulatory transmitters may function as a specific DA neuron signaling to astrocytes. Consistent with this hypothesis, in different brain regions eCBs and DA have been reported to induce intracellular Ca^{2+} elevations in astrocytes mediated by activation of CB1Rs [130, 134, 136, 153] and DA receptors [164], both linked to intracellular IP_3 signaling pathway [150, 168]. It is also noteworthy that D2-type receptors can oligomerize with other receptors, including CB1Rs [173]. To clarify whether eCBs and DA signaling participate in VTA bLTP through astrocyte CB1R and D2R activation, we performed experiments in the presence of the CB1R antagonist AM-251 or the D2-type receptor antagonist eticlopride hydrocloride (Fig. 15).

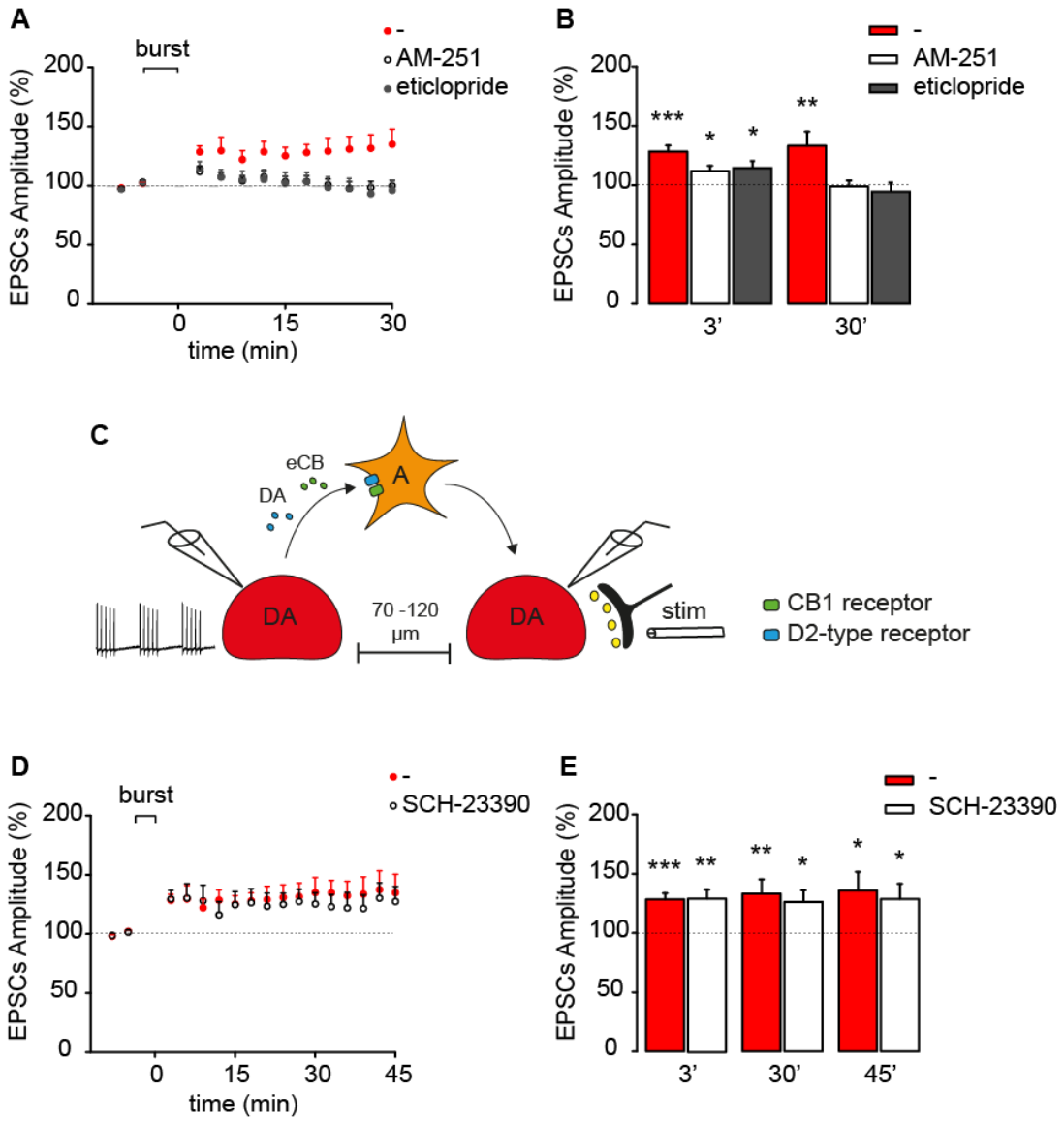

Figure 15: eCBs and DA signaling from the burst firing neuron is involved in bLTP

(A) Mean EPSCs amplitude (%) of female control group (red), AM-251 group (white) and eticlopride hydrocloride group (grey). **(B)** Quantification of the mean EPSCs amplitude (%) at three and thirty minutes after burst firing in control conditions (red), in the presence of AM-251 (white) and in the presence of eticlopride hydrochloride (grey) (AM-251, three min: $p = 0.044$, thirty min: $p = 0.853$; eticlopride hydrochloride, three min: $p = 0.034$, thirty min: $p = 0.495$). **(C)** Schematic drawing showing that bLTP depend on both CB1 and D2-type receptor signaling. **(D)** Mean EPSCs amplitude (%) of female control group (red) and SCH-23390 hydrocloride group (white). **(E)** Quantification of the mean EPSCs amplitude at three, thirty and forty-five minutes after burst firing in control conditions (red) and in the presence of SCH-23390 (white) (SCH-23390, three min: $p = 0.003$, thirty min: $p = 0.026$, forty-five min: $p = 0.05$) *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, p value was calculated between basal and after burst firing induction.

We found that bath perfusion with AM-251 (4 μM; n = 7) or eticlopride hydrochloride (1 μM; n = 10) prevents bLTP in slices from female mice, while a bSTP was still observed under these new conditions (Fig. 15 A, B), suggesting that bLTP, but not bSTP, requires both CB1 and D2-type receptor signaling. To investigate whether DA participates in the mechanism of bLTP by activating not only D2-type receptors but also D1-type receptors, we performed the same experimental procedure in the presence of the D1-type receptor antagonist SCH-23390 hydrochloride (10 μM; n = 10). After inhibiting D1-type receptors bLTP was still evoked by a sustained burst firing, rulling out the possibility that D1-type receptors participate in the mechanism of bLTP generation. Taken toghether, these results suggest that somatodendritic release of both eCBs and DA play a role in the induction of bLTP through activation of CB1R and D2 type receptors which are presumably expressed in VTA astrocytes.

3.6 bLTP requires mGluR1 but not NMDAR signalling

To gain further insights into the molecular mechanism at the basis of bLTP, we investigated the role of the glutamatergic NMDA receptor (NMDAR). Activation of this receptor is widely recognised to mediate plastic changes of excitatory synaptic transmission [174]. We found that in the presence of the NMDAR antagonist D-AP5 (50 μM), bLTP could still be evoked (Fig. 16 A, B; n = 11), indicating that the NMDAR is not involved. We then asked whether the release of astrocytic glutamate and the following activation of the presynaptic metabotropic glutamate receptor type 1 (mGluR1) may be involved, as previously observed in hippocampal circuitries [134, 136]. We found that in the presence of the mGluR1 receptor antagonist LY-367385 (100 μM), bLTP in VTA was abolished (Fig. 6 C, D; n = 12), suggesting that presynaptic mGluR1 is involved in bLTP. Notably, under these conditions the bSTP was still observed.

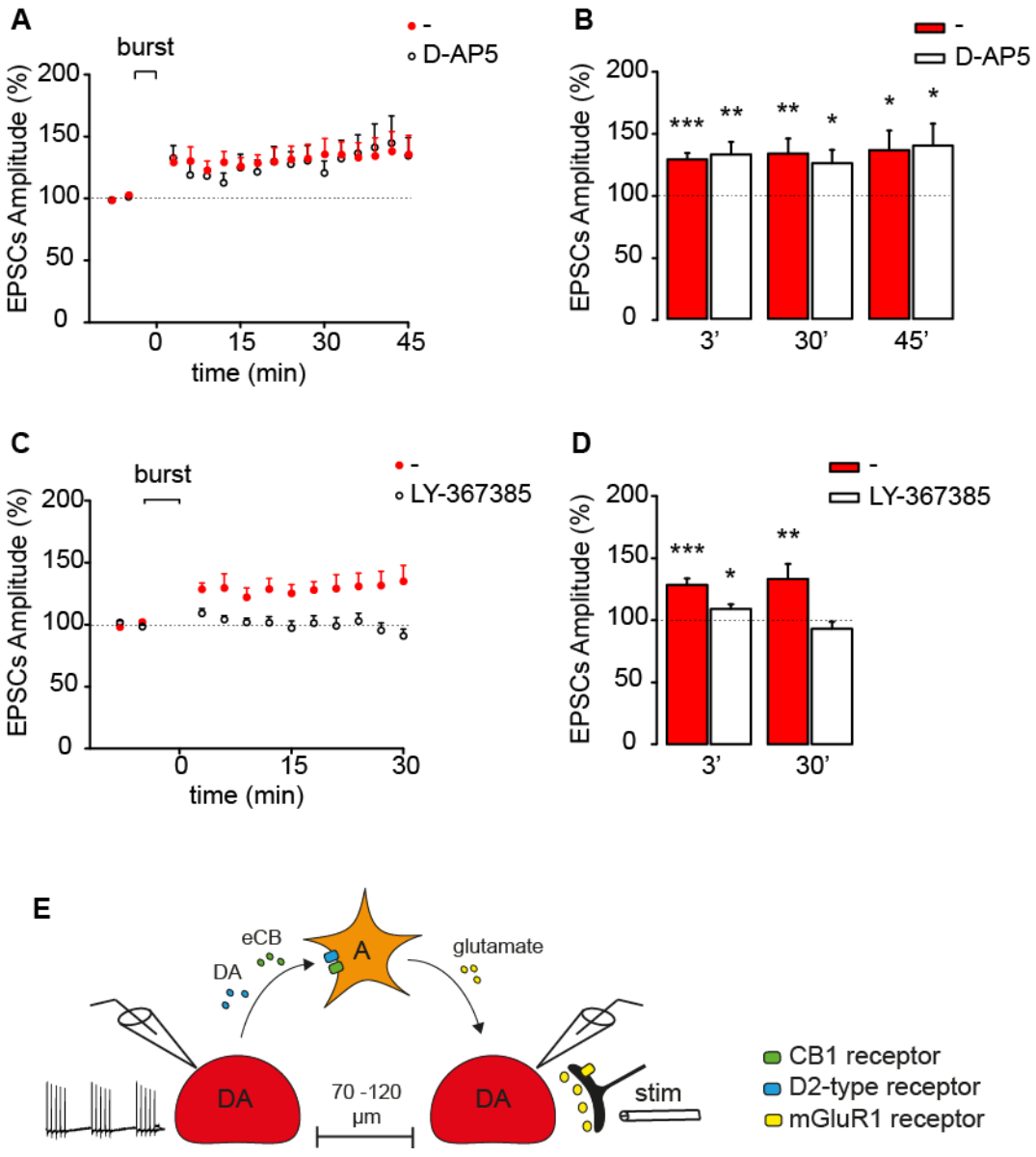

Figure 16: mGluR1 but not NMDA receptor is involved in bLTP

(A) Mean EPSCs amplitude (%) of female control group (red) and D-AP5 group (white). **(B)** Quantification of the mean EPSCs amplitude at three, thirty and forty-five minutes after burst firing in control conditions (red) and in the presence of D-AP5 (white) (D-AP5, three min: p = 0.009, thirty min: p = 0.038, forty-five min: p = 0.024). **(C)** Mean EPSCs amplitude (%) of female control group (red) and LY group (white). **(D)** Quantification of the mean EPSCs amplitude (%) at three and thirty minutes after burst firing in control conditions (red) and in the presence of LY367385 (white) (LY367385, three min: p = 0.040, thirty min: p = 0.249). **(E)** Schematic drawing showing that mGluR1 signaling is involved in bLTP. *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and after burst firing induction.

3.7 bLTP requires nitric oxide

In other brain regions (hippocampus and striatum) the release of glutamate from astrocytes and the activation of presynaptic mGluR1 leads to a transient potentiation of excitatory synaptic transmission [134, 153]. However, in VTA the release of glutamate from astrocytes and the activation of presynaptic mGluR1 after the burst firing of a DA neuron unexpectedly leads to a potentiation of excitatory sinaptic transmission that lasts at least 45 min. This long lasting effect suggests the contribution of other mediators that are released during the burst firing protocol. A likely candidate is nitric oxide (NO) that in other brain circuits has been shown to be produced and released by neurons into the extracellular space to contribute to long-term synaptic plasticity [175]. To investigate whether NO plays a role in the triggering of bLTP, we blocked the synthesis of NO with the bath-perfused NO synthase inhibitor L-NAME. In the presence of L-NAME (100 μM) we found that the burst firing in slices from female mice failed to evoke bLTP and evoked only a bSTP (Fig. 17 A, B; n = 7). Moreover, the cellular source of NO is most probably the burst-firing DA neuron because bLTP was also abolished when L-NAME was introduced through the patch pipette in the burst firing DA neuron (Fig. 17 A, B; n = 8). It is noteworthy that in both these experimental groups, the bSTP lasts more than three minutes after the burst firing induction being presents also at six minutes after the burst firing. Following these results, we propose that bLTP in the VTA circuitry of female mice is evoked by the following sequence of events. Firstly, the burst firing of a DA neuron induces the somatodendritic release of eCBs and DA. Secondly, the activation by these neuromodulators of the CB1 and D2-type receptors in astrocytes triggers in these glial cells IP_3R2-dependent Ca^{2+} elevations and subsequent glutamate release. Thirdly, the activation by astrocytic glutamate of presynaptic mGLU1Rs, coupled with NO production by the burst firing neuron leads to a sustained increase in glutamate release probability in excitatory afferents. Finally, in response to this series of events the excitatory transmission on an adjacent DA neuron exhibit a long-term potentiation (Fig 17, C).

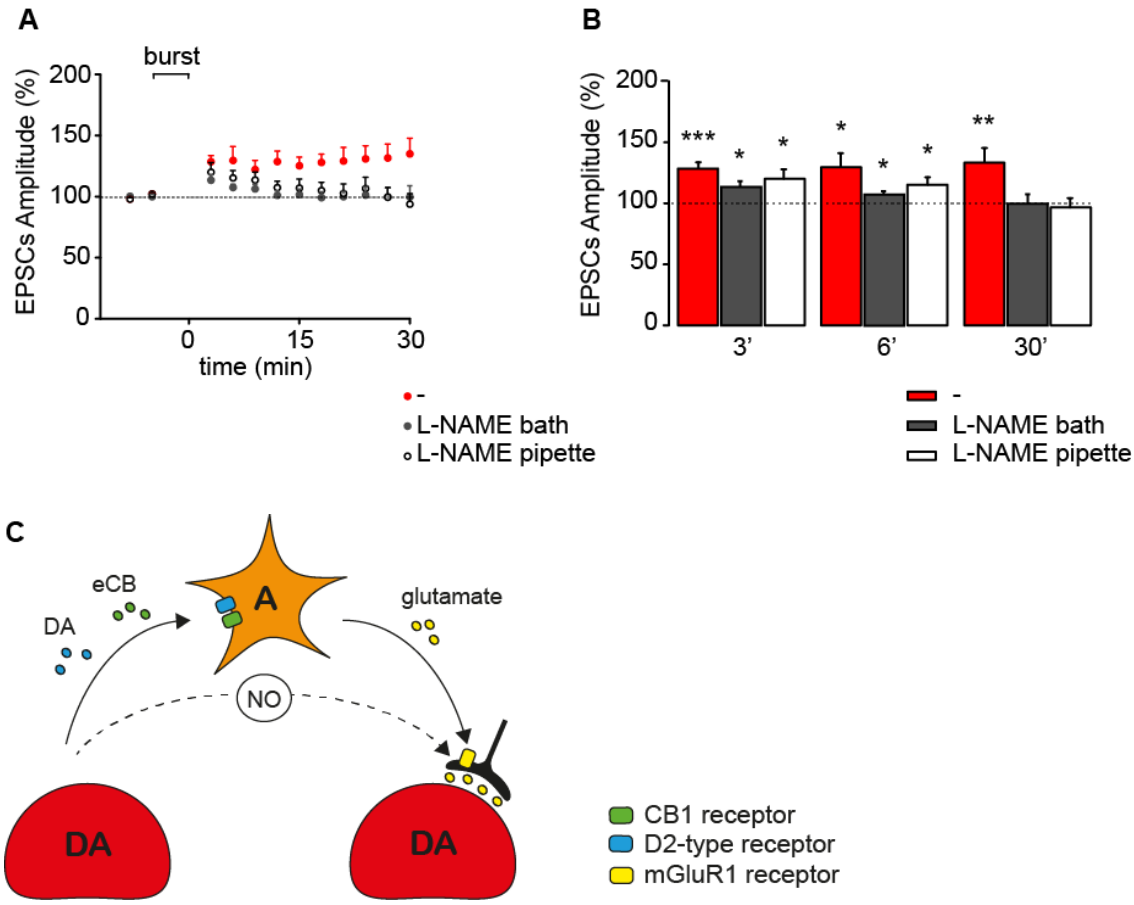

Figure 17: Nitric oxide (NO) from the burst firing neuron is involved in bLTP

(A) Mean EPSCs amplitude (%) of female control group (red), group in which L-NAME was bath perfused (grey) and group in which L-NAME was included in the patch pipette of the burst firing neuron (white). **(B)** Quantification of the mean EPSCs amplitude (%) at three, six and thirty minutes after burst firing in control conditions (red), in the presence of bath-perfused L-NAME (grey) and in the presence of L-NAME in the patch pipette (white) (L-NAME bath-perfused, three min: p = 0,030, six min: p = 0.041, thirty min: p = 0,687; L-NAME patch pipette, three min: p = 0,037, six min: p = 0.039, thirty min: p = 0,665). **(C)** Schematic drawing representing the signaling pathway involved in bLTP. *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and after burst firing induction.

3.8 VTA astrocytes respond with intracellular Ca^{2+} increases to the D2-type receptor agonist quinpirole

To validate our hypothesis, we investigated whether VTA astrocytes possess functional D2-type receptors that mediate Ca^{2+} elevations. To address this issue, we performed Ca^{2+} imaging experiments in VTA slices from young female and male mice (P14-P17).

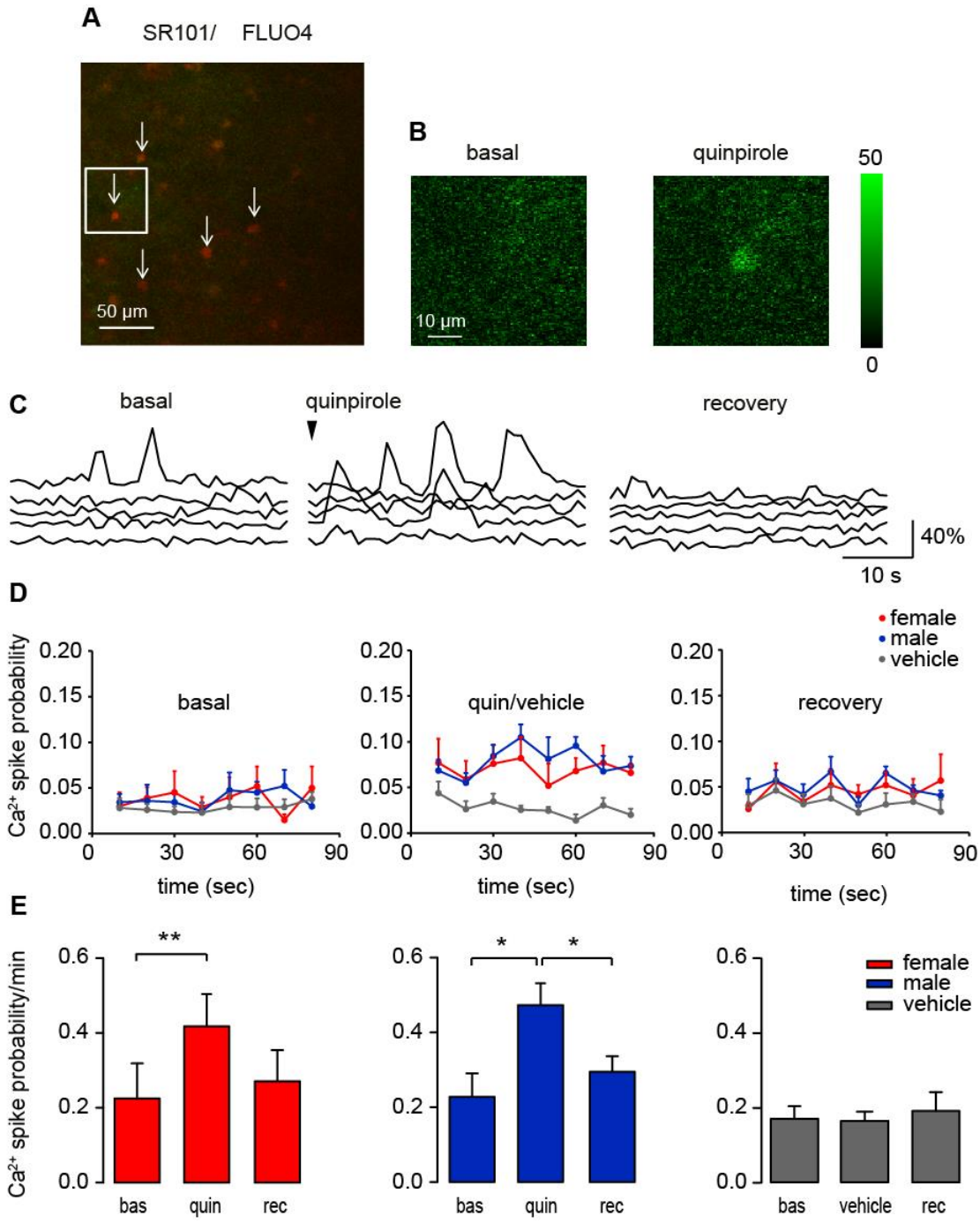

Figure 18: Quinpirole induces long-lasting somatic Ca^{2+} transient in VTA astrocytes

(A) Merged image of SR101 and FLUO-4. **(B)** Pseudo-colored images of a FLUO-4-loaded astrocyte acquired before (left) and after (right) quinpirole puff application. **(C)** Fluorescence signal dynamics ($\Delta F/F_0$) from five astrocytes (arrows in A) in basal conditions, after quinpirole puff application (arrowhead indicates the timing of quinpirole puff application) and during recovery **(D)** Summarizing time courses showing the mean Ca^{2+} spike probability in slices from female (red) (151 astrocytes, 6 slices, 3 animals) and male (blue) (171 astrocytes, 6 slices, 4 animals) mice challenged with quinpirole and in slices challenged with vehicle (106 astrocytes, 5 slices, 3 animals) (grey). **(E)** Summarizing bar charts showing the mean Ca^{2+} spike probability per minute in slices from female (red) and male (blue) mice challenged with quinpirole (female mice, quin vs bas: p = 0.003; male mice quin vs bas: p = 0.015, quin vs rec p = 0.024) and in slices challenged with vehicle (grey). *p<0.05, **p<0.01, ***p<0.001.

We loaded brain slices with the Ca^{2+} fluorescent indicator FLUO-4 and the selective astrocyte marker SR101 (Fig. 18 A, B). We then recorded fluorescence signal variations in astrocytic somata after pressure pulse applications to a pipette containing the D2 type receptor agonist quinpirole (3 mM). We found that quinpirole evoked in VTA astrocytes a sustained Ca^{2+} response with multiple Ca^{2+} peaks (Fig. 18 C, D, E) in slices from both female and male mice. These results suggest that VTA astrocytes express functional D2-type receptors, supporting our hypothesis of the cellular and molecular mechanism at the basis of bLTP. In addition, these results rule out the possibility that in slices from male mice the impairment of bLTP induction in response to the burst firing could be due to a lack of functional D2-type receptors in astrocytes from male mice.

3.9 Heteroneuronal short-term potentiation (bSTP) of synaptic transmission does not require D1-type receptors

We are currently focusing our attention on the mechanism involved in bSTP observed in young male mice. A plausible candidate is the D1-type receptor since this type of receptor is present on presynaptic afferents in VTA and its stimulation has been reported to increase glutamate release in VTA [81] (but see [82]).

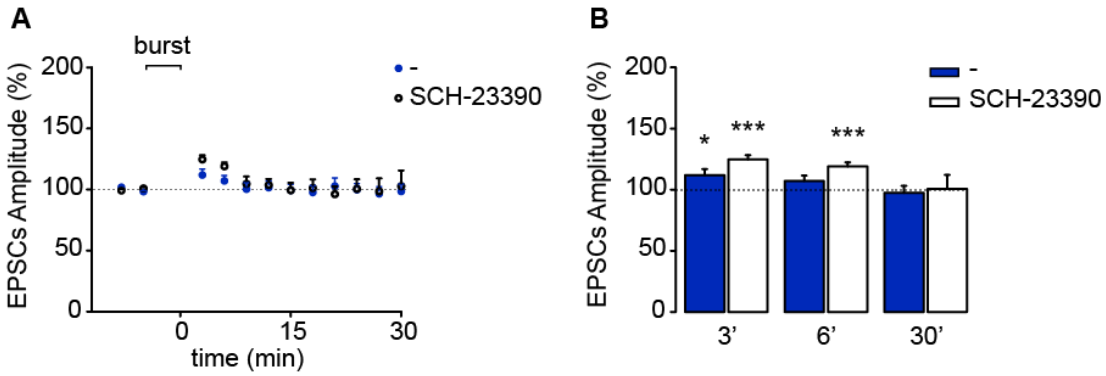

Figure 19: D1 type receptor signaling is not involved in bSTP

(A) Mean EPSCs amplitude (%) of control male mice group (blue) and SCH group (white). **(B)** Quantification of the mean EPSCs amplitude (%) at three, six and thirty minutes after burst firing in control conditions (blue) and in the presence of SCH-23390 (white) (control, three min: p = 0.037, six min: p = 0.146), thirty min: p = 0.664; SCH-23390, three min: p = 0.0001, six min: p = 0.0005, thirty min: p = 0.959). *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and after burst firing induction.

To address this issue, we performed experiments in slices from juvenile VTA male mice in the presence of the D1-type receptor antagonist SCH-23390 hydrochloride (10 μM). We found that, in the presence of SCH, the burst firing of a DA neuron still evoked a bSTP that lasted six minutes after the burst firing (Fig. 19, A, B; n = 9). These results suggest that D1-type receptors are not involved in the mechanism of bSTP triggering.

3.10 Burst firing of DA neurons induces bLTP in both female and male young adult mice

The burst firing in a DA neuron modulates the excitatory synaptic transmission in VTA slice preparations obtained from juvenile mice. Whether the same phenomenon is expressed in older animals is, however, unknown. To address this issue, we performed experiments in slices from young adult mice (P30-70). In young adult female mice, we found that burst firing still induced bLTP of excitatory synaptic transmission (Fig. 20 A, B; n = 7), but, unexpectedly, bLTP was also observed in slices from young adult male mice (Fig. 20 A, B; n = 7). This result is clearly in contrast with that obtained in younger male mice. To verify whether bLTP observed in both male and female young adult mice depends on intracellular Ca^{2+} signaling in astrocytes, we used again the IP_3R2KO mice. Surprisingly, we found that bLTP was still observed in slices from young adult female and male IP_3R2KO mice (Fig. 20 C, D, E; n = 10 female plus 10 male mice). However, even if bLTP in IP3R2KO mice was still significant at 30 minutes after burst firing induction (thirty min: p = 0.045), this potentiation was less consistent compared to the one observed in WT animals (n = 7 female plus 7 male mice, thirty min: p = 0.0004). This latest result suggests that the IP_3R2-dependent astrocyte Ca^{2+} signal is not necessary to evoke bLTP in older animals. This result opens a new perspective in the study of astrocytic modulation of synaptic transmission. One possibility is that, similarly to that described in hippocampus [176], other IP_3R subtypes different from the subtype 2, mediate astrocytes activation and thus the modulation of synaptic transmission. Whether the cellular and molecular mechanism of bLTP is the same in juvenile and older mice remains an open question.

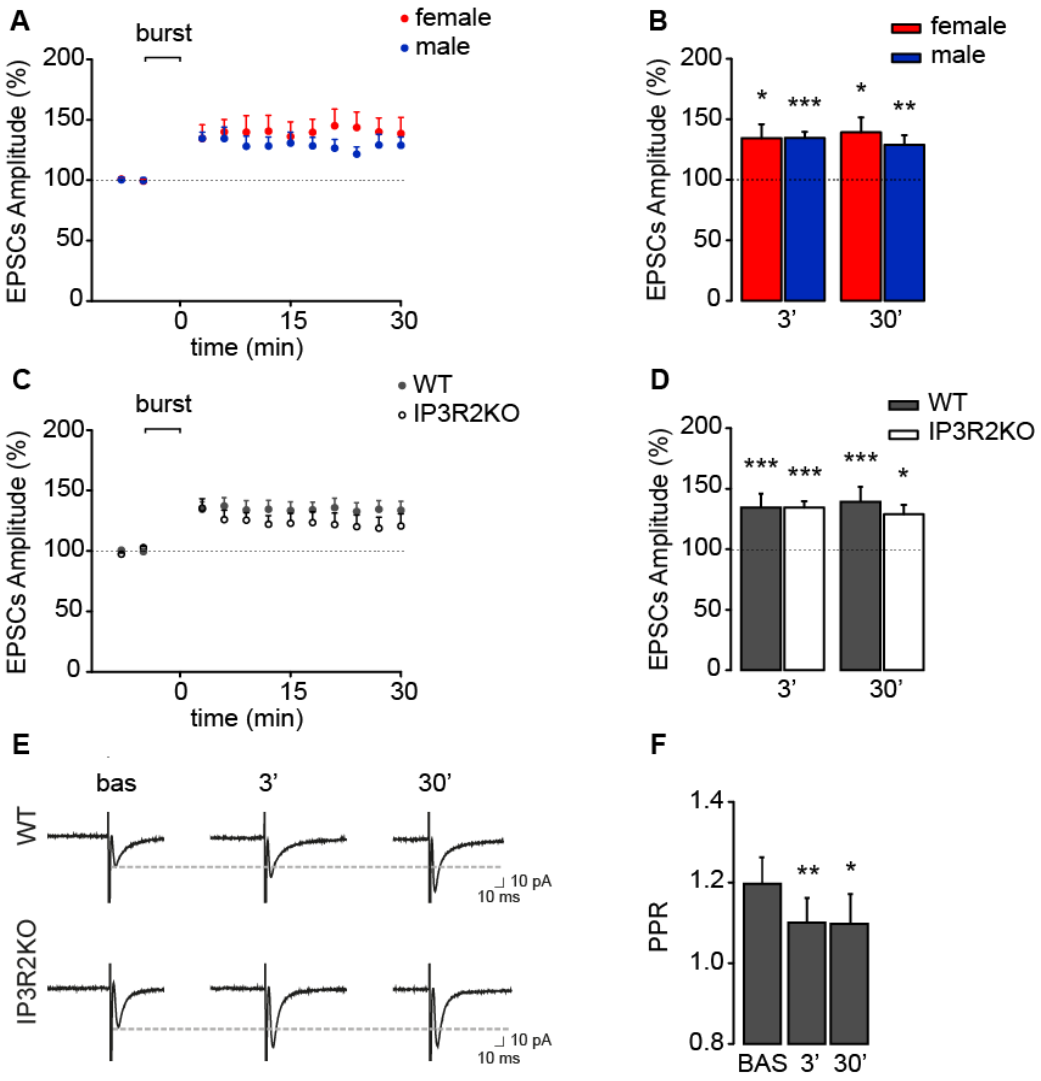

Figure 20: Burst firing induces bLTP of excitatory transmission in both male and female young adult mice

(A) Mean EPSCs amplitude (%) of female (red) and male (blue) young adult mice **(B)** Quantification of mean normalized EPSC amplitude after three and thirty minutes from the burst firing induction in young adult female (red) and male (blue) mice (female mice, three min: p= 0.024, thirty min: p= 0.019; male mice, three min: p= 0.0005, thirty min: p= 0.010). **(C)** Mean EPSCs amplitude (%) of WT (grey) and IP_3R2KO (white) **(D)** Quantification of mean normalized EPSC amplitude after three and thirty minutes from the burst firing induction in young adult WT (grey) and IP3R2KO (white) mice (WT, three min: p= 0.00007, thirty min: p= 0.0004; IP3R2KO, three min: p= 0.0002, thirty min: p= 0.045). **(E)** Averaged EPSCs amplitude (60 stimulations) of representative traces before (basal) and after three and thirty minutes of burst firing induction in young adult WT (upper traces) and IP_3R2KO (lower traces) mice. **(F)** Mean paired-pulses ratio in basal condition and after three (p = 0.003) and thirty (p = 0.045) minutes from the burst firing induction in young adult WT mice. *p<0.05, **p<0.01, ***p<0.001, p value was calculated between basal and all the other conditions.

We have initiated to investigate whether the synaptic site of action of bLTP is the same in young and older mice. We therefore examined paired-pulse ratio (PPR) before and after burst firing induction in slices obtained from young adult WT animals (both female and male) and we found a significant ppr reduction after burst firing induction (Fig. 20 F; n = 14), similarly to that described in juvenile mice. Taken together these results indicate that the burst firing induces bLTP of VTA synaptic transmission in both female and male young adult animals, that this bLTP in older mice is independent on the activation of IP_3R2 signaling pathway and that it seems to be mediated by a presynaptic mechanism.

4. Discussion and conclusions

We identified a novel form of plasticity of glutamatergic synaptic transmission in VTA circuits. We demonstrated that burst firing activity in DA neurons induces a potentiation of glutamatergic signaling to neighbouring DA neurons. This phenomenon exhibits in young animals a remarkable gender difference: in female mice it is long-lasting, whereas in male mice it is short-lasting. Furthermore, the long-lasting potentiation in female mice is absent in IP3R2KO mice, suggesting that it depends on intracellular Ca^{2+} elevations in astrocytes.

We focused our attention on the study of the astrocyte-mediated long-term modulation of VTA synaptic transmission in female mice. We propose that this form of long-term potentiation is generated by the following sequences of events. Firstly, the burst firing of a DA neuron induces the somatodendritic release of both DA and eCBs. Secondly, the activation by these neuromodulators of D2-type and CB1Rs in astrocytes triggers in these cells IP_3R2-dependent Ca^{2+} elevations. Thirdly, astrocytic Ca^{2+} elevations trigger glutamate release which activates the mGluR1 at pre-synaptic excitatory axon terminals. This action, coupled with the release of NO from the burst firing neuron, increases the probability of glutamate release in a long-term manner leading to a heteroneuronal long-term potentiation of the excitatory transmission to DA neurons. We showed that astrocytes have the potential to respond to exogenous applications of both the CB1R agonist WIN and the D2-type receptor agonist quinpirole suggesting the potential of astrocytes to respond with Ca^{2+} elevations to the DA neuron burst firing. Additional experiments are necessary to obtain a direct evidence of these events.

4.1 Burst-induced heteroneuronal long-term potentiation (bLTP) of synaptic transmission in juvenile female mice: role of astrocyte Ca^{2+} signaling

The present results provide evidence for an astrocyte-mediated long-term change of synaptic transmission onto DA neurons in the VTA of young female mice. Specifically, we found that a prolonged burst firing activity in a single DA

neuron can induce a long-lasting potentiation of glutamatergic transmission onto an adjacent DA neuron (bLTP). The burst firing mode in DA neurons is known to be physiologically evoked by the presentation of behaviorally relevant stimuli [9-11, 50]. This is not the first time that a burst firing induction is used in ex vivo preparations to mimic the switch to the modality of the firing discharge that is observed in DA neurons during behavioral relevant events. In a recent work it was found that a sustained burst firing pattern, similar to the one used in the present study, elicited in the VTA DA neuron - in which the burst firing was triggered - a potentiation of the inhibitory postsynaptic currents mediated by G-protein-coupled inwardly rectifying potassium (GIRK) channels. In contrast, a sustained tonic firing elicited a depression of these currents [177]. In our experiments, we found that the tonic and the burst firing exert different modulatory effects on VTA synaptic transmission, demonstrating that the switch to a burst firing mode drives plastic changes of the excitatory synaptic transmission to DA neurons.

The switch from tonic to burst firing activity in DA neurons is under the control of excitatory projections originating from different brain regions [9, 12, 13] and appears to specifically depend on NMDAR activation [9, 50]. Given the crucial role of glutamatergic inputs in DA neuronal circuit-dependent behavior, the plasticity at the excitatory synapses onto DA neurons have been extensively investigated. As already mentioned, plastic changes of excitatory synapses in the VTA can be triggered by high frequency stimulation of excitatory afferents and spike-timing dependent protocols in both *in vitro* [14] and *in vivo* during the acquisition phase of natural cue-reward learning, exposure to drugs of abuse and optogenetic stimulation of VTA DA neurons [84, 86-90].

In our study, we demonstrated that the switch to a reward-related burst firing mode can influence the strength of glutamatergic synaptic transmission onto adjacent neurons by an astrocyte recruitment mediated by D2-type and CB1 receptor signaling. Modulation of synaptic transmission by astrocytes has been observed in other brain regions, such as hippocampus, [129, 133, 134, 136, 140, 147], neocortex [130] and basal ganglia [153]. The modulation by astrocytes of VTA excitatory circuits gives further support to the active role of these glial cells in the modulation of the lateral synaptic transmission as a general mechanism in brain circuits. According to this view, the output of a

specific neuronal population composing a given brain circuit depends on the integrated activity of both neurons and astrocytes. In the case of the lateral posterior excitatory VTA circuit here investigated, we proposed that the potentiation of excitatory transmission mediated by astrocytes induces an increased depolarization of the postsynaptic neuronal membrane that by favouring the removal of the Mg^{2+} block of the NMDAR, enhances the activation of these receptors. Ultimately, the burst firing activity in DA neurons is enhanced leading to a higher DA release in the target structure lateral NAc shell. *In vivo* experiments are necessary to validate the astrocyte modulation of excitatory glutamatergic transmission in VTA circuits that we observed in *ex vivo* slice preparations. Although we mimic in our slices the burst firing pattern observed *in vivo* during reward-related events, the relevance of burst firing duration (3 vs 5 minutes burst firing) in bLTP induction needs to be clarified. There are no evidence in literature of a different burst firing duration *in vivo* that can explain different behaviorally relevant responses to rewarding stimuli. However, *ex vivo* experiments, such as those in brain slices, can hardly reproduce the complex *in vivo* situation, where a large fraction of dopaminergic neurons are coherently activated in a burst firing mode. In fact, it could be possible that the simultaneously activation of a burst firing mode in a large fraction of dopaminergic cells can induce bLTP generation also after a burst firing mode of short durations. To verify this issue it could be important to develop an approach in brain slices in which burst firing discharges are induced in a number of DA neurons, rather than in a single DA neuron only, thereby mimicking the activity of VTA DA neuron circuits during reward. To this aim, we can take advantage of viral injection approaches to selectively express the light-gated channel channelrhodopsin-2 (ChR2) in VTA DA neurons. Through this approach and a suitable protocol of optogenetic light pulse stimulation, we can study whether a burst firing of different durations that can be evoked in a number of ChR2-expressing DA neurons induces heteroneuronal plastic changes of excitatory glutamatergic transmission.

4.2 CB1 and D2-type receptor signaling

By using pharmacological tools, we found that bLTP is mediated by both eCB and DA signaling since it is impaired in the presence of either the CB1R antagonist AM-251 or the D2-type receptor antagonist eticlopride hydrocloride. DA neurons release both eCBs and dopamine at the somatodendritic level [60, 61, 70, 71]. It is noteworthy that eCBs act on CB1Rs at excitatory presynaptic terminals to decrease glutamate release probability [65, 66]. Similarly, the activation of the D2-type autoreceptors in DA neurons is a well-known mechanism of feedback autoinhibition to reduce DA neuronal excitability [76, 77]. In addition, activation of presynaptic D2-type receptors inhibits excitatory transmission onto VTA DA neurons [78]. The well-established inhibitory actions of neuronal CB1 and D2-type receptors in VTA suggest that receptors with a neuronal localization are not the mediators of the bLTP. The fact that eCBs and DA can exert opposite effects on neuronal excitation and synaptic transmission, reveals the high complexity of the VTA circuitry and can have relevant implications for VTA network functions both in the reward mechanism and in addition. It is noteworthy that in the globus pallidus, the cannabinoid-induced suppression of synaptic transmission switches to potentiation when dopaminergic tone is increased upon co-activation of CB1 and D2 receptors [178]. However, in the VTA this scenario is unlikely to occur because in this brain region activation of D2-type receptors favours the depolarization-induced release of eCBs and the subsequent depolarization-induced suppression of excitation [65]. In contrast, we propose that CB1 and D2-type receptors present in astrocytes are the most plausible mediators of bLTP. In support of this hypothesis, low but functionally relevant CB1Rs have been found in astrocytes from different brain regions [155]. Moreover, astrocytes respond with Ca^{2+} elevations to the activation of CB1R and this astrocyte activation leads to a modulation of synaptic transmission in different brain regions [130, 134, 136, 153]. Furthermore, functional D2-type receptors have been described in astrocytes [164, 168]. Accordingly, we have shown that astrocytes from the VTA respond with Ca^{2+} elevations to CB1 and D2-type receptor agonists. To validate the hypothesis of an astrocyte locus for the CB1 and D2-type receptors mediating bLTP, we will perform new experiments to determine whether the

Ca^{2+} activity in VTA astrocytes is increased in response to a sustained burst firing of a DA neuron. If this were the case, we will use the CB1 and D2-type receptor antagonist AM251 and eticlopride hydrocloride, respectively, to determine whether the inhibition of CB1 and D2 receptors affects the burst-induced astrocyte Ca^{2+} response. Moreover, we will use a viral injection approach to generate knockout mice for CB1 and D2R specifically in VTA astrocytes to further validate the role played by CB1 and D2R from VTA astrocytes in bLTP. The impairment of bLTP observed with AM251 or with eticlopride hydrocloride suggests that the activation of both receptors may be necessary to induce adequate Ca^{2+} elevations in astrocyte in response to the neuronal burst firing. In such a case, a functional synergy between the two receptor activation or the formation of CB1/D2R heterodimers could explain the necessity of both types of receptors for bLTP generation. It has been described that D2Rs can heterodimerize with different receptors, including the CB1R [173], suggesting that in astrocytes CB1 and D2Rs can, indeed, form heterodimers that functionally cooperate to elicit a Ca^{2+} increase in response to the neuronal burst firing. To provide direct evidence for the formation of CB1R/D2R heterodimers in astrocytes, we will perform immunoprecipitation studies as well as immunogold electron microscope (EM) experiments with different size gold particles that will allow to evaluate a hypothetical close localization of these receptors in the astrocytic membrane. The formation of heterodimers between receptors for different neurotransmitters or neuromodulators is a characteristic of astrocyte physiology that has been recently addressed in our group. In particular, the structural and functional association of the $GABA_BR2$ with the somatostatin type 4 receptor leads to a different astrocyte response during the activation of different types of interneurons (somatostatin-releasing interneurons and parvalbumin interneurons), in terms of astrocyte response sensitivity and plasticity to subsequent interneuron activations [179]. Interestingly, interfering with these functional heterodimers abolishes the differences observed in the astrocytes response to the two interneuron classes. These results suggest that the formation of functional heterodimers is a characteristic of astrocyte physiology that can significantly shape the astrocyte response to neuronal activity.

4.3 mGluR1 involvement in bLTP and cellular localization

In different brain regions the gliotransmitter glutamate modulates synaptic transmission through activation of presynaptic mGluR1 or NMDARs [129, 130, 133, 136, 153]. In our model of astrocyte modulation of synaptic transmission in VTA, we observed that bLTP requires the activation of mGluRs, but not of NMDARs. We also measured a reduction in the PPR which suggests a presynaptic mGluR location. However, no structural evidence exists for the presence of the mGluR1 at excitatory axon terminals in the VTA and a postsynaptic expression has been rather reported. In contrast to the mGluR1-mediated potentiation of excitatory transmission to DA neurons that we observed, activation of post-synaptic mGluR1s leads to a long-term depression (LTD) of the excitatory transmission due to a switch of the subunit composition of the AMPARs, in particular a switch from a GluA2-lacking, high conductance AMPAR to a GluA2-containing, low conductance AMPAR [180]. Interestingly, during the first postnatal week in mice, excitatory transmission is mainly mediated by GluA2-lacking AMPARs and the activation during postnatal development of the mGluR1 leads to an enhanced insertion of GluA2-containing AMPARs [181]. In addition, the LTP-like potentiation of excitatory transmission evoked by drugs of abuse, due to an increase in the insertion of GluA2-lacking AMPARs in the excitatory synapses, can be reverted by the mGluR-LTD [91]. We here propose that functional presynaptic mGluR1s are expressed at excitatory axon terminals to DA neurons and that, in response to astrocytic glutamate, their activation leads to an increase in the neurotransmitter release probability and, consequently, to an increase in the excitatory transmission. This scheme of opposite effects of postsynaptic versus presynaptic mGluRs activation (depression versus potentiation of synaptic transmission, respectively) [134, 136, 182] has been previously described in the hippocampus and we now propose that it also occurs in the VTA. New EM experiments are needed to corroborate our functional data with direct evidence of the mGluR1 expression at presynaptic glutamatergic axon terminals onto VTA DA neurons.

4.4 Nitric oxide role in the potentiation of synaptic transmission

In hippocampus and striatum the release of eCBs from neuronal dendrites evokes a transient Ca^{2+} response in astrocytes that elicits a transient potentiation of the excitatory transmission. In VTA circuits, we observed a long lasting, rather than a transient, potentiation of glutamatergic transmission. This event is also dependent on astrocyte Ca^{2+} response. Such a long term change might be due to the cooperation of eCBs and DA with other neuromodulators specifically released during the sustained burst firing of DA neurons. One plausible candidate is nitric oxide (NO) which is released by neurons following NMDAR-mediated Ca^{2+} elevations and is known to mediate long term changes in synaptic transmission [183]. In LTP forms generated by high frequency stimulation of afferents, NO is, indeed, a crucial intercellular messenger which controls the strength of excitatory and inhibitory synapses by modulating the probability of neurotransmitter release [175]. Among the various neurotransmitters and neuromodulators of the VTA, NO has been the subject of great interest [184], and, most interestingly, it has been reported to be involved in the rewarding effect of the drugs of abuse [185]. As a further support for a role of NO in bLTP in the VTA, studies in hippocampus demonstrated that the increase in release probability elicited by astrocyte activation is a transient event and that a concomitant NO production evoked by a mild depolarization of the postsynaptic cell, allows the transient increase in release probability to become a long-lasting phenomenon [136]. A similar mechanism appears to be involved in bLTP generation in VTA circuits. By including the NO synthase (NOS) inhibitor L-NAME in the patch pipette, we could demonstrate that the source of NO is the burst firing DA neuron. Although the expression of the NOS has been demonstrated in DA neurons from the VTA [186], to our knowledge this is the first demonstration that a burst firing pattern in a DA neuron elicits NO release.

4.5 Differential modulation of VTA synaptic transmission in male and female mice

It is widely recognized that females are more suscetible than males in the use of drugs of abuse and in the development of addiction [187, 188]. As a matter of fact, while the basic neural system involved in positive and negative reinforcement are similar in males and females, sex differences are present in how these neural system are organized, activated and connected to the rest of the brain, and these are postulated to underlie sex differences in the path of addiction [188]. Converging evidence indicates that also the structure and the functioning of the VTA dopaminergic system are intrinsically different in males and females. Gender differences may be driven by sex differences in the hormonal enviroment during development or in adulthood. Furthermore, also during the early life some gender differences have been observed. For example, stress and stress hormones like glucocorticoids are important factors which interact with the VTA dopaminergic system in order to achieve behavioral adaptation. Male-female differences have been found not only during adulthood, but also in early life when neurobiological programming by stress or glucocorticoid exposure differentialy impacts dopaminergic developmental trajectiories in male and female brain [189]. In line with this early gender difference, we observed that the burst firing-induced potentiation of synaptic transmission is different in male and female juvenile animals. In contrast to the bLTP observed in female mice, in juvenile male mice the burst firing induces a heteroneuronal short-term potentiation of synaptic transmission (bSTP). The reasons for this difference are unclear. Regarding the molecular mechanism mediating this potentiation of synaptic transmission, we observed that bSTP seems not to be mediated by astrocyte Ca^{2+} signaling, because in IP_3R2KO female mice the bLTP was impaired, but a bSTP similar to that observed in male mice could still elicited. Moreover, bSTP is not mediated by CB1R, D2-type receptor or mGluR1 because in the presence of antagonists for these receptors the burst firing failed to elicit an bLTP, but it elicited a STP lasting three minutes. However, bSTP seems strictly related to the burst firing activity because it is absent when we impose a tonic-like firing pattern in DA neurons. For this reason, we investigated other signaling pathways that could be

activated by the burst firing protocol and that could elicit this event. Since D1-type receptors for dopamine are present on excitatory presynaptic terminals [80] and their activation increases glutamate levels in the VTA in vivo [79] and glutamate release in *ex vivo* slice preparation [81] (but see [82]), we have speculated that dopamine release during the burst firing protocol could activate D1-type receptors to trigger a bSTP. However, in the presence of the D1-type antagonist SCH23390, the bSTP is not impaired and its amplitude and durations are even higher. This result is in agrement with the proposed inhibition of glutamate release via presynaptic D1-type receptors [82]. New experiments are needed to clarify the molecular mechanism of the bSTP.

Finally, the reasons for the lack of bLTP in slices from young male mice remain unknown. Although the possibilities are numerous, to explain the gender difference that we observed, we will address in future experiments two different hypotheses. Firstly, we will investigate whether the expression or function of the presynaptic mGluR1 is defective in male mice. EM experiments will be important to address this issue. Secondly, we will clarify whether astrocyte Ca^{2+} signaling in response to the burst-firing protocol is impaired due to a deficit in CB1R and/or D2-type receptor signaling. In our experiments with the D2-type receptor agonist quinpirole, we observed, however, similar astrocyte Ca^{2+} responses in VTA slices from female and male mice. This result rules out the possibility that astrocytes from male mice lack functional D2-type receptors. Whether astrocytes from male mice possess functional CB1R and/or functional CB1R/D2R heterodimers still needs to be investigated.

4.6 Long-term potentiation in young adult mice

At first glance, the gender difference in the potentiation of excitatory transmission in young mice that I described in this thesis could be linked to the higher predisposition of females with respect to males to drugs of abuse. Surprisingly, however, in young adult mice, we found that the burst firing in DA neurons induces a long-term potentiation of synaptic transmission not only in female, but also in male mice. It thus seems that the gender difference observed in juvenile mice may depend on a developmentally regulated circuit mechanism in the VTA of male mice that confers to astrocytes their modulatory

role. To sustain this hypothesis, it is necessary, however, to demostrate that the bLTP observed in young adult mice is mediated by the same cellular and molecular mechanism that operates in juvenile mice. In young adult IP_3R2KO mice a bLTP is still significantly present after 30 min of burst firing protocol, even though this potentiation is statistically less consistent in IP_3R2KO compared to wild type mice. This apparently conflicting result does not preclude the possibility that astrocytes, and astrocytic Ca^{2+} signaling in particular, play a key role in the mechanism of bLTP in young adult mice. In line with this, in *in vivo* and in brain slices preparations from young adult IP_3R2KO mice, spontaneous and G-protein-coupled receptor activated Ca^{2+} events are relatively preserved at the microdomains, whereas Ca^{2+} elevations at the level of the soma are highly compromised [190]. These observations suggest that transmembrane Ca^{2+} fluxes substantially contribute to a detectable proportion of Ca^{2+} microdomains activity. In hippocampus from young adult mice, a standard high-frequency stimulation (HFS) protocol induces a LTP of the Schaffer collateral (SC)–CA1 pyramidal cell synapses that is mediated by a Ca^{2+}-dependent release of D-Serine from astrocytes [140]. Interestingly, this astrocyte-dependent LTP is not impaired in IP_3R2KO mice and it can be reduced by interfering with Ca^{2+} fluxes in astrocytes through the TRPA1 channel [191]. In line with this, our results could be explained if the astrocyte Ca^{2+} signaling that is necessary for triggering bLTP would require an amplification of the Ca^{2+} rise through an activation of IP_3R2 in juvenile mice that is not neccessary in young adult mice. Alternatively, although IP_3R2 is widely believed to be the main functional IP_3R in astrocytes, three IP_3R subtypes (1, 2 and 3) have been identified in vertebrates [170]. Then, we can speculate that in young-adult mice an IP_3R distinct from IP_3R2 binds to IP_3 to induce a Ca^{2+} release from the endoplasmic reticulum (ER) in astrocytes which triggers the gliotransmitter release that elicits bLTP. In line with this, activation of the IP_3R3 subtype has been reported to contribute to astrocytic Ca^{2+} signalling. Furthermore, the astrocyte-mediated HFS-evoked LTP was blocked by heparine, an IP_3R inhibitor, in astrocytes [176]. Finally, in future experiments we will specifically investigate whether the bLTP observed in juvenile and young adult mice shares the same cellular and molecular mechanism. Preliminary experiments revealed that, as observed in juvenile mice, the bLTP in young

adult mice is impaired in the presence of the D2R specific inhibitor L741,626 (data not shown).

4.7 Functional implications of the bLTP

During drug addiction, features such as compulsive seeking, loss of self-control and propensity to relapse lead to high social and health care costs. The acute actions of drugs of abuse cannot explain the development of addictive behaviours. To understand how addiction is generated, we must understand the specific traces left in brain circuits by drug experience. Thanks to the introduction of techniques such as transgenic approaches and optogenetics, our knowledge of these events has greatly improved over the last decade. We now know that the natural cue-reward learning is associated with a transient increase in the excitatory synaptic strength onto VTA DA neurons, due to an increase in the postsynaptic AMPAR function [84] and that a similar increase of AMPAR function underlines the long-lasting potentiation of excitatory transmission onto VTA DA neurons following the use of different addictive drugs [15]. It is noteworthy that the impairment of this potentiation does not affect short-term behavioral effects of drugs (for example, conditioned place preference (CPP) but interferes with later drug-associated behaviors, such as the reinstatement of CCP [192]. All toghether, these results suggest that modifications of excitatory transmission in VTA DA neurons have important role in shaping behavioral adaptations.

In my doctorate thesis, we provide evidence of a functional recruitment of astrocytes to the VTA dopaminergic neural circuits that leads to a fine tuning by gliotransmitter release of the excitatory synaptic transmission in VTA. We found that the phasic, burst firing activity of DA neurons - that is linked to reward and drug addiction behaviour - induces a long-term potentiation of glutamatergic synaptic transmission onto VTA DA neurons through a mechanism mediated by astrocytes. The reciprocal signaling between VTA DA neurons and astrocytes may represent, therefore, a novel mechanism contributing to reward memory formation as well as the development of drug addiction. The limited efficacy of available treatments for addiction makes mandatory to better understand the brain mechanisms involved so new targeted interventions can be developed.

Accordingly, understanding the role played by astrocytes in dopaminergic neural circuits of the reward system could reveal these glial cells as new therapeutic targets for addictive and other pathological reinforcement–dependent behaviours. Interestingly, in dnSNARE mice with a selective impairment in gliotransmission, the cocaine-induced reinstatement of condition place preference and the cue-induced reinstatement of cocaine self-administration are reduced compared to wild type animals [193]. Because in these mice gliotransmission is impaired in the astrocytes from different circuits involved in reward mechanism (VTA or NAc, for example), the imparment of the reinstatement can not be assigned to the astrocyte that belong to a specific reward circuit. Using local AAV injections to selectively delete the signalling of astrocytes from a given region, such as the VTA, we will unambiguously determine whether the bLTP observed in VTA brain slices plays a role in different paradigms of drugs of abuse, and eventually indicate VTA astrocytes as new therapeutic targets in drug addiction.

5. Material and methods

Animals and brain slice preparation

All procedures were conducted in accordance with the Italian and European Communities Council Directive of Animal Care and were approved by the Italian Ministry of Health. Horizontal VTA slices (240 μm) were obtained from C57BL/6J wild type and inositol 1,4,5-triphosphate-type 2 receptor (IP_3R2) knockout mice (IP_3R2KO) [194]; at postnatal days 14-17 (juvenile) or 30-70 (young adult). Animals were anesthetized with isofluorane, the brain removed and transferred into an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, pH 7.4 with 95% O_2-5% CO_2). Slices were cut with a vibratome (Leica Vibratome VT1000S Mannhein, Germany) in the ice-cold solution described in Dugue at al. 2005 [195] containing (in mM): 130 KGluconate, 15 KCl, 0.2 EGTA, 20 HEPES, 25 glucose, 2 Kynurenic acid). Slices were then transferred for 1 minute in a room-temperature solution containing (in mM): 225 D-mannitol, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 0.8 $CaCl_2$, 8 $MgCl_2$, 2 kynurenic acid with 95% O_2-5% CO_2. Finally, slices were transferred in ACSF at 32°C for 20 minutes and then maintained at room temperature for the entire experiment.

Electrophysiological recordings. Brain slices were continuously perfused in a submerged chamber with recording solution containing (in mM): NaCl 120; KCl 2; NaH_2PO4 1; $NaHCO_3$ 26; $MgCl_2$ 1; $CaCl_2$ 2; glucose 10; pH 7.4 (with 95% O_2-5% CO_2). Picrotoxin (50 μM) was added to block $GABA_A$ receptor currents. Cells were visualized with an Olympus FV1000 microscope (Olympus Optical, Tokyo, Japan). Conventional VTA DA neurons were recorded in the lateral part of the region medial to the medial terminal nucleus of the accessory optical tract (MT). DA neurons from the lateral VTA were identified on the basis of their distinct morphology, characterized by large and elongated soma with no particular dendritic orientation and by the presence of the following electrophysiological properties: a low-frequency tonic firing, a large I_h current elicited by hyperpolarizing steps [65] and a slow depolarizing potential during current step injections [47]. Simultaneous electrophysiological whole-cell patch-

clamp recordings from two DA neurons (distance of the somas, 70-120 μm) were made. Patch electrodes had resistances of 3-4 MΩ and were filled with an internal solution containing (in mM): K-gluconate, 135; KCl, 70; Hepes, 10; $MgCl_2$, 1; Na_2ATP 2 (pH 7.4 adjusted with KOH, 280-290 mOsm/L). Recordings were obtained using a multiclamp-700B amplifier (Molecular Device, Foster City, CA, USA). Signals were filtered at 1kHz and acquired at 10 k-Hz sampling rate with a DigiData 1440A interface board and pClamp 10 software. Series and input resistance were monitored throughout the experiment using a 5 mV pulse. Recordings were considered stable when the change of series and input resistances were below 20%. Cells that not meet these criteria were discarded.

Extracellular stimulation Theta capillaries filled with recording solution were used for bipolar stimulations. To stimulate glutamatergic afferents, electrodes were connected to a S-900 stimulator through an isolation unit and placed 100-200 μm rostral to the recording electrode. Paired pulses (50-ms intervals) were delivered at 0.33 Hz. Excitatory post-synaptic currents (EPSCs) were recorded while holding the membrane potential at -70 mV. Stimulus intensity was adjusted to evoke 30-40 % maximal EPSCs. The EPSC amplitude was measured as the peak current amplitude (2-9 ms after stimulus) minus the mean baseline current (100 ms before stimulus). Mean EPSCs were grouped in 3-min bins (i. e. mean EPSCs from 60 stimuli) to illustrate the time course. Paired-pulses ratio was calculated as 2^{nd} EPSC/1^{st} EPSC. Changes in mean EPSCs in the first DA neuron were monitored after imposing a burst or a tonic firing pattern to the second DA neuron (70-120 mm apart). Burst firing pattern was imposed in current-clamp mode, through injections of intracellular current pulses, with 5-pulse 20 Hz burst, every 500 ms for 5 min [177]. When indicated, we imposed a burst firing lasting only 1 (n = 7) or 3 (n = 4) min. Results from 1 and 3 min burst firing experiments were pooled together because no differences were observed in the two groups of data. Tonic firing was imposed with individual current pulses applied at 2 Hz for 5 minutes [177]. For statistical analysis of long-term effects, mean EPSCs from 120 stimuli before (basal) and 30 or 45 min after the firing protocol (burst or tonic firing pattern) were compared. For statistical analysis of short-term effects, mean EPSCs from 120

stimuli before (basal) and mean EPSCs from 60 stimuli 3 min after the firing protocol were compared.

***Dye loading* and Ca^{2+} imaging experiments.** Slices were loaded with the astrocyte specific marker Sulforhodamine 101 (SR101) (0.3 μM, Sigma Aldrich, Milano) in ACSF at 32°C for 15 minutes. Then, slices were loaded with the calcium sensitive dye OGB1-AM (5μM, Life Technologies, Monza, IT). Loading was performed for 15 min at 32°C in an ACSF solution containing sulfinpyrazone (200 μM Sigma Aldrich, Milano, IT; to limit the secretion of the fluorescent dye in its free acid form), pluronic F-127 (0.12% , Sigma Aldrich, Milano, IT; to prevent fluorescent dye aggregations) and kynurenic acid (1 mM, Sigma Aldrich, Milano, IT; to reduce glutamate excitotoxicity) and constantly bubbled with 95% O_2-5% CO_2. Following this procedure, a large number of astrocytes were loaded with the dye, while neurons remained unstained. Alternatively, after loading with SR101, slices were loaded for 45 min at room temperature with the Ca^{2+} sensitive dye Fluo4-AM (7,6 μM, Life Technologies, Monza, IT) in an ACSF solution containing pluronic F-127 (0.0067%, Sigma Aldrich, Milano, IT) and bubbled with 95% O_2-5% CO_2. Ca^{2+} imaging experiments were conducted with a confocal laser scanning microscope TCS-SP5-RS (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with two solid state lasers tuned at 448 nm and 543 nm (to image the astrocyte somatic Ca^{2+} variations and the SR101 fluorescence, respectively) and a 20x objective (NA, 1.0). Images were acquired with a 2-0.5 Hz frame rate. Image sequences were aligned and processed with ImageJ. Regions of interest (ROIs) were drawn around cellular somata using red channel from the SR101 signal. Ca^{2+} events were estimated as changes of the fluorescence signal over baseline ($\Delta F/F_0 = (F(t)-F_0)/(F_0\text{-backgruond})$). A fluorescence increase was considered a significant event when it exceeded three times the standard deviation from the baseline. The CB1R agonist WIN 55,212-2 (1-50 μM) was bath applied and the astrocyte response was monitored by quantifying the percentage of responsive astrocytes and the number of Ca^{2+} peaks per minute. Quinpirole hydrochloride 3 mM (SIGMA, Aldrich, Milano, IT) was locally delivered by using a pressure ejection unit (PDSE, NPI Electronics, Tamm, Germany) that applied pressure pulses (0.5 bar, 2 sec) to a quinpirole-containing pipette. Astrocyte Ca^{2+}

responses were quantified analyzing the probability of occurrence of Ca^{2+} spike by detecting the onset of Ca^{2+} elevations (Ca^{2+} spikes) during the recording period and grouping the Ca^{2+} spikes in 10s bins for all the astrocytes in the field. Value 0 and 1 were assigned for bins showing no response and Ca^{2+} spike, respectively, to obtain the Ca^{2+} spike probability index. A mean time course of the Ca^{2+} spike probability per slice was calculated at basal conditions, after quinpirole challenge and during recovery from three recordings in each condition (5-min time intervals between recordings). The mean time course of the Ca^{2+} spike probability for all the experiments is reported in the Figures. For statistical analysis, the mean Ca^{2+} spike probability/min was calculated for each condition. To verify that the response to quinpirole local applications was not due to mechanical stimulation, 3 slices from female mice and 2 slices from male mice were challenged with a puff application of vehicle (vehicle group, n = 5).

Drugs Picrotoxin 50 μM (SIGMA, Aldrich, Milano, IT); AM-251 4 μM (Abcam, Cambridge, UK); D-AP 5 50 μM (Abcam, Cambridge, UK); LY-367385 100 μM (Abcam, Cambridge, UK); eticlopride hydrochloride 1 μM (Abcam, Cambridge, UK); SCH-23390 hydrochloride 10 μM (Abcam, Cambridge, UK), L-741,626 10 μM (Tocris, Bristol, UK) and WIN 55,212-2 (1, 10, 25, 50 μM) were bath applied. L-NAME 100 μM (SIGMA, Aldrich, Milano, IT) was bath applied or included in the patch pipette. Quinpirole hydrochloride 3 mM (SIGMA, Aldrich, Milano, IT) was locally delivered by using a pressure ejection unit (PDSE, NPI Electronics, Tamm, Germany) that applied pressure pulses (0.5 bar, 2 sec) to a quinpirole-containing pipette.

Data analysis Data analysis was performed with Clampfit 10.5, Origin 8.0 (Microcal Softaware), Microsoft Excel 2010 and ImageJ (NHI).

Statistical analysis Data are expressed as mean ± standard error of the mean (SEM). Normality test (Shaphiro-Wilk test) was applied to the data before running statistical tests. Based on the normality test result, data were analyzed using either parametric (Student's t-test, $\alpha = 0,05$) or nonparametric tests (Wilcoxon signed-rank test) as appropriate. Statistical differences were established with $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

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