# A molecular and functional dissection of the GABAergic neuronal diversity in the ventral tegmental area

S.T. Paauw

## Layman Summary

The ventral tegmental area (VTA) is a region in the brain that is involved in various behaviours and disorders, such as addiction. In the brain, different classes of neurons exist. These neurons are connected to each other in neuronal networks. The VTA contains dopamine neurons, which release dopamine. It also contains GABAergic neurons that 'inhibit' the activity of other neurons, meaning they make the neurons they are connected to less active. There are many subtypes of GABAergic neurons, which all express a different combination of genes and make connections to a variety of other neurons in and outside of the VTA. At the moment, not much is known about exactly how many subtypes of GABAergic neurons exist in the VTA and what the role of each GABAergic subtype is in different behaviours. We here review the currently available literature on GABAergic neurons in the VTA, and attempt to find links between gene expression, connectivity and behaviour, in order to identify different subtypes of GABAergic neurons. Distinguishing these GABAergic subtypes will allow researchers to study more specifically each subtype of GABAergic neurons and which connections they make. This will eventually improve our understanding of the role of GABAergic neurons in health, and give us insights in mechanisms of disease, such as how people get addicted or depressed.

## Abstract

GABAergic neurons in the ventral tegmental area (VTA) are involved in a diversity of behaviours through distinct brain circuits. This heterogeneity in VTA GABAergic function should be encoded at the molecular level. An increasing number of studies have investigated GABAergic diversity in the VTA using different approaches; however, the full extent of this diversity currently remains unknown. Here, we review the currently available literature on GABAergic neurons in the VTA, and attempt to connect molecular, connectivity and functional data in order to elucidate GABAergic diversity in the VTA. We establish a link between molecularly defined cell types, connectivity and behaviour, by linking regional markers and connectivity data with single nucleus RNA sequencing data. However, most behavioural studies remain unassociated with GABAergic subtypes, due to a lack of specific markers used in these studies. The integration of molecular, connective and behavioural literature as performed in this review will improve our comprehension of GABAergic diversity in the VTA. Future research should focus on the identification of molecularly distinct subpopulations of GABAergic neurons using published GABAergic cell type markers, to allow specific (viral) targeting of GABAergic subpopulations in connective and behavioural studies and facilitate comparison of these subtypes to the literature. Ultimately, this will provide the field with a more detailed understanding of VTA GABAergic function in health and disease.

## Introduction

The ventral tegmental area (VTA) is a structure in the ventral midbrain that plays a central role in the reward system, and defects in its function are implicated in several neurodevelopmental, neurodegenerative and psychiatric disorders, as well as in addiction (Morales and Margolis, 2017). Most

studies on the VTA have focused on its dopaminergic neurons (DAergic neurons) that connect to cortical and striatal regions through the mesocortical and mesolimbic pathways. These pathways are involved in motivation, reward and aversion. However, the VTA also contains a diversity of glutamatergic and GABAergic neurons, which are also involved in these behaviours (Root et al., 2020).

Approximately 35% of VTA cells in the rat are GABAergic neurons, which release the inhibitory neurotransmitter γ-Aminobutyric acid (GABA) (Nair-Roberts et al., 2008). These neurons locally target VTA DAergic neurons, but also project to many regions outside the VTA (Morales and Margolis, 2017). VTA GABAergic neurons may release more than one neurotransmitter and play distinct behavioural roles, depending on the GABAergic subpopulation (Root et al., 2020). This diversity in connectivity and function highlights the heterogeneity of the VTA GABAergic population, which should be encoded at the molecular level.

While extensive research has been performed on DAergic diversity in the VTA, few studies have focused on VTA GABAergic neuron diversity. This is mainly due to the lack of well-studied, selective and specific marker genes for distinct GABAergic subpopulations, which are difficult to identify, thus limiting the use of genetic tools such as optogenetics (Kim et al., 2019). Recently, GABAergic molecular diversity in the VTA has become of more interest, due to the rise of high-throughput transcriptional profiling tools, such as single nucleus RNA sequencing (Phillips III et al., 2022).

Although these techniques have generated more data on VTA GABAergic diversity on the molecular, circuit and behavioural level, this data has not been integrated yet. This is attributable to the scarcity of genetic profiling studies on GABAergic diversity, and the lack of clear description of the GABAergic neurons observed in studies. Therefore, the current review provides a comprehensive overview of the current knowledge on GABAergic diversity in the VTA, and aims to unravel VTA GABAergic subtype classifications by connecting molecular, connective and behavioural data from the available literature. This will provide the field with better insight in GABAergic diversity in the VTA, which will improve our understanding of GABAergic function in health and disease. Since very little data from humans exists on this topic, we here focus on data obtained exclusively from rodents.

## Cellular heterogeneity within the VTA GABAergic population

On the cellular level, the GABAergic population in the VTA is highly diverse in many aspects:

i) GABAergic subtypes are located in different regions of the VTA,
ii) are involved in different neural circuits, and
iii) exhibit different molecular markers.

Here, we provide an overview of the currently available information on cellular heterogeneity within the VTA GABAergic population.

## Circuit organisation of GABAergic neurons in the VTA

The VTA is divided into five major subregions: interfascicular (IF), rostral linear (RLi), caudal linear (CLi), parabrachial pigmented (PBP) and paranigral (PN) nuclei. GABAergic neurons are found across all subregions of the VTA, but most VTA GABAergic neurons are found in the rostromedial VTA, as opposed to DAergic neurons, of which the majority are found in the caudolateral VTA (Morales and Margolis, 2017). GABAergic neurons in the VTA can be divided into two subpopulations: local interneurons and projection neurons.

Local interneurons inhibit DAergic neurons in the VTA through $GABA_A$ receptors (Tan et al., 2012). They also innervate other GABAergic and glutamatergic neurons within the VTA (Polter et al., 2018; Tan et al., 2012; Yu et al., 2019). The axons of these local GABAergic neurons are limited in spread within the VTA,

suggesting that different local GABAergic subtypes control distinct subpopulations of DAergic neurons within the VTA, which may manifest in different behaviours (Polter et al., 2018).

GABAergic projection neurons, on the other hand, send inhibitory projections to 35 anatomically distinct regions, which include the medium spiny neurons (MSNs) and cholinergic interneurons (CINs) of the nucleus accumbens (NAc), dorso-medial-striatal-cholinergic interneurons (dmCINs), the lateral habenula (LHb), the substantia nigra compacta, laterodorsal tegmental nucleus (LDTg), medial terminal nucleus of the accessory optic tract, prefrontal cortex (PFC), anterior cingulate cortex, infralimbic cortex, ventral pallidum (VP), dorsomedial pallidum, lateral and magnocellular preoptic nuclei (LPO and MCPO), lateral hypothalamus (LH), basolateral and central amygdala (CeA), mediodorsal thalamus, parafascicular thalamic nucleus (Pf), mediodorsal thalamic nucleus, GABAergic and serotonergic neurons of the dorsal raphe nucleus (DRN), bed nucleus of the stria terminalis (BNST), deep mesencephalic nuclei, interstitial nucleus of the posterior limb of the anterior commissure (IPAC), ventrolateral periaqueductal gray (PAG), ventral nucleus of the lateral lemniscus, pontine reticular nucleus, locus coeruleus, superior colliculus (SC), dentate gyrus, ipsilateral external cortex of the interior commissure (ECIC), piriform cortex, claustrum, endopiriform nucleus, ipsilateral lateral and medial parabrachial nuclei (Breton et al., 2019; Brown et al., 2012; Chen et al., 2020; Chowdhury et al., 2019; Li et al., 2019; Ntamati and Lüscher, 2016; Rizzi et al., 2021; Root et al., 2014a; Stamatakis et al., 2013; Taylor et al., 2014; Tritsch et al., 2014; Yu et al., 2019; Zhou et al., 2019). GABAergic neurons that project to different regions are spatially organised within the VTA, suggesting they exist in distinct subclasses (Breton et al., 2019).

Afferents of VTA GABAergic neurons include GABAergic neurons in the dorsolateral VP, which mainly innervate VTA local interneurons (Li et al., 2021); NAc lateral shell neurons that inhibit local DAergic neuron-innervating interneurons in the lateral VTA (lVTA) (Yang et al., 2018); basal forebrain (BF) somatostatin-positive inhibitory neurons that inhibit VTA local DAergic neuron-innervating interneurons (Wang et al., 2021); glutamatergic afferents from the medial PFC (Carr and Sesack, 2000); glutamatergic inputs from the SC (Zhou et al., 2019); predominantly excitatory input from the PAG onto GABAergic VTA neurons exhibiting a larger $I_h$ amplitude (Ntamati et al., 2018); both GABAergic and glutamatergic afferents from the LH (Nieh et al., 2016); inhibitory and stimulatory inputs from the BNST (Jennings et al., 2013; Kudo et al., 2014); the DRN, VP, LHb and LDTg (Faget et al., 2016); anterior cortex, paraventricular hypothalamus, NAc, dorsal striatum, septum, medial habenula, preoptic area, globus pallidus, zona incerta, parabrachial nucleus and central amygdala (Beier et al., 2015). Notably, VTA GABAergic neurons are innervated by many of the same brain regions as VTA DAergic neurons (Beier et al., 2015). The most important and well-studied connections of VTA GABAergic neurons are depicted in figure 1.

## Molecular markers to identify subtypes of VTA GABAergic neurons

Recently, many researchers have attempted to decipher the molecular phenotypes underlying the GABAergic diversity in the VTA. This has provided the field with new possibilities for the identification of molecular subclasses of VTA GABAergic neurons, which is essential in connecting VTA GABAergic molecular and functional diversity.

VTA GABAergic neurons are usually detected by the presence of glutamate decarboxylase 1 (GAD67 or GAD1), glutamate decarboxylase 2 (GAD65 or GAD2) or the vesicular GABA transporter (VGaT; *Slc32a1*). These are all generic GABAergic markers, which are expressed in different proportions in VTA GABAergic neurons, depending on the neuronal subtype. Only about a third of VTA $VGaT^+$ neurons co-expresses GAD67 mRNA, and a very small percentage (~1%) of VTA GABA neurons expresses only GAD67, but not VGaT mRNA (Chowdhury et al., 2019).

The areas innervated by $GAD65^+$ and $GAD67^+$ VTA neurons mostly overlap, but there are some exceptions. For instance, $GAD67^+$ and $GAD65^+$ VTA neurons each project to different parts of the LHb;

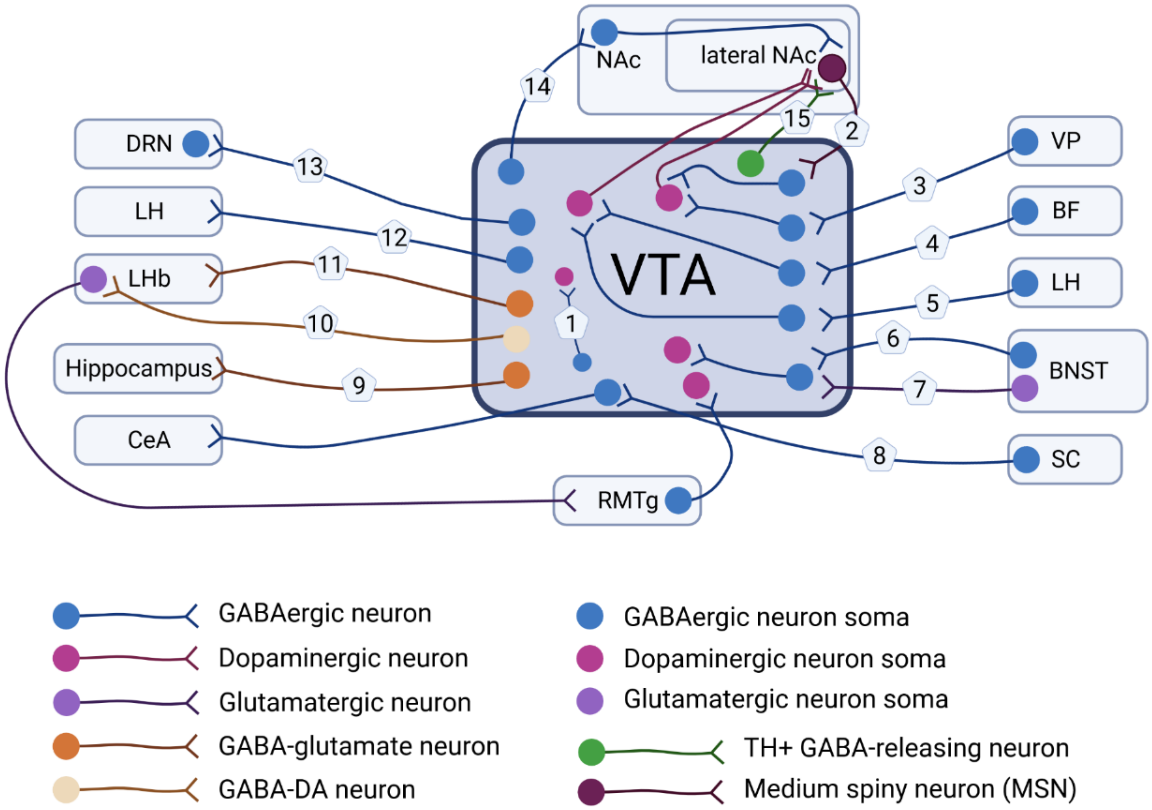


**Figure 1. Connectivity of VTA GABAergic neurons and associated behaviours.** VTA GABAergic neurons receive inputs from the nucleus accumbens (NAc), ventral pallidum (VP), basal forebrain (BF), lateral hypothalamus (LH), bed nucleus of the stria terminalis (BNST) and superior colliculus (SC). They project to the NAc, dorsal raphe nucleus, LH, lateral habenula (LHb), hippocampus and central amygdala (CeA), amongst other, less well-studied regions (see text). Numbers indicate behaviours associated with the circuitry: **1)** Hiding-in-nest behaviour; **2)** Place preference; **3)** Sustained wakefulness and increased locomotor activity, but not anxiety; **4)** Prosocial behaviour; **5)** Real-time place preference; **6)** Rewarding and anxiolytic behaviour; **7)** Aversive and anxiogenic behaviour; **8)** Looming-evoked defensive flight behaviour; **9)** Does not affect sleep; **10)** Conditioned place preference; **11)** Response to aversive and rewarding stimuli, but not cues; **12)** Promotes sleep and increases high-fat food intake; **13)** Place aversion and protective effect on morphine-induced conditioned place preference; **14)** Stimulus-outcome learning, alteration of cue processing but not reward consumption, and encodes adaptation of reward-seeking behaviours; **15)** Reward-seeking behaviour is enhanced when GABAergic release is inhibited in this pathway. The GABAergic somas in the VTA are depicted in a solely schematic fashion and do not accurately reflect the anatomical location of these neurons. Only circuits with a clear behavioural correlate are shown here. The connectivity depicted in this figure corresponds to the molecular subtypes and references from table 1. *This figure was created in BioRender.com.*

GAD67$^+$ fibres innervate the ventrolateral LHb, whereas GAD65$^+$ fibres innervate the medial LHb (Taylor et al., 2014). The VP, MCPO, SC, piriform cortex, claustrum and endopiriform nucleus receive projections from GAD67$^+$ neurons but not from GAD65$^+$ neurons (Taylor et al., 2014). Conversely, the dorsomedial pallidum, LPO, IPAC and ECIC are targeted by GAD65$^+$ but not GAD67$^+$ fibres (Taylor et al., 2014). VTA GAD67$^+$ projections to the NAc shell, NAc core, PFC, amygdala, LHb, VP and MCPO originate predominantly from the ventrolateral VTA (PBP and PN nuclei) (Taylor et al., 2014).

A portion of VTA neurons innervates NAc medium spiny neurons (MSNs) and releases GABA, but does not express any of the generic GABAergic markers. Instead, this population is tyrosine hydroxylase (TH)-positive and co-releases DA and GABA, which is dependent on the vesicular monoamine transporter (VMAT2; *Slc18a2*) and the membrane GABA transporters mGAT1 (*Slc6a1*) and mGAT4 (*Slc6a11*) (Berrios et al., 2016; Tritsch et al., 2014). Furthermore, a subset of TH$^+$ GAD$^+$ neurons projecting from the IF to the LHb release only GABA and not DA (Stamatakis et al., 2013). A variety of studies has found co-expression of GAD65 and TH and/or DAT, but not GAD67 and TH and/or DAT in rat nor mouse (Kim et

al., 2019; Merrill et al., 2015; Steffensen et al., 2018), suggesting that this $TH^{VTA—LHb}$ pathway expresses GAD65 but not GAD67.

A subset of GABAergic VTA cells, most of which reside in the midline VTA (RLi and IF), releases both GABA and glutamate. The major inputs from the VTA to the LHb are provided by these VTA $VGluT2^+$ $VGaT^+$ combinatorial neurons (Root et al., 2014a, 2018, 2020). To a lesser extent, the LHb also receives input from VTA $VGluT2^+$ $VGaT^+$ $TH^+$ (hereafter called 'triple-combinatorial neurons'), which release DA together with GABA and glutamate (Root et al., 2014a). VTA neurons that co-release GABA and glutamate also project to the granule cells of the dentate gyrus and the ventral pallidum (Ntamati and Lüscher, 2016; Yoo et al., 2016).

The VTA triple-combinatorial neuronal subpopulation, which is located in the lVTA (PBP), has been characterised by Poulin et al. (2020), who synthesised data from single-cell profiling studies on DAergic diversity in the midbrain, resulting in the classification of seven distinct DAergic neuron subtypes (Poulin et al., 2020). This integrative approach led to the identification of several marker genes for triple-combinatorial neuronal subpopulations across different developmental stages and methodologies: *Cbln1*, *Calb1*, cholecystokinin (*Cck*), *GAD65*, corticotropin-release factor-binding protein (*Crhbp*) and Wnt7b (Poulin et al., 2020).

A subset of neurons in the VTA express the molecular marker somatostatin (Sst), which is commonly used to identify interneurons. However, in the VTA this marker is also expressed in GABAergic projection neurons, since a subset of $VGaT^+$ $Sst^+$ neurons project to the lateral hypothalamus (LH) (Yu et al., 2022). Based on electrophysiological properties of Sst-expressing neurons, three subtypes of $Sst^+$ neurons were identified in the VTA: neurons with a prominent afterdepolarisation (ADP), high-frequency firing neurons (HFF), and neurons with the 'Delayed' phenotype (Nagaeva et al., 2020). ADP neurons were localised mostly in the lVTA (PBP) and consisted of mostly $VGluT2^+$ $VGaT^+$ and some $VGaT^+$-only neurons. These neurons were found to be interneurons that evoked inhibitory postsynaptic currents in neighbouring VTA DA neurons (Nagaeva et al., 2020). However, it remains to be determined whether these ADP neurons actually release glutamate, as little co-release of glutamate was found at local synapses between VTA GABAergic and DAergic neurons (Polter et al., 2018). Another group of interneurons was identified by expression of neuronal nitric oxide synthase (encoded by *Nos1*), which was expressed in 12% of $VGaT^+$ neurons in the PBP specifically (Paul et al., 2018; Yu et al., 2019). *Nos1* was also expressed in $Sst^+$ neurons (Nagaeva et al., 2020), raising the possibility that these may be ADP neurons. HFF neurons included mostly $VGaT^+$-only and $VGluT2^+$ $VGaT^+$ neurons, and were also found predominantly in the PBP. 'Delayed' neurons, on the other hand, were most prominent in the dorsomedial VTA (PIF and PN), and all 'Delayed' $Sst^+$ neurons expressed DAergic molecular markers (Nagaeva et al., 2020).

In the caudal VTA, neither Sst nor parvalbumin (Pv) was expressed; Pv was expressed in $GAD^+$ cells in the rostromedial VTA (Merrill et al., 2015; Ngolab et al., 2015; Olson and Nestler, 2007). VTA $GAD^+$ cells expressed calbindin (CB, encoded by *Calb1*) and calretinin (CR, encoded by *Calb2*) in the PBP and midline nuclei and the lateral PBP, respectively (Merrill et al., 2015; Olson and Nestler, 2007). CB and Pv were never coexpressed in $GAD^+$ neurons (Merrill et al., 2015). CB was also expressed by VTA $VGluT2^+$ $VGaT^+$ and $VGluT2^+$ $VGaT^+$ $TH^+$ neurons (Mongia et al., 2019), although an earlier study had not detected CB expression in $TH^+$ $GAD65^+$ neurons (Korotkova et al., 2003). VTA $GAD^+$ neurons further expressed neuropeptide Y (NPY), Cck in the rostral and caudal VTA and ventral PBP, and a subset (28%) expressed Crhbp in the lVTA (Korotkova et al., 2003; Merrill et al., 2015; Olson and Nestler, 2007; Wang and Morales, 2008). $GAD67^+$ VTA neurons contained mRNA for endocannabinoid biosynthetic enzymes (DAGLα, NAPE-PLD and 12LO) and mGluR1/5 (*Grm1*/*Grm5*), which were often co-expressed (Merrill et al., 2015).

Expression of opioid receptors by VTA GABAergic neurons has been an area of great interest, as these receptors play an important role in opioid addiction. Most studies in this area have focused on the μ-opioid receptor (MOR, encoded by *Oprm1*). This receptor is expressed by subsets of GABAergic ($VGluT2^-$ $VGaT^+$), glutamatergic ($VGluT2^+$ $VGaT^-$) and $VGluT2^+$ $VGaT^+$, but not DAergic cells in the VTA (Kudo et al., 2014; Miranda-Barrientos et al., 2021). Interestingly, the MOR selective agonist DAMGO did not affect the membrane potential of most $VGluT2^+$ $VGaT^+$ neurons, whereas it hyperpolarized $VGluT2^-$ $VGaT^+$ and

VGluT2$^+$ VGaT$^-$ neurons (Miranda-Barrientos et al., 2021). In another study, DAMGO inhibited a significant portion, but not all of GAD65/67$^+$ neurons in the VTA; strikingly, neurons that were sensitive to inhibition through MOR activation did not respond to dopamine receptor D2 (D2R; *Drd2*) activation, whereas D2R-activated neurons were insensitive to DAMGO (Margolis et al., 2012). Similar effects were observed in VTA GAD67$^+$ neurons (Chieng et al., 2011). GAD67$^+$ MOR$^+$ neurons included VTA neurons that were targeted by the BNST (Kudo et al., 2014). Also, rostral VTA (rVTA) GAD65$^+$ neurons that innervate DRN interneurons express MOR (Li et al., 2019). Both GAD65/67$^+$ local interneurons and GABAergic projections onto NAc CINs were sensitive to DAMGO, but not DA (Matsui et al., 2014). On the other hand, VTA GABA-releasing projections onto NAc MSNs were inhibited by the D2-like receptor agonist quinpirole, leading the authors to believe that this GABAergic input from VTA onto NAc MSNs most likely originates from TH$^+$ GABA-releasing neurons (Matsui et al., 2014; Tritsch et al., 2014).

Nicotinic acetylcholine receptors (nAChRs), which are functionally expressed on VTA GABA neurons, play an important role in nicotine reward. GAD65$^+$ neurons in the posterior VTA that target the LHb express the α4*-nAChR (*Chrna4*); these neurons do not express Pv, Sst, CB, or CR (Ngolab et al., 2015). GAD67$^+$ VTA neurons express α4-, α6- (*Chrna6*) and β2 (*Chrnb2*) nAChR subunit mRNA (Steffensen et al., 2018). Local GAD67$^+$ interneurons are amongst the neurons that express the β2 nAChR subunit (Tolu et al., 2013), as well as VGluT2$^+$ GAD65$^+$ neurons from the medial VTA (mVTA) that project to the lVTA (Yan et al., 2019).

**Genetic profiling studies on GABAergic diversity in the VTA**

An increasing number of studies has attempted to unravel the molecular heterogeneity within the GABAergic population in the VTA through genetic screening approaches. A transcriptional profiling study identified the markers CBLN4 (medial PBP and PN), *RXFP3* (rostromedial VTA), *NRP2* (caudal VTA), *TRH*, *GPR101* and *RORA* (sparsely dispersed) on VTA VGaT$^+$ cells (Paul et al., 2019). Importantly, gene selection was based on availability of cre-driver mouse lines, and may thus have excluded good candidate markers (Paul et al., 2019). Another study prepared and analysed whole cell RNA from P3 mice for gene expression, and identified coexpression of VGaT and Ntf3 in the PBP through in situ hybridization (Viereckel et al., 2016). Moreover, VGaT$^+$ neurons showed dispersed expression of TrpV1 in the VTA (Viereckel et al., 2016). The marker *Six3* was also ranked highly in this analysis, but was excluded from further analysis due to riboprope nonselectivity (Viereckel et al., 2016). Expression of this marker was also discovered in a developmental study in the E18.5 mouse in GAD67$^+$ interneurons in the PBP, in addition to *Zfpm2*, which was expressed in lVTA GAD67$^+$ interneurons (Lahti et al., 2016).

A different approach was used by Kim and colleagues, who identified specific genes for each type of neuron in the VTA (GABAergic, glutamatergic, DAergic) based on in situ hybridisation data in the Allen Brain Atlas (Kim et al., 2019). GAD67 was used as a marker for GABAergic neurons and GAD67$^+$ cells were preferentially located in the more lateral regions of the VTA. Alternative marker genes for GAD67, that correlated with GAD67 expression were deemed GAD67-like genes. Based on correlation patterns, GAD67-, TH-, and VGluT2-like genes were categorised into 11 subclusters, of which four clusters correlated with GAD67. However, none of the alternative marker genes showed expression patterns similar to that for GAD67 based on ISH data (Kim et al., 2019).

A very recent study investigated the molecular architecture of the VTA through single-nucleus RNA sequencing, a high-throughput method resulting in an atlas of transcriptionally defined cell populations in the rat VTA (Phillips III et al., 2022). With this approach, they identified 11 neuronal subpopulations, based on expression of genes involved in the synthesis and transport of GABA (GAD65, GAD67, GAT1 and VGaT), glutamate (VGluT2 and Grm2) and DA (TH, Ddc, DAT and VMAT): one glutamate-only population, three GABA-only populations marked by high levels of *Slc32a1* (VGaT) and GAD67, one DA-only population, four GABA-glutamate combinatorial populations and two GABA-glutamate-DA combinatorial populations (Phillips III et al., 2022). For two clusters, among which a GABA-glutamate-DA combinatorial population, cell-type-specific marker genes were identified. Furthermore, the expression pattern of opioid receptors was assessed for each cell cluster. Interestingly, two GABA-only populations

strongly expressed both *Sst* and *Htr2c*, which encodes the serotonin 2C receptor, whereas the other GABA-only population negatively correlated with *Htr2c*, but was the only population strongly enriched in the parvalbumin interneuron-associated marker *Kit* and *Oprm1* (Phillips III et al., 2022). For each subpopulation, the transcriptional profiling data was made available online. These molecularly distinct populations may (at least in part) overlap with functionally different populations in the VTA. Therefore, this atlas is a useful tool in elucidating GABAergic diversity in the VTA on the molecular level.

## Diverse VTA GABAergic neuronal effects on behaviour

GABAergic neurons in the VTA are involved with a variety of behaviours. There are two ways VTA GABAergic neurons contribute to behaviour: through local inhibition of VTA DAergic neurons, as well as independently (**Figure 1**). In this section, we describe in detail how the heterogeneous VTA GABAergic neuron population affects a wide variety of behaviours in rodents.

### VTA GABAergic influence on reward and aversion

**Reward- and aversion-associated behaviour elicited by GABAergic interneuron (dis)inhibition of VTA DAergic neurons**

GABAergic neurons in the VTA play an important role in the processing of reward and aversion. $VGaT^+$ VTA neurons signal cue-evoked anticipation of reward, as they are active during the delay period for an expected reward (Cohen et al., 2012; Eshel et al., 2015). Consistent with this, optogenetic activation of VTA $VGaT^+$ neurons during a reward-predictive cue did not alter conditioned anticipatory licking behaviour, whereas activation of this GABAergic population five to ten seconds after cue-preceded reward delivery or during free-reward consumption resulted in a decrease in consummatory licking behaviour (Van Zessen et al., 2012). Similar results were observed after chemogenetic (and therefore chronic) activation of $GAD67^+$ VTA neurons, which disrupted the responding accuracy and latency to incentive cues, but did not alter motivation for the reward (Wakabayashi et al., 2019). In a slightly different experimental paradigm, continuous bilateral stimulation of VTA $VGaT^+$ neurons also reduced anticipatory licking to conditioned odours and thus altered the motivation for reward (Eshel et al., 2015). The increased latency to incentive cues after chemogenetic stimulation of GABA neurons is in correspondence with the finding that chemogenetic activation of GAD67+ VTA neurons disrupts time perception, particularly when the timing is set by cues (Shields et al., 2021). These effects were not mediated by VTA-NAc GABAergic projection neurons, but instead were proposed to occur through local inhibition of VTA DA neurons (Wakabayashi et al., 2019; Van Zessen et al., 2012).

Place aversion could also be elicited by local GABAergic inactivation of VTA DA neurons. Optogenetic activation of $GAD65^+$ as well as $VGaT^+$ VTA neurons elicited conditioned place aversion in mice by inactivating VTA DA neurons (Galaj et al., 2020; Tan et al., 2012). Conversely, $VGaT^+$ VTA neurons were excited by aversive stimuli, and optogenetic inhibition of $VGaT^+$ VTA neurons prevented itch-associated conditioned place aversion (Cohen et al., 2012; Su et al., 2019). Real-time and conditioned place aversion could also be induced by optogenetic stimulation of VTA astrocytes that facilitate excitation of the local GABA ($VGaT^+$)—DA circuit (Gomez et al., 2019). Astrocytes modulate afferent glutamatergic drive on VTA GABA neurons and could only increase excitation of $VGaT^+$ neurons and inhibition in DA neurons during concurrent activity of neuronal afferents (Gomez et al., 2019). Strikingly, conditional knockout of the glutamate transporter GLT-1 selectively in VTA astrocytes, which impedes the ability of VTA astrocytes to excite local VTA GABAergic DA neuron-innervating interneurons, abolished avoidance behaviour, but preserved preference for reward (Gomez et al., 2019). Together, these studies suggest that local VTA GABAergic neurons alter the motivational salience of a cue and promote aversion, but do not affect motivation for reward. These behaviours are dependent on a local circuit in the VTA, where

astrocytes modulate stimulatory input onto GABAergic neurons, which subsequently inhibit VTA DAergic neurons.

The influence of VTA astrocytes on reward-associated behaviours stresses the importance and dependency of the local GABA—DA projection in the VTA on afferents to VTA GABAergic neurons. The lateral NAc (NAcLat) sends inhibitory projections to predominantly GABAergic neurons in the lVTA. Photoinhibition of $GAD65^+$ neurons in the lVTA that receive this inhibitory input from the NAcLat resulted in the disinhibition of VTA DA neurons, and mice showed robust real-time place preference for the compartment where they received stimulation of NAcLat—lVTA projections (Yang et al., 2018). Thus, place preference can be mediated through inhibitory projections from the NAcLat to lVTA $GAD65^+$ neurons. Optogenetic stimulation of GABAergic, but not glutamatergic efferents from the LH to VTA $VGaT^+$ neurons increased DA release in the NAc, which led to real-time place preference as well as social interaction and novel object investigation (Nieh et al., 2016). Similarly, rewarding and anxiolytic behaviour could be elicited by photostimulation of BNST GABAergic efferents to VTA $VGaT^+$ DA neuron-inhibiting neurons, whereas photostimulation of BNST glutamatergic efferents to these neurons produced the opposite effect (Jennings et al., 2013). In line with this finding, mice showed anxiety-like behaviour in an open field test, elevated plus maze test, and marble-burying test during optogenetic stimulation of VTA $VGaT^+$ neurons (Chen et al., 2020).

Thus, VTA GABAergic local interneurons inhibit VTA DAergic neurons. Inactivation of GABAergic local interneurons leads to disinhibition of DAergic neurons and produces rewarding behaviours, whereas their inactivation generates aversive behaviours, but leaves motivation for reward intact.

**Reward- and aversion-associated behaviour elicited by VTA GABAergic projection neurons**

Although most reward- and aversion-associated behaviours are influenced by local GABAergic inhibition of VTA DAergic neurons, they may also occur independently of this local circuit. In such manner, contrary to the inhibiting function of local VTA GABAergic interneurons on DAergic neurons, activation of VTA GABAergic neurons may lead to DAergic neuron activation and rewarding behaviour.

For instance, activation of $TH^+GAD65^+$ neurons projecting from the rostromedial VTA (lF) to the LHb increases the firing rate of VTA DAergic neurons, by inhibiting LHb glutamatergic projections to RMTg GABAergic neurons that inhibit VTA DAergic neurons (Stamatakis et al., 2013). This activation resulted in conditioned place preference behaviour and reinforcing behaviour that was specifically dependent on $GABA_A$, but not DA, signalling in the LHb (Stamatakis et al., 2013). Furthermore, impairment of GABA co-release from $TH^+$ GABA-releasing neurons that do not express generic GABAergic markers enhanced optical self-stimulation, a measure of reward-seeking behaviour (Berrios et al., 2016).

Another example of different effects of GABAergic subtypes on behaviour was shown by Root et al. (2020), who discovered that the response to cues predicting reward, aversion and the absence of reward, or the delivery of a reward or aversive stimulus, differed between VTA $VGluT2^-$ $VGaT^+$ and VTA $VGluT2^+$ $VGaT^+$ combinatorial neurons. VTA $VGluT2^-$ $VGaT^+$ neurons, which project mainly to the BNST, peduncular part of the LH, and DRN, responded slightly but not significantly to cues predicting reward. Instead, they were robustly activated by cues predicting the absence of reward and the delivery of an aversive stimulus, as well as by the rewarding and aversive outcomes. On the other hand, VTA $VGluT2^+$ $VGaT^+$ neurons, which project to the LHb, were found to respond to both rewarding and aversive stimuli, but not to cues predicting these outcomes or predicting the absence of reward (Root et al., 2020). Photostimulation of VTA $GluT2^+$ $VGaT^+$ combinatorial neurons promotes conditioned aversive behaviour through activation of glutamate receptors in the LHb (Root et al., 2014b, 2014a).

The importance of different VTA GABAergic circuits in different behaviours was further stressed by the finding that rVTA $GAD65^+$ projection neurons synapse mainly onto dorsal raphe nucleus (DRN) GABAergic neurons, whereas caudal VTA (cVTA) $GAD65^+$ neurons directly innervate DRN serotonergic neurons. Photostimulation of rVTA GABAergic neurons disinhibited DRN serotonergic neurons, resulting in aversive behaviour (Li et al., 2019). Conversely, activation of cVTA $GAD65^+$ neurons promoted reward

(Li et al., 2019). Furthermore, despite findings from Wakabayashi et al. (2019) that GAD67$^+$ projection neurons from the VTA to the NAc were not involved in incentive cue processing, the same group discovered that this VTA-NAc pathway alters cue processing for rewards (Wakabayashi et al., 2021). This discrepancy with earlier findings could be explained by the difference in behavioural paradigms used in both studies. The GABAergic VTA-NAc projection namely encodes the adaptation of reward-seeking behaviours, as stimulation of this trajectory decreased responses to incentive cues associated with smaller-than-expected rewards, but did not affect the consumption of the reward (Wakabayashi et al., 2021). Global VTA GABAergic (GAD67+) neuron activation, in contrast, caused a uniform decrease in responding to incentive cues irrespective of changes in the size of the reward (Wakabayashi et al., 2021).

In conclusion, the activation of VTA GABAergic neurons may result in opposing behaviours, depending on the neuronal circuit it takes part in, but importantly, also based on molecular subtype.

**VTA GABAergic influence on motivated learning processes**

When looking at the studies above, it is striking that many studies investigating VTA GABAergic influence on reward and aversion use behavioural tasks that involve conditioning to cues. The role of VTA GABAergic neurons can also differ depending on whether rewards are expected or not. This raises the question what the specific influence of VTA GABAergic neurons is in learning.

VTA GAD65$^+$ projection neurons connect to NAc cholinergic interneurons (CINs). Stimulation of this pathway enhanced stimulus-outcome learning, specifically when the outcome is aversive (Brown et al., 2012). Furthermore, VGaT$^+$ neurons in the lVTA connect to dmCINs through direct projections and via the Pf. When GABAergic terminals in the DMS were inhibited, mice performed worse than controls in discriminating conditioned aversive stimuli (Rizzi et al., 2021). On the other hand, stimulus discrimination was enhanced by inhibition of lVTA VGaT$^+$-Pf projections (Rizzi et al., 2021). Thus, VTA GABAergic neurons have diverse functions in associative learning.

Local VTA GABAergic neurons have been associated with prediction-error learning, due to their role in inhibiting VTA DA neurons that are thought to encode reward prediction error, which is the discrepancy between expected and actual rewards (Cohen et al., 2012). VTA VGaT$^+$ neurons were identified as neurons that signal expected reward to VTA DA neurons, and inhibition of VTA VGaT$^+$ neurons changed the response of VTA DA neurons to expected reward as if it were less expected (Cohen et al., 2012; Eshel et al., 2015). The influence of VTA VGaT$^+$ neurons in associative learning was further confirmed by the finding that photostimulation of this population during a four-odour classical conditioning task resulted in aversion for the odour associated with the stimulation. This aversion persisted even in the absence of stimulation. The authors concluded that the activation of VTA VGaT$^+$ neurons reflects a decrease in the expected value of this odour (Eshel et al., 2015). More specifically, reward expectations are signalled by VTA VGluT2$^-$ VGaT$^+$ neurons (Root et al., 2020). VTA VGluT2$^+$ VGaT$^+$ combinatorial neurons, on the other hand, signal violations of reward expectation (Root et al., 2020). These results suggest that VTA GABAergic neurons not only signal aversion, but also expected value through conditioning.

**VTA VGaT$^+$ neurons control the coordination of motivated movements**

It was recently found that VTA VGaT$^+$ neurons play a role in the coordination of motivated head movements along the three principal axes of rotation: yaw (left–right), roll (clockwise–counterclockwise) and pitch (up– down; Hughes et al., 2019). For each axis of rotation, head movement correlated with the spiking activity of two populations of VTA VGaT$^+$ neurons. During a reward-tracing task, optogenetic modulation of the activity of either of these populations produced opposite head movements, depending on whether the population was stimulated or inhibited (Hughes et al., 2019). Eventually, the manipulations led the mice unable to execute the task appropriately, resulting in decreased reward delivery. Many studies investigating the effect of VTA VGaT$^+$ stimulation on reward- and aversion-associated behaviour have employed tasks that require head rotation control (such as eating). Therefore,

the authors proposed that the aversive behaviour resulting from VTA VGaT$^+$ stimulation is a reflection of difficulty with movement control, or is caused by the aversive experience of vestibular disturbance and resulting disorientation and nausea, instead of an inherently aversive role of VTA VGaT$^+$ neurons in motivated behaviour (Hughes et al., 2019).

## VTA GABAergic neuron functions not associated with reward

Recently, an increasing number of studies has focused on the involvement of VTA GABAergic neurons in non-reward-related functions, such as sleep and food intake.

Different populations of VTA GABAergic neurons are implicated in the regulation of sleep and wakefulness. Chemogenetic activation of both VTA VGaT$^+$ and VTA GAD67$^+$ neurons promoted the non-rapid eye movement (NREM) sleep phase but did not affect REM sleep duration (Chowdhury et al., 2019; Yu et al., 2019). Inhibition of each of these cell populations resulted in sustained wakefulness (Chowdhury et al., 2019; Yu et al., 2019). Interestingly, specific activation of VGaT$^+$ Nos1$^+$ neurons in the PBP region of the VTA did not generate sleep nor wakefulness, whereas activation of VTA Sst$^+$ and Pv$^+$ neurons each lead to three hours of NREM sleep (Yu et al., 2019). The effects of VTA VGaT$^+$ neuron activation could therefore not be attributed to one cell type. Paradoxically, while lesioning VTA VGaT$^+$ neurons extended wakefulness, the same neurons were active during wake and REM sleep (Yu et al., 2019). On the other hand, VTA GAD67$^+$ neurons showed the highest activity during NREM sleep and the lowest during REM sleep, and this population activity was opposite to that of VTA DA neuronal activity (Chowdhury et al., 2019). Further experiments showed that the wakefulness that resulted from inhibition of VTA VGaT$^+$ neurons was partially due to the local disinhibition of VTA DA neurons. Local inhibition of VTA DA, glutamate and DA/glut neurons elicited by VTA VGaT$^+$ neuron stimulation, as well as activation of the VTA$^{VGaT}$-LH pathway (orexin neurons), accounted for the sustained NREM sleep (Yu et al., 2019). Similar promotion of NREM sleep was achieved when LH orexin neuron-targeting VTA GAD67$^+$ neurons were stimulated (Chowdhury et al., 2019). Interestingly, VGaT$^+$ VTA neuron projections to the LH that expressed Sst induced restorative sleep after social defeat stress (SDS), but not through connections to LH orexin neurons (Yu et al., 2022).

Inhibition or lesion of VTA VGaT$^+$ neurons produces not only sustained wakefulness, but also mania-like behaviours, such as increased locomotor activity (hyperactivity), reduced anxiety in the elevated plus maze test, decreased immobility times on the tail suspension and forced swim tests, heightened sensitivity to amphetamine and sucrose preference (Yu et al., 2021). These effects were mainly due to local disinhibition of VTA DA neurons and disinhibition of the VTA$^{VGaT}$-LH projection (Yu et al., 2021).

Sleep and wakefulness are also controlled by the VP, through projections to the VTA (Li et al., 2021). VP GABAergic neurons showed increased activity during wake and REM sleep, and activation of these neurons established sustained wakefulness (Li et al., 2021). VP GABAergic neurons predominantly targeted VTA GAD67$^+$ neurons, and stimulation of this pathway resulted in disinhibition of VTA TH$^+$ neurons. VP GABAergic neurons also inhibited VTA TH$^+$ neurons to a lesser extent through $GABA_B$ receptors. Interestingly, chemogenetic activation of VP GABAergic neurons increased locomotor activity, but did not influence behaviour in the elevated plus-maze or tail-suspension test (Li et al., 2021). Therefore, the behavioural effects of VP GABAergic neuron activation do not completely overlap with the behaviours observed when stimulating VTA VGaT$^+$ neurons.

VTA GABAergic neurons further influence (palatable) food intake. In juvenile rats, underdeveloped GAD67$^+$ neurons in the VTA were proposed to promote energy intake in juvenile rats, as a mechanism to stimulate growth (Maejima et al., 2019). Lesion of VTA GAD67$^+$ neurons in adult rats increased the consumption of standard chow, but not high-fat (palatable) food (Maejima et al., 2019). In contrast, optogenetic activation of VTA VGaT$^+$ neurons increased palatable food consumption in satiated mice, but reduced intake of standard chow in food-deprived mice (Chen et al., 2020). The overconsumption of

palatable food could be attributed to the activation of the VTA$^{VGaT+}$-LH pathway (Chen et al., 2020). Stimulation of LH neurons leads to overeating as well; however, lesion of VTA VGaT$^{+}$ neurons did not disrupt this effect (Marino et al., 2020).

Together, these studies provide evidence that VTA GABAergic neurons are involved in more behaviours than those related to reward and aversion, namely sleep and food intake. As both of these are related to VTA DAergic neuron (dys)function, it would be interesting to investigate whether VTA GABAergic neurons affect other DAergic neuron-associated behaviours, such as executive functions and working memory.

## VTA GABAergic neurons in stress and addiction

The influence of VTA GABAergic neurons on reward- and aversion-associated behaviour makes them potent regulators of stress and addiction. For instance, VTA GAD65$^{+}$ neurons that target the CeA regulate looming-evoked defensive flight behavior, through glutamatergic stimulation from the SC. Hiding-in-nest behaviour, on the other hand, could be evoked by sustained, but not short-term, inhibition of VTA DAergic neurons, which the authors hypothesised to occur through VTA local GABAergic inhibition (Zhou et al., 2019).

VTA VGaT$^{+}$ neurons are also activated by social stress (Koutlas et al., 2022), and abolish prosocial behaviour when disinhibited through inhibition of BF GABAergic afferents (Wang et al., 2021). Stress can even decrease reward seeking through the VTA$^{VGaT+}$-NAc pathway, and disrupts VTA GABAergic encoding of reward anticipation (Lowes et al., 2021). Moreover, a relationship exists between stress and addiction, as stress can increase ethanol self-administration. This is caused by disordered GABAergic signalling: stress alters the response of VTA GABAergic neurons to GABAergic input, leading to excitation, instead of inhibition of VTA GABAergic neurons (Ostroumov et al., 2016).

The effects of morphine, nicotine, cocaine and THC can be modulated by VTA GABAergic neurons. Morphine-induced conditioned place preference can be disrupted through chronic stimulation of the rVTA$^{GAD65+}$—DRN projection (Li et al., 2019). Sensitivity to nicotine reward is increased by functional upregulation of the α4*-nAChR in VTA GAD65$^{+}$ neurons (Ngolab et al., 2015). Photostimulation of VTA VGaT$^{+}$ neurons inhibited cocaine, but not heroin self-administration (Galaj et al., 2020). Furthermore, intra-NAc shell administration of THC was associated with decreased activity of VTA GABAergic neurons, and produced fear memory salience (Fitoussi et al., 2018). In conclusion, VTA GABAergic neurons can be modulated by stress and have a protective function in addiction behaviour.

## Discussion

A few aspects of GABAergic diversity in the VTA stand out after reading the currently available literature summarised above. Firstly, GABAergic neuronal identity based on gene expression is most related to connectivity, which in turn is dependent on specific location in the VTA. Moreover, many studies that investigated the behaviours associated with VTA GABAergic neurons have employed generic GABAergic markers and as and as a result of that, study results may be difficult to interpret and are sometimes paradoxical. For instance, activation of VTA VGaT$^{+}$ neurons increased high-fat food intake (Chen et al., 2020), but in a different study, lesion of this population had no effect on overeating (Marino et al., 2020). On the other hand, distinct GABAergic subtypes can also have similar effects on behaviour; for example, activation of both VGaT$^{+}$ VTA local DA neuron-inhibiting interneurons and rVTA GAD65$^{+}$ projections to DRN interneurons produced place aversion (Li et al., 2019; Tan et al., 2012). This makes it challenging to assign a specific effect on behaviour to a particular GABAergic population. Besides, the question remains

whether optogenetic activation of cells, as used in these studies, is representative of their activation *in vivo*.

An often-used technique to obtain more specificity in the GABAergic population studied, is by targeting a specific projection; however, this is only possible in the case of projection neurons. Due to the lack of subtype-specific markers for interneurons, the GABAergic local neurons that innervate DA neurons are lumped together. Many studies do not take into account that GABAergic projection neurons may also be targeted unintentionally through this approach, for example when targeting interneurons through VGaT, which is also expressed by LHb-projecting VGluT2$^+$VGaT$^+$ neurons. This confounds the results of these studies and stresses the importance of single cell- and nucleus-sequencing studies specifically in the VTA, through which molecular markers for GABAergic subpopulations can be identified.

Currently, the only published single cell-sequencing data from the VTA specifically has been obtained by Phillips et al. (2022), who created an atlas of transcriptionally defined cell populations in the rat VTA from single nucleus RNA sequencing data. Nine clusters were considered GABAergic, based on their differential gene expression profile: cluster 1, 4 and 6 were exclusively GABAergic, cluster 0, 7, 8 and 10 were GABAergic and glutamatergic, and cluster 2 and 9 were GABAergic, glutamatergic and DAergic. We have mapped potential new molecular markers for GABAergic subpopulations, obtained from the studies described above, against the GABAergic clusters proposed by Phillips et al. (2022) (**Table 1**). Because mGAT1 (*Slc6a1*) has not been used as a marker gene for GABAergic neurons in the VTA in the literature, we have excluded clusters that did not express generic GABAergic markers or were classified as GABAergic based purely on expression of mGAT1 (cluster 0, 2, 7 and 10). In the following discussion, we propose that (at least) six GABAergic subpopulations exist, and we link these to the data on VTA GABAergic molecular marker profiles, circuit and behaviour summarized in the previous sections of this review. This literature synthesis is summarised in table 1.

## Converging VTA GABAergic cellular and functional heterogeneity

### GABA-only subpopulations

Three subpopulations of neurons, clusters 1, 4 and 6, were GABA-only, meaning they express generic markers for GABA (VGaT, GAD65 and/or GAD67) but not glutamate (VGluT2) or DA (TH, DAT). All these subpopulations expressed VGaT. Interestingly, cluster 1 and 6 showed comparable gene expression: both expressed *GAD65* as well as *GAD67*, *Sst*, *Htr2c* and *Zfpm2* (**Table 1**). Thus, these populations likely contain LH-projecting VGaT$^+$Sst$^+$ VTA neurons (Yu et al., 2022), as well as DA neuron-inhibiting VGaT$^+$Sst$^+$ VTA interneurons (Nagaeva et al., 2020). *Nos1*, a marker for GABAergic interneurons (Paul et al., 2018), was only differentially expressed by neurons of cluster 4, which was also the only population expressing *Oprm1*, *Kit* and mGluR1 (*Grm1*). Cluster 4 did not express *GAD65*, *Sst*, *Htr2c* or *Zfpm2*. This suggests that at least two different populations of GABAergic interneurons exist in the VTA, which can be distinguished by their transcriptional profile. Both Nos1$^+$ and Sst$^+$ interneurons were found in the PBP (Nagaeva et al., 2020; Yu et al., 2019), in correspondence with their function in inhibiting DA neurons, which are mostly located in the caudolateral VTA (Morales and Margolis, 2017).

The finding that VGaT$^+$ neurons were active during wake and REM sleep, but activation of VTA VGaT$^+$Nos1$^+$ neurons did not affect sleep (Yu et al., 2019), suggests that neurons from cluster 4 are not involved in sleep regulation. On the other hand, activation of VGaT$^+$Sst$^+$ neurons is involved in NREM sleep and restorative sleep after social defeat stress (SDS) (Yu et al., 2019, 2022). However, activation of Sst$^+$ cells did not account for the full effect on NREM sleep induction obtained by VTA VGaT$^+$ neuron activation (Yu et al., 2019). Furthermore, VGaT$^+$ VTA neurons connecting to LH orexin neurons promote NREM sleep and VTA VGaT$^+$Sst$^+$ neurons drive sleep through the LH, but do not affect restorative sleep (after SDS) through LH orexin neurons (Yu et al., 2019, 2022). Thus, sleep is regulated by different pathways and neuronal subpopulations.

Two GABAergic populations identified by transcriptional profiling were $Sst^+$: cluster 1 and 6. Nagaeva et al. (2020) also identified two $Sst^+$ subpopulations in the PBP, one of which appeared to be interneurons (Nagaeva et al., 2020). It would be interesting to examine whether these two populations match the two $Sst^+$ subpopulations identified by Phillips et al. (2022). If this is the case, cluster 6 (likely GABAergic projection neurons) may contain the $VGaT^+Sst^+$ neurons projecting to the LH that promote sleep. These LH-projecting GABAergic neurons may be the same neurons that increase high-fat food intake when activated (Chen et al., 2020).

We suggest that cluster 1 neurons are GABAergic interneurons that inhibit DAergic neurons, since neurons from exclusively cluster 1 express the $GABA_A$ receptor (Gabra1) and GABAergic interneurons in the VTA are mostly innervated by GABAergic afferents. $GAD65^+$ VTA interneurons that are inhibited by the lateral NAc, resulting in place preference (Yang et al., 2018), are therefore probably amongst the neurons in cluster 1. Also, the $GAD67^+$ VTA interneurons innervated by VP GABAergic neurons (Li et al., 2021), $VGaT^+$ neurons targeted by the LH (Nieh et al., 2016) and GABAergic neurons innervated by the BF (Wang et al., 2021) could belong to this subpopulation. In contrast, VTA $GAD65^+$ neurons targeted by SC glutamatergic neurons that project to the CeA and produce looming-evoked defensive flight behaviour potentially belong to cluster 6, whereas the hiding-in-nest behaviour elicited by activation of $GAD65^+$ DA neuron-inhibiting interneurons may represent general aversive behaviour produced by cluster 1 interneurons (Zhou et al., 2019). A selective marker for cluster 1 may be *Six3*, which, in the developing mouse brain, represents a small subset of GABAergic neurons in the PBP that are of different developmental origin than other GABAergic neurons, namely outside rhombomere 1 (r1) (Lahti et al., 2016). However, neurons of this subpopulation also expressed *Zfpm2*, which does arise from r1 (Lahti et al., 2016). This suggests that within cluster 1, different subpopulations from distinct developmental origins exist.

$GAD67^+Oprm1^+$ neurons are inhibited by BNST afferents (Kudo et al., 2014). These are likely the same neurons as the $VGaT^+$ DA neuron-inhibiting neurons that can produce rewarding and anxiolytic or aversive and anxiogenic behaviour through activation of BNST GABAergic or glutamatergic neurons, respectively (Jennings et al., 2013). However, *Oprm1* is not only expressed by interneurons, as it is also expressed by VTA GABA neurons that innervate DRN interneurons and NAc cholinergic interneurons (Brown et al., 2012; Li et al., 2019; Matsui et al., 2014). Therefore, we propose that neuronal cluster 4 contains both interneurons and projection neurons. Through connections with DRN and NAc, these neurons influence distinct aspects of reward and aversion (**Table 1**).

Many behaviour associated with interneurons cannot be classified into a subcluster, due to the use of generic markers such as VGaT, GAD67 and GAD65 that target more than one subpopulation. Nevertheless, it is clear that the majority of VTA GABAergic subpopulations is involved in reward and aversion, in a variety of ways, and some but not all VTA GABAergic neurons affect sleep.

**GABA-glutamate combinatorial populations**

It is difficult to connect combinatorial neurons to the transcriptional profiles found by Phillips et al. (2022), because none of the clusters showed differential expression of both VGlut and VGaT within one cluster, although many other studies proved the existence of these combinatorial neurons. Cluster 8 from Phillips et al. (2022) was considered GABA-glutamate combinatorial, but did not show differential expression of VGaT, GAD65 or VGluT2, which may be due to comparison with GABA-only and Glut-only populations that very strongly express VGaT and GAD65 or VGluT2, respectively.

$VGluT2^+VGaT^+$ VTA neurons project to the LHb (Root et al., 2020) VP (Yoo et al., 2016), dentate gyrus (Ntamati and Lüscher, 2016) and even to VTA DA neurons (Nagaeva et al., 2020; Yan et al., 2019). Most VTA GABAergic projection neurons innervating the LHb have been shown to co-release glutamate (Root et al., 2020). Therefore, the $GAD65^+ChrnA4^+$ neurons in the caudal VTA that innervate the LHb and do not express CB (Ngolab et al., 2015) are expected to be GABA-glutamate combinatorial neurons. However, cluster 8 showed differential expression of CB (*Calb1*). CB expression in $VGluT2^+VGaT^+$ neurons

was also found by Mongia et al. (2019), suggesting that the $Gad^+CB^+Pv^-$ neurons in the PBP and midline nuclei (Merrill et al., 2015; Olson and Nestler, 2007) may be GABA-glutamate combinatorial neurons.

Regarding behaviour, not much is known about the $VGluT2^+VGaT^+$ population, except for the involvement of the VTA $VGluT2^+VGaT^+$-LHb tract in response to, but not prediction of reward and aversion (Root et al., 2020). Furthermore, stimulation of VTA $VGaT^+$ efferents to the dentate granule cells of the hippocampus did not affect sleep (Yu et al., 2019).

### GABA-glutamate-DA combinatorial populations

Cluster 9 was identified as GABA-glutamate-DA combinatorial population, although no markers for glutamate were expressed differentially. Interestingly, *Slc26a7*, which encodes the anion exchange transporter, was expressed by all cells of this subpopulation ($p = 0$) (Phillips III et al., 2022). Based on the transcriptional profile, this population includes $Th^+GAD65^+$ neurons from the rostromedial VTA which target LHb glutamatergic neurons (Stamatakis et al., 2013). Stimulation of this population led to conditioned place preference (Stamatakis et al., 2013). These neurons could also be distinguished by their lack of hyperpolarisation-activated inward rectifying current ($I_h$), which is typically expressed by most $TH^+$ but not $GAD^+$ neurons in the mouse (Morales and Margolis, 2017; Stamatakis et al., 2013). Other information about connectivity and behaviour of these combinatorial neurons remains elusive.

### $TH^+$ $VGaT^-$ GABA-releasing populations

One $TH^+$ subpopulation in the VTA co-releases DA and GABA onto NAc MSNs, but does not express any generic GABAergic markers (Tritsch et al., 2014). These neurons release GABA through a non-canonical pathway, which is dependent on the vesicular monoamine transporter (VMAT2; *Slc18a2*). $TH^+$ $VGaT^-$ GABA-releasing neurons do not produce GABA, but rely on the membrane GABA transporters mGAT1 (*Slc6a1*) and mGAT4 (*Slc6a11*) for GABA uptake (Berrios et al., 2016; Tritsch et al., 2014). Suppression of GABA release from these neurons resulted in reward-seeking behaviour (Berrios et al., 2016). More data on this subpopulation is currently not available.

## Concluding remarks and future perspectives

The GABAergic population in the VTA is highly diverse and contributes to a variety of behaviours. However, much is still unknown about the exact number of GABAergic subtypes that exist within the VTA, and through which connections each subpopulation contributes to behaviour. The lack of selective markers for GABAergic subpopulations challenges research on individual subtypes. Currently, only one study has attempted to elucidate markers for VTA GABAergic subpopulations through single nucleus RNA sequencing of the rat VTA (Phillips III et al., 2022). It is crucial that more single cell gene expression profiling studies are performed, also in the mouse, in order to create a more robust atlas of GABAergic subpopulations in the rodent VTA. For now, as long as validated selective and specific markers for GABAergic subpopulations are lacking, studies investigating behavioural effects of VTA GABAergic neurons should focus on specific GABAergic projections, to limit confounding variables.

When comparing the single nucleus RNA sequencing study from Phillips III et al. (2022) with other studies that employed optogenetics, immunohistochemistry or in situ hybridisation, some discrepancies stand out. For example, the GABA-glutamate subpopulation from Phillips III et al. (2022) did not observe any differential expression of VGaT and VGluT2 in this population, whereas many studies have shown the existence of this combinatorial subpopulation (Ntamati and Lüscher, 2016; Root et al., 2014a; Yoo et al., 2016). Understandably, the authors may have tried to fit one of their identified subpopulations to these combinatorial GABA-glutamate neurons, since they are aware of its existence. It is important to

realise that there are also some limitations for single nucleus studies: since only neuronal nuclei were sequenced, RNA expression in axon terminals is missing from the data. Moreover, gene expression as depicted in table 1 shows only *differential* gene expression. Lack of differential gene expression of a gene in a certain neuronal population is not necessarily an indication that this gene is not at all expressed in this population; its expression may just not be significantly higher relative to other populations. Thus, VGaT and VGluT2 could still be expressed in cluster 8, albeit less than in GABA-only or glutamate-only populations. Besides this, many of the functional data on GABAergic cells in the VTA originates from mouse data, whereas the single nucleus RNA profiling study was in rat. Although rat and mouse data show much overlap (Merrill et al., 2015), there are some discrepancies between the two, for example in their electrophysiological properties (Morales and Margolis, 2017). This also arises the question whether these studies will translate well to humans.

In conclusion, future research should focus on single cell profiling studies, in order to elucidate GABAergic diversity in the VTA and identify specific molecular marker genes for GABAergic subpopulations. This data should be combined with immunohistochemistry, spatial transcriptomics and single molecular in situ hybridisation techniques, defining GABAergic subtypes in their specific location in the VTA. This will result in a complete picture of the gene and protein expression in both soma/nucleus and axons. Based upon these molecularly distinct subpopulations of GABAergic neurons, tools (e.g. viruses) can be generated and used to target specific GABAergic subpopulations in connective and behavioural studies, enabling a clearer connection between GABAergic subpopulations and the circuitry and behaviour they are associated with. Future literature synthesis approaches could further look into the cellular properties that may define GABAergic subpopulations, which we did not take into account in the current study.

**Table 1. Synthesis of the currently available literature on GABAergic diversity in the VTA.**

| GABAergic population | Marker genes | Circuit | Behaviour |
|---|---|---|---|
| **Cluster 1** ***(Phillips III et al., 2022)*** | Slc32a1, GAD65, GAD67, Sst, Htr2c, CBLN4, Six3, Zfpm2, Pnoc, Gabra1 | VTA GABA --\| VTA DA | Hiding-in-nest behaviour ***(Zhou et al., 2019)*** |
| | | Lateral NAc --\| lVTA GABA --\| VTA DA | Place preference ***(Yang et al., 2018)*** |
| | | VP GABA --\| VTA GABA --\| VTA DA | Sustained wakefulness; increased locomotor activity; no anxiety behaviours ***(Li et al., 2021)*** |
| | | BF --\| VTA GABA --\| VTA DA → NAc | Prosocial behaviour ***(Wang et al., 2021)*** |
| | | LH --\| VTA GABA --\| VTA DA → NAc | Real-time place preference; social interaction; novel object investigation ***(Nieh et al., 2016)*** |
| **Cluster 4** ***(Phillips III et al., 2022)*** | Slc32a1, GAD67, Slc6a1, Nos1, Oprm1, Kit, Grm1, ChrnA6, NRP2, RXFP3, <u>*GAD65*</u> | VTA GABA --\| VTA DA | $VGaT^{+}Nos1^{+}$ neurons: no effect on sleep ***(Yu et al., 2019)*** |
| | | BNST --\| VTA GABA --\| VTA DA | Rewarding and anxiolytic behaviour ***(Kudo et al., 2014)*** |
| | | BNST → VTA GABA --\| VTA DA | Aversive and anxiogenic behaviour ***(Kudo et al., 2014)*** |
| | | VTA GABA --\| NAc cholinergic interneurons | Stimulus-outcome learning ***(Brown et al., 2012)***; alters cue processing for but not consumption of reward, and encodes adaptation of reward-seeking behaviours ***(Wakabayashi et al., 2021; Van Zessen et al., 2012)*** |
| | | VTA GABA (rVTA) --\| DRN interneurons | Place aversion; protective effect on morphine-induced conditioned place preference ***(Li et al., 2019)*** |
| **Cluster 6** ***(Phillips III et al., 2022)*** | Slc32a1, GAD65, GAD67, Slc6a1, Slc6a11, Sst, Htr2c, Crhbp, NRP2, Zfpm2, Drd2 | SC → VTA GABA --\| CeA | Looming-evoked defensive flight behaviour ***(Zhou et al., 2019)*** |
| | | VTA GABA --\| LH | Promotes sleep ***(Yu et al., 2022)***; increases high-fat food intake ***(Chen et al., 2020)*** |
| **Cluster 8** ***(Phillips III et al., 2022)*** | GAD67, Slc6a1, Slc6a11, Calb1, TRH, <u>*VGaT, VGluT2, GAD65, Oprm1, ChrnB2*</u> | VTA $VGluT2^{+}VGaT^{+}$ --\| LHb | Respond to rewarding and aversive stimuli, but not cues ***(Root et al., 2020)*** |
| | | VTA $VGluT2^{+}VGaT^{+}$ --\| dentate granule hippocampal cells | No effect on sleep ***(Yu et al., 2019)*** |
| **Cluster 9** ***(Phillips III et al., 2022)*** | Slc32a1, GAD65, Th, Slc6a3, Cck, Crhbp, ChrnA6, CBLN1, Slc26a7, Ddr2, <u>*Calb1, Wnt7b, NPY*</u> | VTA $TH^{+}GAD65^{+}$ --\| LHb → RMTg --\| DA | Conditioned place preference ***(Stamatakis et al., 2013)*** |
| **$TH^{+}$ GABA-releasing** ***(Tritsch et al., 2014)*** | Th, Slc6a3, Slc18a2, Slc6a1, Slc6a11, Drd2 | $TH^{+}$ GABA-releasing --\| NAc MSNs | Loss of GABA co-release enhances reward-seeking ***(Berrios et al., 2016)*** |

Marker genes not in italics are significant and positive correlations from the single nucleus RNA profiling study by Phillips III et al. (2022). Marker genes depicted underlined and in italics represent our own interpretation of the underlying data. 'X → Y': activating (glutamatergic) interaction, where X activates Y; 'X --| Y': inhibiting (GABAergic) interaction, where X inhibits Y. BF, basal forebrain; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; DA, dopamine; DRN, dorsal raphe nucleus; LH, lateral hypothalamus; LHb, lateral habenula; lVTA, lateral ventral tegmental area; MSNs, medium spiny neurons; NAc, nucleus accumbens; RMTg, rostromedial tegmental nucleus; rVTA, rostral ventral tegmental area; SC, superior colliculus; VP, ventral pallidum; VTA, ventral tegmental area.

# List of abbreviations

| Abbreviation | Definition |
|---|---|
| ADP | Afterdepolarisation |
| BF | Basal forebrain |
| BNST | Bed nucleus of the stria terminalis |
| CB | Calbindin |
| Cck | Cholecystokinin |
| CeA | Central amygdala |
| CIN | Cholinergic interneuron |
| CLi | Caudal linear nucleus |
| CR | Calretinin |
| Crhbp | Corticotropin-release factor-binding protein |
| dmCINs | Dorso-medial-striatal cholinergic interneurons |
| D2R | Dopamine receptor 2 |
| DRN | Dorsal raphe nucleus |
| ECIC | External cortex of the interior commissure |
| GAD65/GAD2 | Glutamate decarboxylase 2 |
| GAD67/GAD1 | Glutamate decarboxylase 1 |
| HFF | High-frequency firing |
| IF | Interfascicular nucleus |
| IPAC | Interstitial nucleus of the posterior limb of the anterior commissure |
| LDTg | Laterodorsal tegmental nucleus |
| LH | Lateral hypothalamus |
| LHb | Lateral habenula |
| LPO | Lateral preoptic nucleus |
| MCPO | Magnocellular preoptic nuclei |
| MOR | μ-opioid receptor |
| MSN | Medium spiny neuron |
| mVTA | Medial VTA |
| NAc | Nucleus accumbens |
| nAChR | Nicotinic acetylcholine receptor |
| NAcLat | Lateral nucleus accumbens |
| NPY | Neuropeptide Y |
| NREM | Non-rapid eye movement |
| PAG | Periaqueductal gray |
| PBP | Parabrachial pigmented nucleus |
| Pf | Parafascicular thalamic nucleus |
| PFC | Prefrontal cortex |
| PN | Paranigral nucleus |
| Pv | Parvalbumin |
| RLi | Rostral linear nucleus |
| rVTA | Rostral VTA |
| SC | Superior colliculus |
| Sst | Somatostatin |
| TH | Tyrosine hydroxylase |
| VGAT | Vesicular GABA transporter |
| VMAT2 | Vesicular monoamine transporter |
| VP | Ventral pallidum |

# References


Beier, K.T., Steinberg, E.E., Deloach, K.E., Xie, S., Miyamichi, K., Schwarz, L., Gao, X.J., Kremer, E.J., Malenka, R.C., and Luo, L. (2015). Circuit Architecture of VTA Dopamine Neurons Revealed by Systematic Input-Output Mapping. Cell *162*, 622–634.

Berrios, J., Stamatakis, A.M., Kantak, P.A., McElligott, Z.A., Judson, M.C., Aita, M., Rougie, M., Stuber, G.D., and Philpot, B.D. (2016). Loss of UBE3A from TH-expressing neurons suppresses GABA co-release and enhances VTA-NAc optical self-stimulation. Nat. Commun. *7*, 1–8.

Breton, J.M., Charbit, A.R., Snyder, B.J., Fong, P.T.K., Dias, E. V., Himmels, P., Lock, H., and Margolis, E.B. (2019). Relative contributions and mapping of ventral tegmental area dopamine and GABA neurons by projection target in the rat. J. Comp. Neurol. *527*, 916–941.

Brown, M.T.C., Tan, K.R., O'Connor, E.C., Nikonenko, I., Muller, D., and Lüscher, C. (2012). Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning. Nature *492*, 452–456.

Carr, D.B., and Sesack, S.R. (2000). Projections from the rat prefrontal cortex to the ventral tegmental area: Target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. J. Neurosci. *20*, 3864–3873.

Chen, L., Lu, Y.P., Chen, H.Y., Huang, S.N., Guo, Y.R., Zhang, J.Y., Li, Q.X., Luo, C.Y., Lin, S.W., Chen, Z.N., et al. (2020). Ventral tegmental area GABAergic neurons induce anxiety-like behaviors and promote palatable food intake. Neuropharmacology *173*, 108114.

Chieng, B., Azriel, Y., Mohammadi, S., and Christie, M.J. (2011). Distinct cellular properties of identified dopaminergic and GABAergic neurons in the mouse ventral tegmental area. J. Physiol. *589*, 3775–3787.

Chowdhury, S., Matsubara, T., Miyazaki, T., Ono, D., Fukatsu, N., Abe, M., Sakimura, K., Sudo, Y., and Yamanaka, A. (2019). GABA neurons in the ventral tegmental area regulate non-rapid eye movement sleep in mice. Elife *8*, 1–26.

Cohen, J.Y., Haesler, S., Vong, L., Lowell, B.B., and Uchida, N. (2012). Neuron-type-specific signals for reward and punishment in the ventral tegmental area. Nature *482*, 85–88.

Eshel, N., Bukwich, M., Rao, V., Hemmelder, V., Tian, J., and Uchida, N. (2015). Arithmetic and local circuitry underlying dopamine prediction errors. Nature *525*, 243–246.

Faget, L., Osakada, F., Duan, J., Ressler, R., Johnson, A.B., Proudfoot, J.A., Yoo, J.H., Callaway, E.M., and Hnasko, T.S. (2016). Afferent Inputs to Neurotransmitter-Defined Cell Types in the Ventral Tegmental Area. Cell Rep. *15*, 2796–2808.

Fitoussi, A., Zunder, J., Tan, H., and Laviolette, S.R. (2018). Delta-9-tetrahydrocannabinol potentiates fear memory salience through functional modulation of mesolimbic dopaminergic activity states. Eur. J. Neurosci. *47*, 1385–1400.

Galaj, E., Han, X., Shen, H., Jordan, C.J., He, Y., Humburg, B., Bi, G.H., and Xi, Z.X. (2020). Dissecting the role of GABA neurons in the VTA versus SNr in opioid reward. J. Neurosci. *40*, 8853–8869.

Gomez, J.A., Perkins, J.M., Beaudoin, G.M., Cook, N.B., Quraishi, S.A., Szoeke, E.A., Thangamani, K., Tschumi, C.W., Wanat, M.J., Maroof, A.M., et al. (2019). Ventral tegmental area astrocytes orchestrate avoidance and approach behavior. Nat. Commun. *10*, 1–13.

Hughes, R.N., Watson, G.D.R., Petter, E.A., Kim, N., Bakhurin, K.I., and Yin, H.H. (2019). Precise Coordination of Three-Dimensional Rotational Kinematics by Ventral Tegmental Area GABAergic Neurons. Curr. Biol. *29*, 3244-3255.e4.

Jennings, J.H., Sparta, D.R., Stamatakis, A.M., Ung, R.L., Pleil, K.E., Kash, T.L., and Stuber, G.D. (2013). Distinct extended amygdala circuits for divergent motivational states. Nature *496*, 224–228.

Kim, H.J., Kim, M., Kang, B., Yun, S., Ryeo, S.E., Hwang, D., and Kim, J.H. (2019). Systematic analysis of expression signatures of neuronal subpopulations in the VTA. Mol. Brain *12*, 1–9.

Korotkova, T.M., Sergeeva, O.A., Eriksson, K.S., Haas, H.L., and Brown, R.E. (2003). Excitation of Ventral Tegmental Area Dopaminergic and Nondopaminergic Neurons by Orexins / Hypocretins. J. Neurosci. *23*, 7–11.

Koutlas, I., Linders, L.E., van der Starre, S.E., Wolterink-Donselaar, I.G., Adan, R.A.H., and Meye, F.J. (2022). Characterizing and TRAPing a Social Stress-Activated Neuronal Ensemble in the Ventral Tegmental Area. Front. Behav. Neurosci. *16*.

Kudo, T., Konno, K., Uchigashima, M., Yanagawa, Y., Sora, I., Minami, M., and Watanabe, M. (2014). GABAergic neurons in the ventral tegmental area receive dual GABA/enkephalin-mediated inhibitory inputs from the bed nucleus of the stria terminalis. Eur. J. Neurosci. *39*, 1796–1809.

Lahti, L., Haugas, M., Tikker, L., Airavaara, M., Voutilainen, M.H., Anttila, J., Kumar, S., Inkinen, C., Salminen, M., and Partanen, J. (2016). Differentiation and molecular heterogeneity of inhibitory and excitatory neurons associated with midbrain dopaminergic nuclei. Dev. *143*, 516–529.

Li, Y., Li, C.Y., Xi, W., Jin, S., Wu, Z.H., Jiang, P., Dong, P., He, X. Bin, Xu, F.Q., Duan, S., et al. (2019). Rostral and Caudal Ventral Tegmental Area GABAergic Inputs to Different Dorsal Raphe Neurons Participate in Opioid Dependence. Neuron *101*, 748-761.e5.

Li, Y.D., Luo, Y.J., Xu, W., Ge, J., Cherasse, Y., Wang, Y.Q., Lazarus, M., Qu, W.M., and Huang, Z.L. (2021). Ventral pallidal GABAergic neurons control wakefulness associated with motivation through the ventral tegmental pathway. Mol. Psychiatry *26*, 2912–2928.

Lowes, D.C., Chamberlin, L.A., Kretsge, L.N., Holt, E.S., Abbas, A.I., Park, A.J., Yusufova, L., Bretton, Z.H., Firdous, A., Enikolopov, A.G., et al. (2021). Ventral tegmental area GABA neurons mediate stress-induced blunted reward-seeking in mice. Nat. Commun. *12*, 1–13.

Maejima, Y., Yokota, S., Horita, S., and Shimomura, K. (2019). The undeveloped properties of GABA neurons in the ventral tegmental area promote energy intake for growth in juvenile rats. Sci. Rep. *9*, 1–12.

Margolis, E.B., Toy, B., Himmels, P., Morales, M., and Fields, H.L. (2012). Identification of rat ventral tegmental area GABAergic neurons. PLoS One *7*.

Marino, R.A.M., McDevitt, R.A., Gantz, S.C., Shen, H., Pignatelli, M., Xin, W., Wise, R.A., and Bonci, A. (2020). Control of food approach and eating by a GABAergic projection from lateral hypothalamus to dorsal pons. Proc. Natl. Acad. Sci. U. S. A. *117*, 8611–8615.

Matsui, A., Jarvie, B.C., Robinson, B.G., Hentges, S.T., and Williams, J.T. (2014). Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. Neuron *82*, 1346–1356.

Merrill, C.B., Friend, L.N., Newton, S.T., Hopkins, Z.H., and Edwards, J.G. (2015). Ventral tegmental area dopamine and GABA neurons: Physiological properties and expression of mRNA for endocannabinoid biosynthetic elements. Sci. Rep. *5*, 1–16.

Miranda-Barrientos, J., Chambers, I., Mongia, S., Liu, B., Wang, H.L., Mateo-Semidey, G.E., Margolis, E.B., Zhang, S., and Morales, M. (2021). Ventral tegmental area GABA, glutamate, and glutamate-GABA neurons are heterogeneous in their electrophysiological and pharmacological properties. Eur. J. Neurosci. *54*, 4061–4084.

Mongia, S., Yamaguchi, T., Liu, B., Zhang, S., Wang, H., and Morales, M. (2019). The Ventral Tegmental Area has calbindin neurons with the capability to co-release glutamate and dopamine into the nucleus accumbens. Eur. J. Neurosci. *50*, 3968–3984.

Morales, M., and Margolis, E.B. (2017). Ventral tegmental area: Cellular heterogeneity, connectivity and behaviour. Nat. Rev. Neurosci. *18*, 73–85.

Nagaeva, E., Zubarev, I., Gonzales, C.B., Forss, M., Nikouei, K., de Miguel, E., Elsilä, L., Linden, A.M., Hjerling-Leffler, J., Augustine, G.J., et al. (2020). Heterogeneous somatostatin-expressing neuron population in mouse ventral tegmental area. Elife *9*, 1–29.

Nair-Roberts, R.G., Chatelain-Badie, S.D., Benson, E., White-Cooper, H., Bolam, J.P., and Ungless, M.A. (2008). Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. Neuroscience *152*, 1024–1031.

Ngolab, J., Liu, L., Zhao-Shea, R., Gao, G., Gardner, P.D., and Tapper, A.R. (2015). Functional upregulation of α4* nicotinic acetylcholine receptors in VTA GABAergic neurons increases sensitivity to nicotine reward. J. Neurosci. *35*, 8570–8578.

Nieh, E.H., Vander Weele, C.M., Matthews, G.A., Presbrey, K.N., Wichmann, R., Leppla, C.A., Izadmehr, E.M., and Tye, K.M. (2016). Inhibitory Input from the Lateral Hypothalamus to the Ventral Tegmental Area Disinhibits Dopamine Neurons and Promotes Behavioral Activation. Neuron *90*, 1286–1298.

Ntamati, N.R., and Lüscher, C. (2016). VTA projection neurons releasing GABA and glutamate in the dentate gyrus. ENeuro *3*, 1–11.

Ntamati, N.R., Creed, M., Achargui, R., and Lüscher, C. (2018). Periaqueductal efferents to dopamine and GABA neurons of the VTA. PLoS One *13*, 1–11.

Olson, V.G., and Nestler, E.J. (2007). Topographical Organization of GABAergic Neurons Within the Ventral Tegmental Area of the Rat. Synapse *61*, 87–95.

Ostroumov, A., Thomas, A.M., Kimmey, B.A., Karsch, J.S., Doyon, W.M., and Dani, J.A. (2016). Stress Increases Ethanol Self-Administration via a Shift toward Excitatory GABA Signaling in the Ventral Tegmental Area. Neuron *92*, 493–504.

Paul, E.J., Kalk, E., Tossell, K., Irvine, E.E., Franks, N.P., Wisden, W., Withers, D.J., Leiper, J., and Ungless, M.A. (2018). nNOS-expressing neurons in the ventral tegmental area and substantia nigra pars compacta. ENeuro *5*.

Paul, E.J., Tossell, K., and Ungless, M.A. (2019). Transcriptional profiling aligned with in situ expression image analysis reveals mosaically expressed molecular markers for GABA neuron sub-groups in the ventral tegmental area. Eur. J. Neurosci. *50*, 3732–3749.

Phillips III, R.A., Tuscher, J.J., Black, S.L., Andraka, E., Fitzgerald, N.D., Ianov, L., and Day, J.J. (2022). An atlas of transcriptionally defined cell populations in the rat ventral tegmental area. Cell Rep. *39*, 110616.

Polter, A.M., Barcomb, K., Tsuda, A.C., and Kauer, J.A. (2018). Synaptic function and plasticity in identified inhibitory inputs onto VTA dopamine neurons. Eur. J. Neurosci. *47*, 1208–1218.

Poulin, J.F., Gaertner, Z., Moreno-Ramos, O.A., and Awatramani, R. (2020). Classification of Midbrain Dopamine Neurons Using Single-Cell Gene Expression Profiling Approaches. Trends Neurosci. *43*, 155–169.

Rizzi, G., Li, Z., Hogrefe, N., and Tan, K.R. (2021). Lateral ventral tegmental area GABAergic and glutamatergic modulation of conditioned learning. Cell Rep. *34*, 108867.

Root, D.H., Mejias-Aponte, C.A., Zhang, S., Wang, H.L., Hoffman, A.F., Lupica, C.R., and Morales, M. (2014a). Single rodent mesohabenular axons release glutamate and GABA. Nat. Neurosci. *17*, 1543–1551.

Root, D.H., Mejias-Aponte, C.A., Qi, J., and Morales, M. (2014b). Role of glutamatergic projections from ventral tegmental area to lateral Habenula in aversive conditioning. J. Neurosci. *34*, 13906–13910.

Root, D.H., Zhang, S., Barker, D.J., Miranda-Barrientos, J., Liu, B., Wang, H.L., and Morales, M. (2018). Selective Brain Distribution and Distinctive Synaptic Architecture of Dual Glutamatergic-GABAergic Neurons. Cell Rep. *23*, 3465–3479.

Root, D.H., Barker, D.J., Estrin, D.J., Miranda-Barrientos, J.A., Liu, B., Zhang, S., Wang, H.L., Vautier, F., Ramakrishnan, C., Kim, Y.S., et al. (2020). Distinct Signaling by Ventral Tegmental Area Glutamate, GABA, and Combinatorial Glutamate-GABA Neurons in Motivated Behavior. Cell Rep. *32*, 108094.

Shields, A.K., Suarez, M., Wakabayashi, K.T., and Bass, C.E. (2021). Activation of VTA GABA neurons disrupts reward seeking by altering temporal processing. Behav. Brain Res. *410*, 113292.

Stamatakis, A.M., Jennings, J.H., Ung, R.L., Blair, G.A., Weinberg, R.J., Neve, R.L., Boyce, F., Mattis, J., Ramakrishnan, C., Deisseroth, K., et al. (2013). A Unique Population of Ventral Tegmental Area Neurons Inhibits the Lateral Habenula to Promote Reward. Neuron *80*, 1039–1053.

Steffensen, S.C., Shin, S.I., Nelson, A.C., Pistorius, S.S., Williams, S.B., Woodward, T.J., Park, H.J., Friend, L., Gao, M., Gao, F., et al. (2018). A6 Subunit-Containing Nicotinic Receptors Mediate Low-Dose Ethanol Effects on Ventral Tegmental Area Neurons and Ethanol Reward. Addict. Biol. *23*, 1079–1093.

Su, X.Y., Chen, M., Yuan, Y., Li, Y., Guo, S.S., Luo, H.Q., Huang, C., Sun, W., Li, Y., Zhu, M.X., et al. (2019). Central Processing of Itch in the Midbrain Reward Center. Neuron *102*, 858-872.e5.

Tan, K.R., Yvon, C., Turiault, M., Mirzabekov, J.J., Doehner, J., Labouèbe, G., Deisseroth, K., Tye, K.M., and Lüscher, C. (2012). GABA Neurons of the VTA Drive Conditioned Place Aversion. Neuron *73*, 1173–1183.

Taylor, S.R., Badurek, S., Dileone, R.J., Nashmi, R., Minichiello, L., and Picciotto, M.R. (2014). GABAergic and glutamatergic efferents of the mouse ventral tegmental area. J. Comp. Neurol. *522*, 3308–3334.

Tolu, S., Eddine, R., Marti, F., David, V., Graupner, M., Pons, S., Baudonnat, M., Husson, M., Besson, M., Reperant, C., et al. (2013). Co-activation of VTA da and GABA neurons mediates nicotine reinforcement. Mol. Psychiatry *18*, 382–393.

Tritsch, N.X., Oh, W.J., Gu, C., and Sabatini, B.L. (2014). Midbrain dopamine neurons sustain inhibitory transmission using plasma membrane uptake of GABA, not synthesis. Elife *2014*, 1–20.

Viereckel, T., Dumas, S., Smith-Anttila, C.J.A., Vlcek, B., Bimpisidis, Z., Lagerström, M.C., Konradsson-Geuken, A., and Wallén-Mackenzie, A. (2016). Midbrain Gene Screening Identifies a New Mesoaccumbal Glutamatergic Pathway and a Marker for Dopamine Cells Neuroprotected in Parkinson's Disease. Sci. Rep. *6*, 1–16.

Wakabayashi, K.T., Feja, M., Baindur, A.N., Bruno, M.J., Bhimani, R. V., Park, J., Hausknecht, K., Shen, R.Y., Haj-Dahmane, S., and Bass, C.E. (2019). Chemogenetic activation of ventral tegmental area GABA neurons, but not mesoaccumbal GABA terminals, disrupts responding to reward-predictive cues. Neuropsychopharmacology *44*, 372–380.

Wakabayashi, K.T., Feja, M., Leigh, M.P.K., Baindur, A.N., Suarez, M., Meyer, P.J., and Bass, C.E. (2021). Chemogenetic Activation of Mesoaccumbal Gamma-Aminobutyric Acid Projections Selectively Tunes Responses to Predictive Cues When Reward Value Is Abruptly Decreased. Biol. Psychiatry *89*, 366–375.

Wang, H., and Morales, M. (2008). Corticotropin-Releasing Factor Binding Protein Within the Ventral Tegmental Area Is Expressed in a Subset of Dopaminergic Neurons. J. Comp. Neurol. *509*, 302–318.

Wang, J., Li, J., Yang, Q., Xie, Y.K., Wen, Y.L., Xu, Z.Z., Li, Y., Xu, T., Wu, Z.Y., Duan, S., et al. (2021). Basal forebrain mediates prosocial behavior via disinhibition of midbrain dopamine neurons. Proc. Natl. Acad. Sci. U. S. A. *118*.

Yan, Y., Beckley, N.A., Kim, V.J., and Drenan, R.M. (2019). Differential nicotinic modulation of glutamatergic and GABAergic VTA microcircuits. ENeuro *6*, 1–12.

Yang, H., de Jong, J.W., Tak, Y.E., Peck, J., Bateup, H.S., and Lammel, S. (2018). Nucleus Accumbens Subnuclei Regulate Motivated Behavior via Direct Inhibition and Disinhibition of VTA Dopamine Subpopulations. Neuron *97*, 434-449.e4.

Yoo, J.H., Zell, V., Gutierrez-Reed, N., Wu, J., Ressler, R., Shenasa, M.A., Johnson, A.B., Fife, K.H., Faget, L., and Hnasko, T.S. (2016). Ventral tegmental area glutamate neurons co-release GABA and promote positive reinforcement. Nat. Commun. *7*, 1–13.

Yu, X., Li, W., Ma, Y., Tossell, K., Harris, J.J., Harding, E.C., Ba, W., Miracca, G., Wang, D., Li, L., et al. (2019). GABA and glutamate neurons in the VTA regulate sleep and wakefulness. Nat. Neurosci. *22*, 106–119.

Yu, X., Ba, W., Zhao, G., Ma, Y., Harding, E.C., Yin, L., Wang, D., Li, H., Zhang, P., Shi, Y., et al. (2021). Dysfunction of ventral tegmental area GABA neurons causes mania-like behavior. Mol. Psychiatry *26*, 5213–5228.

Yu, X., Zhao, G., Wang, D., Wang, S., Li, R., Li, A., Wang, H., Nollet, M., Chun, Y.Y., Zhao, T., et al. (2022). A specific circuit in the midbrain detects stress and induces restorative sleep. Science *377*, 63–72.

Van Zessen, R., Phillips, J.L., Budygin, E.A., and Stuber, G.D. (2012). Activation of VTA GABA Neurons Disrupts Reward Consumption. Neuron *73*, 1184–1194.

Zhou, Z., Liu, X., Chen, S., Zhang, Z., Liu, Y., Montardy, Q., Tang, Y., Wei, P., Liu, N., Li, L., et al. (2019). A VTA GABAergic Neural Circuit Mediates Visually Evoked Innate Defensive Responses. Neuron *103*, 473-488.e6.