

Strikingly different neurotransmitter release strategies in dopaminergic subclasses

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eLife Assessment

This study presents **solid** evidence for distinct neurotransmitter release modalities between two subclasses of dopaminergic neurons in the olfactory bulb, highlighting an **important** finding that dendritic neurotransmitter release in anaxonic neurons and axonal neurotransmitter release in axon-bearing neurons, and GABAergic self-inhibition in anaxonic neurons emphasizes the functional differences between these neuronal subtypes. However, some experiments looked **incomplete** with a relatively small sample size (low n). The conclusion would benefit significantly from additional validations.

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Abstract

Neuronal function is intimately tied to axodendritic polarity. Neurotransmitter release, for example, is usually the role of the axon. There are widespread exceptions to this rule, however, including many mammalian neuronal types that can release neurotransmitter from their dendrites. In the mouse olfactory bulb, closely related subclasses of dopaminergic interneuron differ markedly in their polarity, with one subtype lacking an axon entirely. These axon-bearing and anaxonic dopaminergic subclasses have distinct developmental profiles and sensory responses, but how their fundamental polarity differences translate to functional outputs remains entirely unknown. Here, we provide anatomical evidence for distinct neurotransmitter release strategies among these closely related dopaminergic subtypes: anaxonic cells release from their dendrites, while axon-bearing neurons release exclusively from their intermittently myelinated axon. These structural differences are linked to a clear functional distinction: anaxonic, but not axon-bearing dopaminergic neurons are capable of self-inhibition. Our findings suggest that variations in polarity can produce striking distinctions in neuronal outputs, and that even closely related neuronal subclasses may play entirely separate roles in sensory information processing.

Introduction

Neurons are one of the most striking examples of cell polarisation. Most neurons have canonical axodendritic polarity, defined by the presence of a somatodendritic domain and a single axon (Banker, 2018 [🔗](#)). The morphological and molecular specialisation of these distinct compartments gives them unique functional properties, with dendrites usually receiving most synaptic inputs, and axons sending outputs to other neurons by releasing neurotransmitters. However, exceptions to this neuronal polarity dogma are common. In the mammalian brain, there are neurons – including midbrain dopaminergic cells (Pucak and Grace, 1994 [🔗](#); Falkenburger et al., 2001 [🔗](#)) and cortical bitufted cells (Zilberter et al., 1999 [🔗](#)) – that release neurotransmitter from their dendrites. There are also neurons – including retinal amacrine cells (Wu et al., 2011 [🔗](#); Goillard et al., 2020 [🔗](#)) – that lack canonical axons altogether. A remarkable hub for such unconventional neuronal polarity is the olfactory bulb (OB), the first part of the brain that processes information about the sense of smell. Dendritic release of neurotransmitters is a widespread phenomenon in the OB, even in projection neurons and other cells with classically defined axodendritic polarity (Schoppa and Urban, 2003 [🔗](#); Hayar et al., 2004 [🔗](#); Imamura et al., 2020 [🔗](#)). In addition, non-canonical polarity is extremely common in the OB, where almost all GABAergic interneurons lack an axon entirely (Tufo et al., 2022 [🔗](#)).

Neuronal polarity in the OB can be completely different even in closely related neuronal subtypes. In the glomerular layer, a major class of interneurons releases both dopamine and GABA in order to modulate neurotransmitter release from olfactory sensory neuron terminals and to regulate downstream processing in local circuits (Wachowiak and Cohen, 1999 [🔗](#); Berkowicz and Trombley, 2000 [🔗](#); Borisovska et al., 2013 [🔗](#); Liu et al., 2013 [🔗](#); Banerjee et al., 2015 [🔗](#); Vaaga et al., 2017 [🔗](#)). We recently found that these dopaminergic (DA) neurons can be classified into two subtypes depending on a binary morphological distinction: the presence or absence of an axon. This fundamental divergence in polarity is correlated with different excitability profiles and odour response properties (Chand et al., 2015 [🔗](#); Galliano et al., 2018 [🔗](#); Lau et al., 2024 [🔗](#)). However, the specific function of these two neuron subtypes within OB glomerular networks is not fully understood. A crucial outstanding question is how the structural differences in these two neuron subtypes translate into strategies for influencing bulbar circuits via neurotransmitter release.

Anaxonic DA neurons only have a soma and dendrites (Chand et al., 2015 [🔗](#); Galliano et al., 2018 [🔗](#)) making these the sole available domains for neurotransmitter release. Indeed, dendritic presynaptic structures are present in DA neurons (Kiyokage et al., 2017 [🔗](#)), and are associated with these cells' functional ability to self-inhibit via local GABA release (Smith and Jahr, 2002 [🔗](#); Murphy et al., 2005 [🔗](#); Maher and Westbrook, 2008 [🔗](#)). On the other hand, axon-bearing DA neurons are known to be the sole source of long-range interglomerular connections in the OB (Kosaka and Kosaka, 2008 [🔗](#)), with functional studies showing that these cells release neurotransmitters over these long-distance axonal projections (Liu et al., 2013 [🔗](#); Whitesell et al., 2013 [🔗](#); Banerjee et al., 2015 [🔗](#)). However, in a brain region where dendritic release is the norm rather than the exception, what remains unknown is whether axon-bearing DAs combine this distal, interglomerular signalling with local, intraglomerular inhibition via release from their dendrites. Answering this question has important implications for how information is processed in early olfactory circuits.

Here, we provide anatomical evidence showing that axon-bearing OB DA neurons have axons with neurotransmitter release sites, while their dendrites lack these sites almost entirely. On the other hand, we found that all anaxonic DA neurons have release sites on their dendrites. These structural differences are associated with a striking functional distinction: while all anaxonic

neurons can self-inhibit, there is an almost total absence of self-inhibition responses in the axon-bearing subpopulation. This polarity-based dichotomy suggests that even these closely related interneuron subtypes may play entirely separate roles in the processing of sensory information.

Results

Labelling putative presynaptic structures in individual olfactory bulb dopaminergic neurons

We first developed a structural approach for labelling putative presynaptic sites in individual OB DA neurons. We observed that synaptophysin, a membrane protein located in vesicles at the presynapse, is expressed in OB DA cells at both the mRNA (**Figure 1A**) and protein (**Figure 1B**) levels. Given the high synaptic density and complexity of OB glomeruli, it is not possible to reliably assign specific synaptophysin label to individual DA neurons using widespread staining of endogenous protein. We therefore chose a sparse labelling approach based on the injection of an AAV-DJ-hSyn-FLEX-mGFP-T2A-Synaptophysin-mRuby construct (Beier et al., 2015; Kathe, et al., 2022). This vector allows the expression in individual cre-expressing cells of a membrane-bound GFP for cell morphology, and fluorophore-tagged full-length synaptophysin for the visualisation of putative presynaptic puncta. There is strong evidence showing that the overexpression of fluorophore-tagged synaptophysin is functionally benign (Granseth et al., 2006; Li and Tsien, 2012).

We performed *in utero* injections in embryonic day 14 (E14) vGAT (vesicular GABA transporter)-cre mice. This timepoint ensures the effective labelling of axon-bearing and anaxonic DA neurons, since both cell types are generated during embryonic development. (**Figure 1C**, Batista-Brito et al., 2008; Galliano et al., 2018). Dopaminergic neurons were then identified via immunostaining against tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine (Galliano et al., 2018). We were able to detect well-defined synaptophysin-mRuby puncta along the processes of TH+ GFP+ neurons (**Figure 1D**).

To assess whether this method labels reliable presynaptic release sites in DA neurons, we co-stained the injected tissue against vGAT and observed high co-localisation between endogenous vGAT and mRuby label (**Figure 1E**; 39/53 = 74 % synaptophysin-mRuby puncta co-localised with vGAT; n = 9 cells from N = 4 mice). These results demonstrate that a large proportion of synaptophysin-mRuby puncta are likely to reflect putative presynaptic release sites in OB DA neurons.

Anaxonic DA neurons have dendritic neurotransmitter release sites

Given that anaxonic DA neurons lack an axon, and are known to release both GABA and dopamine, to be able to release neurotransmitter they must have presynaptic release sites on their dendrites. We identified this neuronal subtype in our population of GFP+/TH+ neurons by the lack of the proximal axonal marker TRIM46 (**Figure 2A**, van Beuningen et al., 2015; Galliano et al., 2018).

As predicted, we found that all anaxonic neurons had synaptophysin-mRuby puncta in their dendrites (**Figure 2B, C**; n=9 anaxonic DA cells from N=4 mice). The thin slices necessary for puncta detection precluded any comprehensive analysis of dendritic morphology or synaptic distributions. Nevertheless, we saw no consistent evidence for spatial patterns, clusters, or privileged branches; instead, puncta were distributed throughout each cell's dendritic arbour (**Figure 2C**). These results provide anatomical evidence for the presence of dendritic neurotransmitter release sites in anaxonic DA neurons.

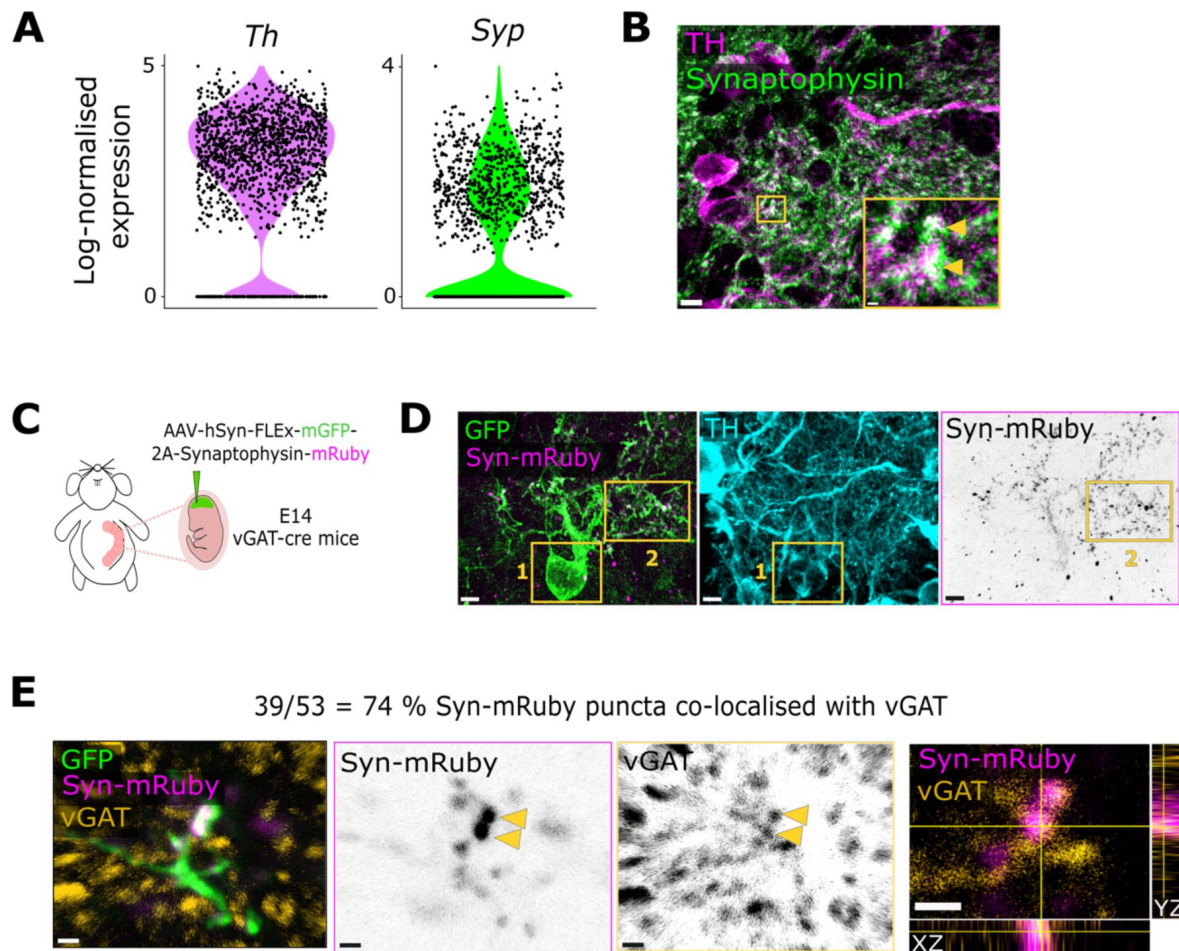


Figure 1.

Labelling putative presynaptic structures in individual olfactory bulb dopaminergic neurons.

(**A**) Log₂-normalised expression of *Th* and *Syn* mRNA in DA neurons from single cell RNA sequencing data (Brann et al., 2020). (**B**) Example snapshot of a 3D confocal stack of endogenous immunostaining for synaptophysin (green) and TH (magenta) in the glomerular layer of the OB. Yellow arrowheads point to small clusters where TH and synaptophysin co-localise. Scalebars: 5 μ m (main) and 0.5 μ m (inset). (**C**) Strategy to label putative presynaptic release sites in individual DA neurons. (**D**) Example snapshot of a 3D confocal stack of a successfully labelled DA cell. Inset 1 reveals the TH+ dopaminergic identity of the neuron (cyan), and inset 2 highlights the Syn-mRuby puncta (magenta, black). Scalebars, 5 μ m. (**E**) Example snapshot of a 3D confocal stack of a GFP+ (green), Syn-mRuby+ (magenta, black) neuronal process co-stained with vGAT (orange, black). Yellow arrowheads indicate examples where Syn-mRuby and vGAT puncta co-localise. The last panel shows the orthogonal views of the bottom puncta. Scalebars, 1 μ m.

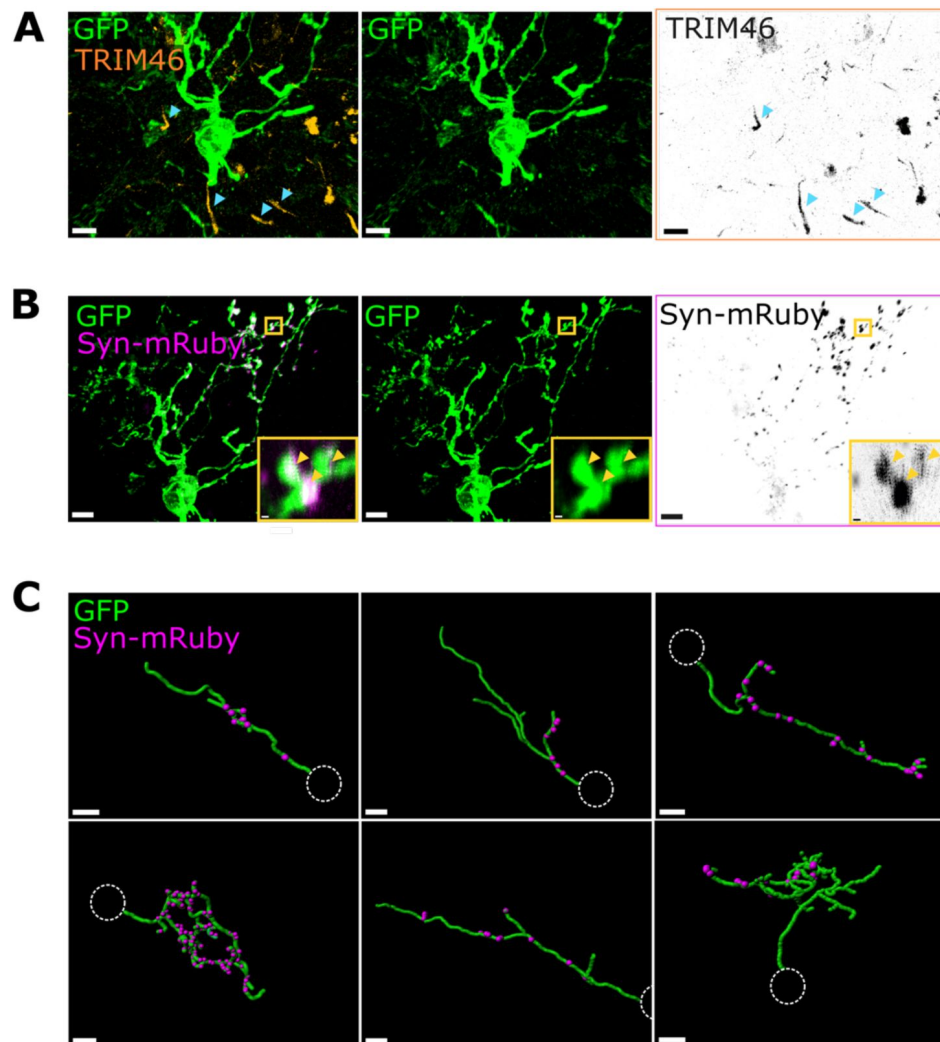


Figure 2.

Anaxonic DA neurons have dendritic neurotransmitter release sites.

(A) Example snapshot of a 3D confocal stack of a TRIM46-anaxonic DA neuron. Blue arrowheads point to examples of other TRIM46+ AISs (orange, black) in the same region which do not co-localise with this neuron's GFP signal. Scalebars, 5 μ m. **(B)** Snapshot of the same neuron in (A) showing Synaptophysin-mRuby Puncta (magenta, black) on the dendrites. Yellow inset highlights a region of the neuron with multiple mRuby+ puncta within the GFP+ (green) processes (yellow arrows). Scalebars, 5 μ m and 0.5 μ m. **(C)** Example snapshots from 3D dendritic reconstructions (green, GFP) and presynaptic puncta detection (magenta, Syn-mRuby) of anaxonic DA neurons. Note: these are not full dendritic reconstructions, but example dendrites. Dotted white circle represents the soma location. Scalebars, 5 μ m.

Axonic DA neurons have release sites on their intermittently myelinated axons, but not on their dendrites

Long-range GABA release has been functionally demonstrated in dopaminergic neurons (Liu et al., 2013 [↗](#); Whitesell et al., 2013 [↗](#); Banerjee et al., 2015 [↗](#)), strongly suggesting that axon-bearing DA neurons have axonal release sites. It was not possible to follow TRIM46+ DA axons for long distances from the soma in thin brain slices, so instead we used the distal axon marker myelin basic protein (MBP). In the first description of myelination in this cell type, we found that DA axons are myelinated in an intermittent pattern, a common trait in other brain regions (**Figure 3A** [↗](#), Tomassy et al., 2014 [↗](#)). Using MBP as indicator of axon identity, we were then able to locate synaptophysin-mRuby puncta in unmyelinated sections of GFP+ TH+ axons (**Figure 3B** [↗](#); n=2 axon-bearing DA cells from N=1 mouse). As predicted, axon-bearing DA neurons therefore have putative presynaptic release sites on their axons.

Most neurons with canonical axodendritic polarity in the olfactory bulb release neurotransmitters from both axons and dendrites (Castro and Urban, 2009 [↗](#)). To assess if this scenario also holds true for DA axon-bearing cells, we explored the dendritic localisation of synaptophysin-mRuby-labelled presynaptic release sites in GFP+ neurons with clear TRIM46+ proximal axonal domains (**Figure 3C** [↗](#)). As expected, these axon-bearing OB DA cells had larger somas than their anaxonic neighbours (**Figure 3D** [↗](#); soma area: $110.3 \pm 7.72 \mu\text{m}^2$ for axon-bearing DA cells, $61.99 \pm 4.41 \mu\text{m}^2$ for anaxonic DA neurons; n = 11 axon-bearing cells and n = 9 anaxonic neurons from N = 4 mice; unpaired t-test with Welch's correction, $p < 0.0001$). Given previous findings that axon-bearing DA cells have more widely ramified dendritic trees (Galliano et al., 2018 [↗](#)), we ensured that our reconstructions of TRIM46-negative dendritic processes were equally extensive in both cell subtypes (maximum length of reconstructed dendrites: $67.64 \pm 8.96 \mu\text{m}$ for axon-bearing DA cells, $74.88 \pm 11.68 \mu\text{m}$ for anaxonic DA neurons; n = 11 axon-bearing cells and n = 9 anaxonic neurons from N = 4 mice; unpaired t-test with Welch's correction, $p = 0.6299$). We found that, unlike their anaxonic counterparts, almost all TRIM46+ neurons (10/11 cells from N = 4 mice, = 90.9 %) did not have any synaptophysin-mRuby puncta on their dendrites (**Figure 3E, F** [↗](#); dendritic puncta density: $0.02 \pm 0.02 (\mu\text{m})^{-1}$ for axon-bearing cells, $0.19 \pm 0.04 (\mu\text{m})^{-1}$ for anaxonic neurons; n = 11 axon-bearing cells and n = 9 anaxonic neurons from N = 4 mice; Mann-Whitney test; $p = 0.0001$). We did find one single axon-bearing DA neuron with apparent dendritic puncta; however, this cell had dramatically higher levels and unusual patterns of expression as compared to all other GFP+ neurons (**Supplementary Figure 1** [↗](#)) and we include it in our sample only through an abundance of caution. These findings provide a structural basis for dendritic neurotransmitter release in anaxonic DA neurons, but not in the axon-bearing population of DA neurons in the OB.

Anaxonic DA neurons are capable of GABAergic self-inhibition

The fact that dendritic release sites are likely located near dendritic receptors (Kiyokage et al., 2017 [↗](#)) might have the functional consequence that anaxonic DA cells can signal to themselves and influence their own activity. We found that OB DA neurons do not express DA receptors at the mRNA level (**Figure 4A** [↗](#)), making it unlikely that they could influence their own activity via DA signalling. However, GABAergic auto-evoked inhibition (AEI) or self-inhibition has been described in OB periglomerular cells in general, and in DA neurons in particular (Smith and Jahr, 2002 [↗](#); Murphy et al., 2005 [↗](#); Maher and Westbrook, 2008 [↗](#); Borisovska et al., 2013 [↗](#)). In addition, we observed that the postsynaptic protein gephyrin can be in very close proximity to synaptophysin-mRuby puncta in the dendrites of anaxonic DA neurons (**Figure 4B** [↗](#)), suggesting that self-inhibition is possible in this specific cell subtype.

To address if anaxonic DA neurons can indeed self-inhibit, we used the DAT-tdT mouse line to specifically label DA neurons (Bäckman et al., 2006 [↗](#); Madisen et al., 2009; Galliano et al., 2018 [↗](#)), and performed whole-cell patch clamp in tdT+ cells to evoke AEI responses. We identified the anaxonic DA population by their characteristic 'monophasic' phase-plane plot profile (**Figure**

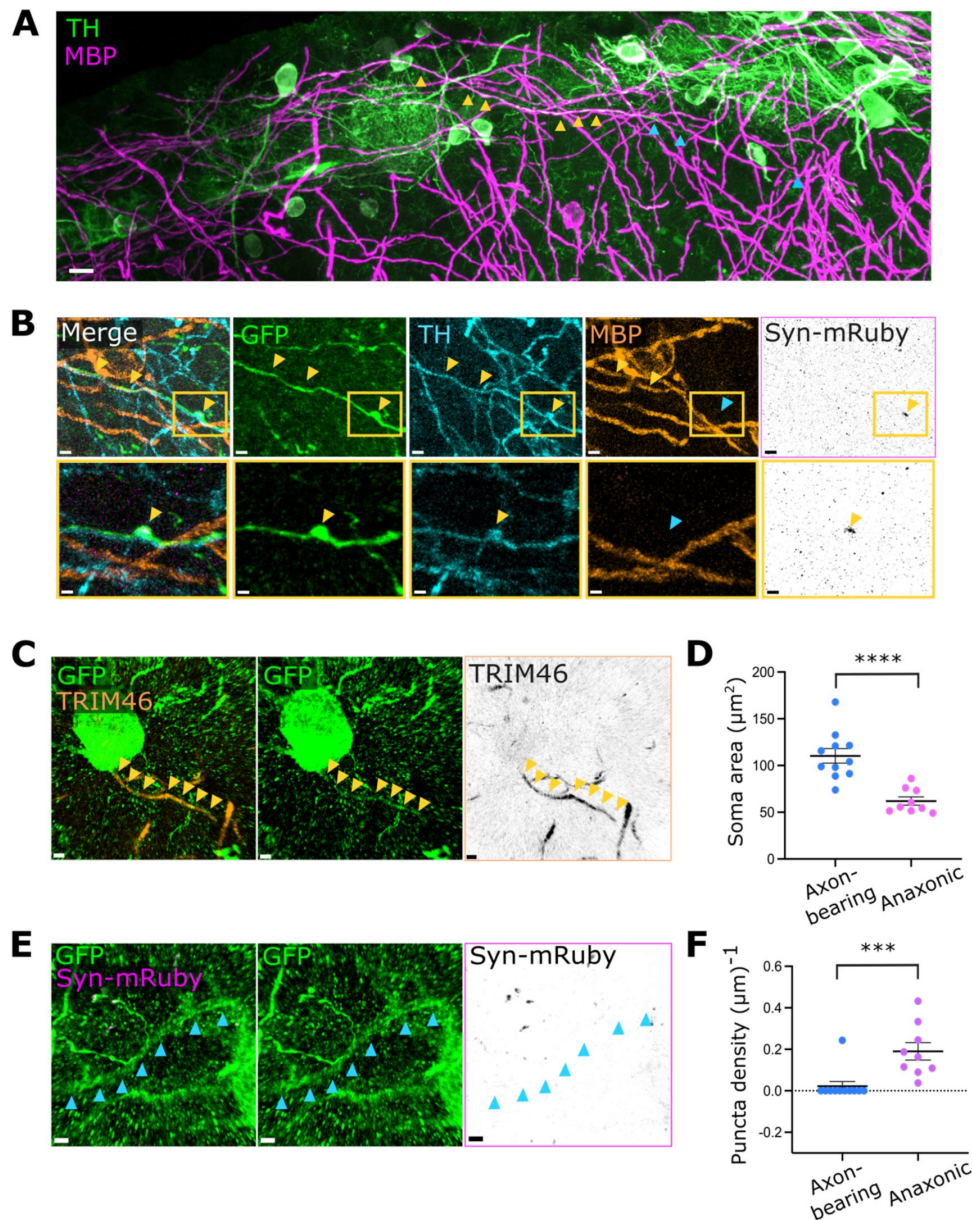


Figure 3.

Axon-bearing DA neurons have release sites on their intermittently myelinated axons, but not on their dendrites.

(A) Stitching of individual confocal stacks processed for maximum intensity projections of OB DA neurons co-stained with TH (green) and myelin basic protein (MBP, magenta). Yellow arrowheads point to myelinated parts of the axon, blue arrowheads show unmyelinated areas. Scalebar, 10 μm . (B) Snapshot of a 3D confocal stack of a distal DA axon stained with GFP (green), TH (cyan), MBP (orange) and synaptophysin-mRuby (magenta, black). Yellow inset highlights the location of the presynaptic bouton. Yellow arrowheads point to co-localised regions, blue arrowheads show non-co-localisation. Scalebars, 2 μm and 1 μm . (C) Snapshot of a 3D confocal stack of an axon-bearing TRIM46+ DA neuron. Yellow arrowheads show co-localised staining for GFP (green) and TRIM46 (orange, black). Scalebars, 2 μm . (D) Soma area of axon-bearing and anaxonic DA neurons. Each dot shows one cell; lines show mean $\pm$ SEM; $n = 11$ axon-bearing cells and $n = 9$ anaxonic neurons from $N = 4$ mice; unpaired t-test with Welch's correction; ****, $p < 0.0001$. (E) Snapshot of a 3D confocal stack of the same axon-bearing DA neuron showed in (C), co-stained with GFP (green) and synaptophysin-mRuby (magenta, black). Blue arrows show dendritic segments lacking mRuby label, despite the presence of clear mRuby+ puncta in neighbouring non-GFP+ processes. Scalebars, 2 μm . (F) Dendritic puncta density in axon-bearing and anaxonic DA neurons. All conventions as in D; $n = 11$ axon-bearing cells and $n = 9$ anaxonic neurons from $N = 4$ mice; Mann-Whitney's t-test; **, $p = 0.0001$.

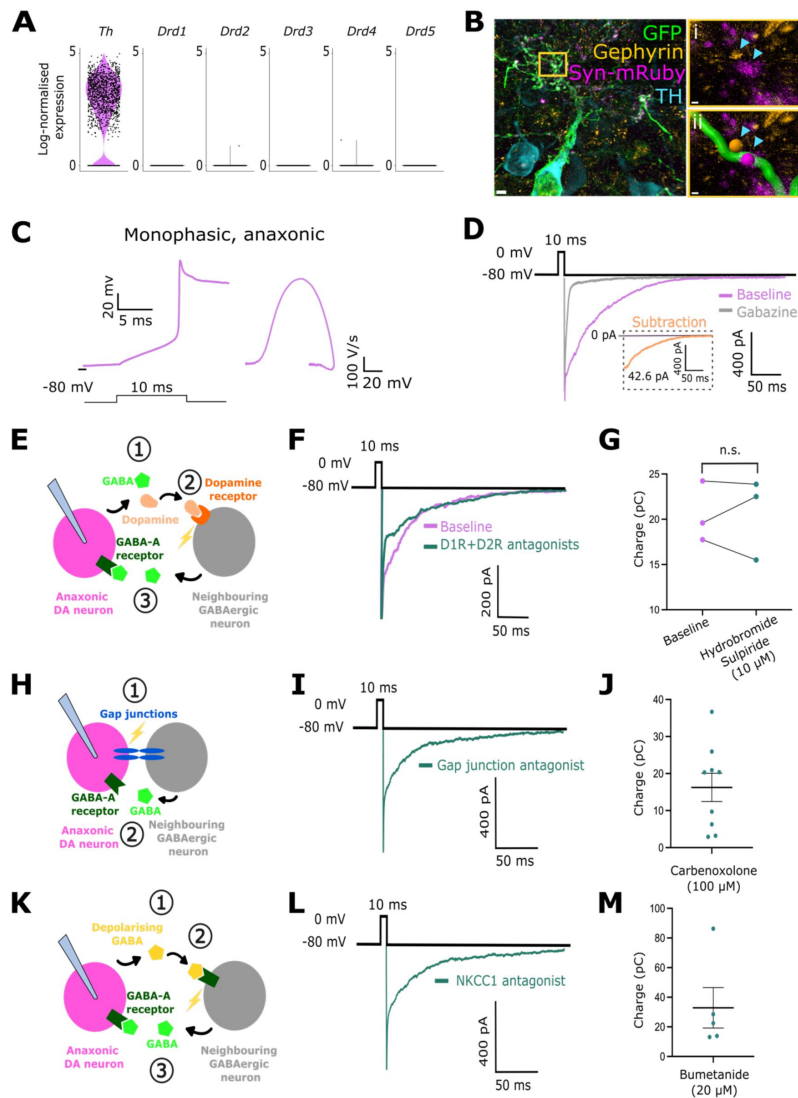


Figure 4.

Anaxonic DA neurons are capable of self-inhibition.

(A) Log₂-normalised expression (of *Th*, *Drd1*, *Drd2*, *Drd3*, *Drd4* and *Drd5* mRNA in OB DA cells. **(B)** Snapshot of a 3D confocal stack of a virally labelled (GFP+, green) anaxonic DA (TH+, cyan) neuron stained against gephyrin (orange) and synaptophysin-mRuby (magenta). **i** and **ii** magnification of the boxed area; green shows reconstructed dendrite; blue arrowheads point to two neighbouring puncta, one mRuby+ and one gephyrin+. Scalebars, 3 μ m and 0.5 μ m. **(C)** Example trace of an action potential fired by a putative anaxonic DA neuron (left) and its monophasic phase-plane plot profile (right). Note the prolonged repolarisation due to Cs-based internal solution. **(D)** Example traces of an AEI response recorded before (magenta) and after (grey) the application of gabazine. The subtraction is shown in the orange inset trace. **(E)** Schematic showing the potential involvement in the AEI response of neighbouring GABAergic neurons activated by dopamine released from the patched DA cell. **(F)** Example traces of an AEI response before (purple) and after (green) applying D1-like and D2-like receptor blockers (hydrobromide and sulpiride, each at 10 μ M). **(G)** AEI charge before (purple) and after (green) applying dopamine receptor antagonists; $n = 3$ cells from $N = 3$ mice; unpaired t-test with Welch's correction: n.s., non-significant. **(H)** Schematic showing the potential involvement in the AEI response of neighbouring GABAergic neurons activated via gap junctions. **(I)** Example trace of an AEI response in the presence of the gap junction blocker carbenoxolone at 100 μ M. **(J)** AEI charge in the presence of carbenoxolone at 100 μ M. Each dot shows one cell; lines show mean $\pm$ SEM; $n = 9$ cells from $N = 4$ mice. **(K)** Schematic showing the potential involvement in the AEI response of neighbouring GABAergic neurons activated by depolarising GABA released from the patched cell. **(L)** Example trace of an AEI response in the presence of the NKCC1 channel blocker bumetanide at 20 μ M. **(M)** AEI charge in the presence of bumetanide at 20 μ M. All conventions as in F; $n = 5$ cells from $N = 3$ mice.

4C [Galliano et al., 2018](#)). After evoking transmitter release with transient depolarisation, we observed a prolonged inward current which was blocked by adding the GABA-A receptor antagonist gabazine at 10 μ M (**Figure 4D** [Galliano et al., 2018](#); AEI charge: 44.11 ± 6.02 pC; $n = 31$ cells from $N = 18$ mice). These data reveal that anaxonic DA neurons display AEI responses, consistent with the co-existence of presynaptic release sites and postsynaptic targets in the dendrites of these cells.

Although the AEI response is initiated by the depolarisation of the recorded anaxonic DA neuron, it has always been assumed, but not demonstrated, that the response is the direct result of GABA released from the same cell. However, given the presence of other GABAergic cells in local glomerular circuits ([Kiyokage et al., 2017](#); for review see [Tufo et al., 2022](#)), there are other potential indirect ways in which depolarising a DA neuron might produce a GABA response in that same cell.

DA neurons in the OB release GABA and dopamine ([Borisovska et al., 2013](#)), and D1-like and D2-like dopamine receptors are expressed in the glomerular layer ([Coronas et al., 1997](#)). When we depolarise an anaxonic DA neuron to evoke neurotransmitter release, it is therefore possible that the dopamine that is co-released with GABA activates the dopamine receptors of neighbouring neurons, evoking secondary GABA release that then drives the AEI response in the recorded cell (**Figure 4E** [Galliano et al., 2018](#)). To rule out this possibility, we showed that the AEI response was unaffected by the application of D1-like and D2-like receptor antagonists (10 μ M SKF 83566-hydrobromide and 10 μ M sulpiride, respectively; **Figure 4F, G** [Galliano et al., 2018](#); AEI charge before blockers: 20.52 ± 1.93 pC, charge after blockers: 20.63 ± 2.59 pC; $n = 3$ cells from $N = 3$ mice; unpaired t-test with Welch's correction, $p=0.71$). These results show that the AEI response is not mediated by dopamine acting through other neurons.

Glomerular neurons are electrically coupled via gap junctions ([Schoppa and Westbrook, 2002](#); [Kosaka et al., 2005](#); [Banerjee et al., 2015](#)). To exclude the possibility that the electrical activation of neighbouring, GABA-releasing neurons is mediating the AEI response (**Figure 4H** [Galliano et al., 2018](#)), we showed that AEI was still present after gap junctions were pharmacologically blocked with 100 μ M carbenoxolone (**Figure 4I, J** [Galliano et al., 2018](#); AEI charge after blockers: 16.27 ± 3.83 pC; $n = 9$ cells from $N = 4$ mice).

Finally, another source of activation of neighbouring GABA-releasing neurons could be GABA itself: GABA can have depolarising effects on glomerular layer neurons ([Parsa et al., 2015](#)). To show that the GABA released by the recorded neuron is not activating neighbouring GABA-releasing cells (**Figure 4K** [Galliano et al., 2018](#)), we recorded strong AEI responses when depolarising GABA responses were prevented in the presence of the Na-K-Cl cotransporter 1 (NKCC1) blocker bumetanide used at 20 μ M (**Figure 4L, M** [Galliano et al., 2018](#); AEI charge after blockers: 32.86 ± 13.66 ; $n = 5$ cells from $N = 3$ mice). Altogether, these data robustly demonstrate that the AEI response in anaxonic DA neurons indicates true self-inhibition.

Axon-bearing DA neurons do not self-inhibit

We next wanted to assess whether axon-bearing DA neurons can self-inhibit. In light of our anatomical data showing the presence of release sites exclusively on the distal axon, we predicted that these cells do not exert synaptic inputs onto themselves. Using the same DAT-tdTomato mouse line to label DA neurons, we identified tdT+ axon-bearing DA neurons by their characteristic 'biphasic' action potential waveform (**Figure 5A** [Galliano et al., 2018](#)). As predicted, GABA-mediated AEI currents were lacking in almost all axon-bearing DA neurons (**Figure 5B, C** [Galliano et al., 2018](#); 8/9 cells from $N = 5$ mice, = 89 %; mean $\pm$ SEM AEI charge for axon-bearing neurons: 3.8 ± 2.8 pC; $n = 9$ cells from $N = 5$ mice; anaxonic cells: 44.11 ± 6.02 pC; $n = 31$ cells from $N = 18$ mice; Mann-Whitney test, $p < 0.0001$) despite these neurons having functional GABA-A receptors as shown by the presence of prominent gabazine-sensitive spontaneous IPSCs (**Figure 5D** [Galliano et al., 2018](#); $n = 9$ axon-bearing neurons from $N = 5$ mice; $n = 31$ anaxonic cells from $N = 18$ mice). Even when we attempted to maximise AEI amplitude via a paired-pulse protocol ([Bouhours et al., 2011](#)) or prolonged

depolarisation, we still saw no evidence of self-inhibition in this cell subtype (**Figure 5E, F** [↗](#); AEI charge for single response: 5.4 ± 2.61 pC; $n = 8$ cells from $N = 7$ mice; AEI charge for paired response: 1.51 ± 2.91 pC; $n = 8$ cells from $N = 7$ mice; Wilcoxon matched-pairs signed rank test, $p = 0.06$. AEI charge for 10 ms response: 0.26 ± 0.21 pC, $n = 7$ cells from $N = 6$ mice; AEI charge for 100 ms response: 7.75 ± 7.51 pC; $n = 7$ cells from $N = 6$ mice; Wilcoxon matched-pairs signed rank test, $p = 0.30$). Axon-bearing DA neurons did produce a tail current in response to the AEI protocol; however, this was insensitive to gabazine and was instead abolished by adding the voltage-gated calcium channel blocker cadmium at 200 μ M (**Figure 5G** [↗](#); cadmium sensitive current: 24.1 ± 6.69 pC; $n = 2$ cells from $N = 2$ mice).

In conclusion, these findings provide both structural and functional evidence showing that anaxonic DA neurons in the OB can signal locally, even to the point of self-inhibition. In contrast, axon-bearing DA neurons can – unlike all other major cell types in this brain region – only release neurotransmitters distally from their axon.

Discussion

Our results show that different polarity-defined dopaminergic (DA) subtypes in the olfactory bulb use distinct anatomical compartments for neurotransmitter release. While anaxonic DA neurons release from the dendrites, axon-bearing DA cells release nearly exclusively from the axon. These anatomical findings correlate strongly with different function: anaxonic DA neurons can self-inhibit, but this ability is absent in almost all of the axon-bearing DA population.

The link between neuronal polarity and neurotransmitter release

Our anatomical data indicate that anaxonic DA neurons have release sites on their dendrites, while the presynaptic sites of almost all the axon-bearing DA cells are located exclusively on their intermittently myelinated axons. These findings are entirely consistent with previous descriptions of release site localisation in similar OB cell types. Early ultrastructural work showed the absence of release sites in the dendrites of axon-bearing neurons in the glomerular layer, defined morphologically as ‘short-axon cells’ (Pinching and Powell, 1971 [↗](#)). It was also shown that axon-bearing DA neurons located in the external plexiform layer do not have dendritic release sites (Liberia et al., 2012 [↗](#)). In contrast, further ultrastructural work revealed the presence of release sites in the dendrites of small DA periglomerular neurons, presumably anaxonic (Kiyokage et al., 2017 [↗](#)). Our results agree with all of these findings while specifically assigning different neurotransmitter release strategies to axon-bearing and anaxonic DA neurons.

We have shown that anaxonic DA neurons use their only available anatomical compartment, the dendrites, to release neurotransmitters. This paradigm of anaxonic structural and functional polarity is unconventional but is found in other neuron types, including amacrine cells in the retina and OB granule cells (Wu et al., 2011 [↗](#); Marshak, 2016 [↗](#); Nunes and Kuner, 2018 [↗](#)). In fact, dendritic release is not an uncommon phenomenon in the brain and is observed even in many axon-bearing neurons. Dopaminergic neurons in the midbrain, for example, are one of the most studied examples of dendritic release (Cheramy et al., 1981 [↗](#); Hikima et al., 2021 [↗](#)). In the olfactory bulb, most neurons with axodendritic polarity can release from both the axon and the dendrites, including mitral cells and external tufted cells (Isaacson and Strowbridge, 1998 [↗](#); Schoppa and Urban, 2003 [↗](#); Hayar et al., 2004 [↗](#)). In this context, axon-bearing OB DA neurons are unique: they do not have dendritic release despite their dopaminergic nature, and despite their identity as OB neurons with classic axodendritic polarity.

OB DA neurons co-release both GABA and dopamine (Wachowiak and Cohen, 1999 [↗](#); Berkowicz and Trombley, 2000 [↗](#); Borisovska et al., 2013 [↗](#); Liu et al., 2013 [↗](#); Vaaga et al., 2017 [↗](#); Banerjee et al., 2015 [↗](#)), so do their distinct release strategies apply to both transmitters? Our anatomical

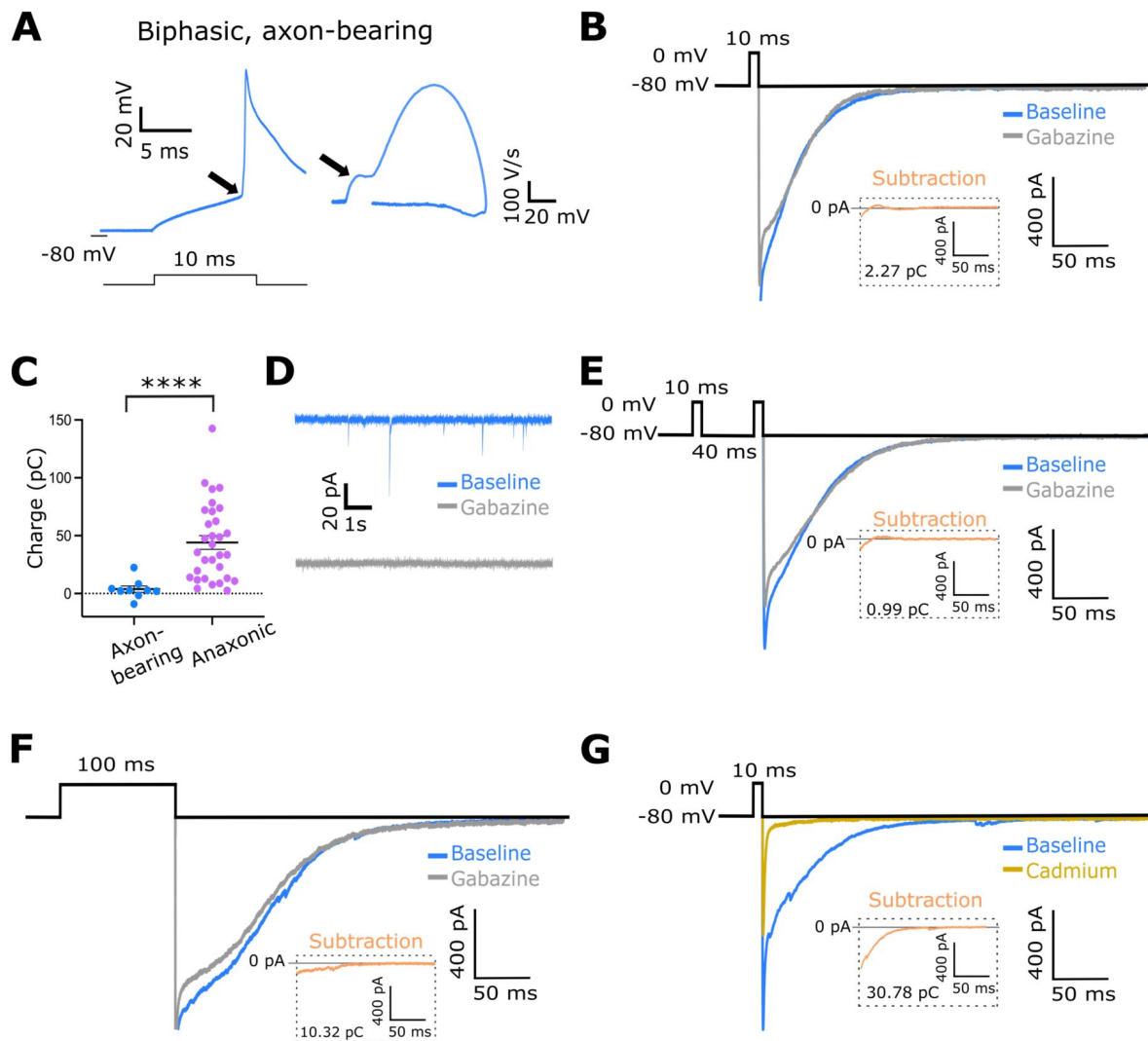


Figure 5.

Axon-bearing DA neurons do not self-inhibit.

(A) Example trace of an action potential fired by a putative axon-bearing DA neuron (left) and its biphasic phase-plane plot profile (right). Arrows point to spike onset kink (left) and initial segment bump (right). Note the prolonged repolarisation due to Cs-based internal solution. (B) Example traces showing tail current responses in an axon-bearing DA neuron before (blue) and after (grey) the application of gabazine. The subtraction is shown in the orange inset trace. (C) AEI charge in axon-bearing and anaxonic DA neurons. Each dot shows one cell; lines show mean $\pm$ SEM; $n = 9$ axon-bearing cells, $n = 31$ anaxonic cells from $N = 18$ mice; Mann-Whitney test; ****, $p < 0.0001$. (D) Example traces showing spontaneous IPSCs in an axon-bearing DA neuron before (blue) and after (grey) the application of gabazine. (E, F) Example traces showing tail current responses in axon-bearing DA neurons before (blue) and after (grey) the application of gabazine using a paired-pulse protocol (E) and prolonged depolarisation (F). Subtractions are shown in the orange inset traces. (G) Example traces showing that the tail current recorded in axon-bearing DA neurons (blue) is blocked by cadmium (ocre). The subtraction is shown in the orange inset trace.

approach reliably detects GABA presynaptic sites, with a high percentage of co-localisation between synaptophysin-mRuby and vGAT (**Figure 1**). However, not all synaptophysin-mRuby puncta are vGAT-positive. This could reflect technical limitations in release site detection (Risher et al., 2015; Heise et al., 2016), but also indicates that vGAT-negative spots may represent specific dopamine release sites (Borisovska et al., 2013; Liu et al., 2013; Vaaga et al., 2017). Indeed, in other brain regions synaptophysin-mRuby puncta have been used to reveal sites of dopamine release (Li et al., 2005). In OB DA neurons GABA and dopamine release are associated with different functions (Lyons-Warren et al., 2023) and the two transmitters are known not to be released from the same vesicles (Borisovska et al., 2013). However, this does not necessarily imply that they are released from different active zones. Addressing this question in the current study was unfortunately not possible due to notorious difficulties in staining the dopamine vesicular transporter 2 (VMAT2; Zhang et al., 2015; Jain et al., 2023). Whether anaxonic and axon-bearing DA neurons release dopamine and GABA from the same sites remains an important question for future studies.

In light of our findings, many fascinating questions about anaxonic DA neurons remain to be explored. How is dendritic release developed and maintained throughout the life of the neuron? Specific mechanisms have been identified that drive the establishment and maintenance of neuronal polarity in neurons with a classic axodendritic configuration (for reviews see Rasband, 2010; Lalli, 2014; Bentley and Banker, 2016). Do some of these molecular events, like cytoskeleton reorganisation and/or trafficking processes, differ in the dendrites of anaxonic neurons versus the ‘standard’ dendrites of axon-bearing neurons? Future work will answer these and further questions on the developmental and functional intricacies of unconventional neurons in the brain.

Does dendritic release equal self-inhibition?

Self-inhibition in the olfactory bulb was initially discovered in a subset of periglomerular cells (Smith and Jahr, 2002) and later described specifically in dopaminergic neurons (Maher and Westbrook, 2008). Here, we find that self-inhibition is not a universal OB DA neuron feature, but instead is an almost exclusive property of anaxonic DA cells. By ruling out potential external sources of GABA in the glomerular circuit, we have also provided the clearest evidence to date for true self-inhibition in OB periglomerular cells.

The link between dendritic release of neurotransmitters and self-inhibition exists in other brain regions as well. Neurons in the substantia nigra release dopamine that binds to auto-receptors and subsequently inhibits the neuron (Pucak and Grace, 1994; Falkenburger et al., 2001). Other examples include noradrenergic neurons in the locus coeruleus and serotonergic cells in the nucleus raphe dorsalis (Aghajanian et al., 1977; Liu et al., 2005). However, while these neurons have a classic axodendritic polarity and are able to release from their axons as well, anaxonic DA cells constitute a distinct model as dendrites are the solely available compartment for such function.

Finally, does dopamine released from an anaxonic OB DA neuron also modulate that same neuron? Our own data demonstrate that the auto-evoked inhibition current is purely GABAergic: it is suppressed after the application of gabazine, but unaffected after blocking dopamine receptors (**Figure 5**). In addition, our transcriptomic data clearly show that OB DA neurons do not express dopamine receptors at all (**Figure 4**). It is therefore unlikely there is a dopaminergic component to the self-inhibition response. Previous reports of dopamine-driven responses in OB DA neurons (Liu, 2020) may instead have occurred via well-described gap junction connectivity between DA cells and dopamine-responsive ETCs (Liu et al., 2013; Banerjee et al., 2015).

Implications for information processing

Anaxonic DA neurons release neurotransmitters from their dendrites, which are confined to local areas of the glomerular layer. In contrast, axon-bearing DA cells release from their axons, which spread for long distances across the glomerular layer (Kosaka and Kosaka, 2008 [↗](#); Galliano et al., 2018 [↗](#)). Our findings lead to the prediction of a clear division of circuit function for very closely related cell subtypes: anaxonic DA neurons take part in local, intraglomerular inhibition, while axon-bearing DA cells participate in distal, interglomerular inhibition.

Functionally, intraglomerular inhibition acts as ‘high pass filter’ for weak odour signals detected in a glomerulus, enhancing the contrast between different olfactory stimuli (Shao et al., 2012 [↗](#); Gire and Schoppa, 2009 [↗](#); Zak and Schoppa, 2021). Anaxonic DA neurons are therefore expected to contribute to intraglomerular inhibition via different pathways which have been generally described for periglomerular cells. This includes the exclusively intraglomerular presynaptic inhibition of olfactory sensory neurons (Hsia et al., 1999 [↗](#); Ennis et al., 2001 [↗](#); McGann et al., 2005 [↗](#); Korshunov et al., 2017 [↗](#); McGann 2013 [↗](#); Vaaga et al., 2017 [↗](#)), as well as the dendrodendritic inhibition of multiple neighbouring glomerular cell types, including interneurons and projection neurons (Murphy et al., 2005 [↗](#); Gire and Schoppa, 2009 [↗](#)).

Self-inhibition would be expected to decrease the capacity of anaxonic DA neurons to release GABA over time, therefore reducing inhibitory influence within glomerular circuits. Self-inhibition could therefore control the timing of intraglomerular inhibition, allowing subsequent stimuli to activate glomerular networks. Self-inhibition could also aid the high-pass filter function of glomerular networks (Gire and Schoppa, 2009 [↗](#)). If it occurs exclusively in the context of strong DA neuron activation, it could decrease intraglomerular inhibition to promote glomerular output when input strength goes past a certain threshold. There is also the possibility that self-inhibition is more of a bug than a feature. Perhaps if anaxonic DA neurons have to release GABA from their dendrites in order to perform their intraglomerular circuit functions, the chances are high that their nearby postsynaptic receptors cannot escape some of that GABAergic influence. Further work is needed to investigate how these mechanisms influence overall intraglomerular inhibition and glomerular output.

Glomeruli in the olfactory bulb create a spatial map of odour identity, where different odours specifically activate different combinations of glomeruli (Soucy et al., 2009 [↗](#); Murthy, 2011 [↗](#)). Interglomerular inhibition acting between different glomeruli selectively enhances the signal contrast in between individual stimuli, boosting odour identification and/or discrimination (Uchida et al., 2000 [↗](#); Urban, 2002 [↗](#)). This circuit function must be exclusively undertaken by axon-bearing DA neurons, due to their unique capacity for long-range axonal release of neurotransmitters (this paper, Liu et al., 2013 [↗](#); Whitesell et al., 2013 [↗](#); Banerjee et al., 2015 [↗](#)).

Several key traits are shared across the entire population of OB DA neurons, including their dopaminergic and GABAergic identities and their glomerular location in the olfactory circuitry. Despite these similarities, we have found fundamental differences between OB DA subtypes in terms of their anatomy, neurotransmitter release compartments and the presence/absence of self-inhibition. These are strong foundations to suggest entirely different sensory processing functions in even very closely related interneuron sub-populations.

Materials and Methods

Animals

We used mice of either sex and housed them under a 12h light-dark cycle in an environmentally controlled room with access to water and food *ad libitum*. Wild-type C57/Bl6 mice (Charles River) were used either as experimental animals, or to back-cross each generation of transgenic animals. The founders of our transgenic mouse lines – vGAT-Cre (Slc32a1^{tm2(cre)Low}/J, Jax stock 016962), DAT-Cre (B6.SJL-Slc6a3^{tm1.1(cre)Bkmn}/J, Jax stock 006660) and flex-tdTomato (B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze}/J, Jax stock 007909) were purchased from The Jackson Laboratory. Immunohistochemistry experiments with vGAT-Cre mice were performed at postnatal day (P)28. Patch-clamp electrophysiology experiments with DAT-tdT mice were performed between P21-P35. All experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986, under the auspices of Home Office personal and project licenses held by the authors.

Viral injections

To sparsely label neuronal morphology together with presynaptic release sites, vGAT-Cre mice were injected *in utero* via intraventricular injection with AAV-DJ-hSyn-FLEX-mGFP-T2A-Synaptophysin-mRuby (3 x 10¹² GC/ml, AAV DJ GVVC-AAV-100, Stanford Vector Core Facility) (Beier et al., 2015 [DOI](#); Kathe et al., 2022 [DOI](#)) at embryonic day (E)14. Time-mated pregnant mice were anaesthetised with isoflurane, used at 4% for general anaesthesia induction and at 2% for anaesthesia maintenance. Injections (~1µl) were performed with a glass-pulled pipette (1B150-F4, World Precision Instruments) through the uterine wall into one of the lateral ventricles of the embryos. The uterine horns were then returned into the abdominal cavity, the wall and the skin were sutured, and the embryos were allowed to continue their development *in utero* and born naturally until experimental use. Post-surgery, mice recovered in a 32°C chamber and were housed individually.

Immunohistochemistry

P28 and P33 mice were anaesthetised with an overdose of pentobarbital and then perfused with 20-25 ml of PBS with heparin (A16198, Thermo Fisher Scientific) (20 units/mL), followed by 20-25 ml of 4% paraformaldehyde (PFA; P001, TAAB Laboratories) in 3% sucrose, 60 mM PIPES, 25 mM HEPES, 5 mM EGTA, and 1 mM MgCl₂ (all purchased from Sigma-Aldrich). The olfactory bulbs were dissected and post-fixed in 4% PFA overnight (4°C), then embedded in 5% agarose and sliced at 50 µm using a vibratome (VT1000S, Leica). Free-floating slices were washed with PBS 1x and permeabilised and blocked in PBS with 5% normal goat serum (NGS, G9023, Sigma-Aldrich) and 0.25% Triton (Triton X100, Sigma-Aldrich) for 2 hr at room temperature (RT). Slices were then incubated in primary antibody solution (antibodies diluted in PBS with 5% and 0.25% Triton) for 2-5 days at 4°C. Primary antibodies, working dilutions and sources are indicated in **Table 1** [DOI](#). Slices were then washed three times for 10 minutes with PBS, before being incubated in secondary antibody solution (species-appropriate Life Technologies Alexa Fluor-conjugated; all at a 1:1000 dilution in PBS with 5% NGS and 0.25% Triton) for 2h at RT. After washing 3 times for 10 minutes with PBS, slices were mounted on glass slides (J2800AMNZ, EpreDia) with FluorSave (345789, Merck).

All immunostainings involving multiple targets were performed by incubating the primary antibodies simultaneously, except for the combination of mouse anti-TH and rat anti-MBP, where a sequential incubation was necessary to avoid cross-reactivity (first TH, then MBP).

Image acquisition, analysis, and illustration

Target	Host	Dilution	Supplier	Reference	Identifier
TH	Mouse	1/500, 1/5000, 1/10000 and 1/50000	Merck	MAB318	RRID:AB_2201528
GFP	Chicken	1/1000	Abcam	ab13970	RRID:AB_300798
TRIM46	Guinea pig	1/500	Synaptic Systems	377005	RRID:AB_2721101
mCherry	Rat	1/500	Thermo Fisher	M11217	RRID:AB_2536611
vGAT	Rabbit	1/1000	Synaptic Systems	131002	RRID:AB_887871
MBP	Rabbit	1/1000	Thermo Fisher	PA5-78397	RRID:AB_2736178
MBP	Rat	1/500	Millipore	MAB386	RRID:AB_94975
Gephyrin	Rabbit	1/500	Synaptic Systems	147008	RRID:AB_2619834
Synaptophysin	Guinea pig	1/500	Synaptic Systems	101004	RRID:AB_1210382

Table 1.

Primary antibodies.

Acquisition

All images were acquired using a laser scanning confocal microscope (Zeiss LSM 710, equipped with Zen 2010 B-SP1 software) using appropriate excitation and emission filters, a pinhole of 1 AU and 40x or 63x oil immersion objectives. Laser power and gain were set to prevent fluorescence saturation and allow acquisition with an optimal signal-to-noise ratio, and z-stacks were acquired at software-defined optimal thickness and step sizes. For puncta detection and dendritic reconstruction, stacks were acquired with 1x zoom (0.033 $\mu\text{m}/\text{pixel}$), 2048 x 2048 pixels, and in z-stacks with 0.37 μm steps. The dopaminergic identity of the cells was assessed qualitatively by taking a single plane snapshot of the TH and GFP channels that included the soma. Axon-bearing neurons displayed at least one clear elongated TRIM46-positive region (of approximately 10 to 25 μm in length) that belonged to a GFP+ process. For co-localisation analyses, stacks were acquired with 2x zoom (0.033 $\mu\text{m}/\text{pixel}$), 2048 x 2048 pixels, and in z-stacks with 0.37 μm steps. Due to the low penetration of the vGAT antibody, and following similar studies (Heise et al., 2016 [DOI](#)), these stacks were thinner (1.1-1.2 μm total depth) to capture most of the signal and perform reliable co-localisation assessment.

Analysis

Puncta detection and dendritic tracing in 3D stacks were performed using Imaris (version 9.1.2). Synaptophysin-mRuby belonging to specific GFP processes were detected based on their signal overlap with GFP. To automate puncta detection, synaptophysin-mRuby and gephyrin puncta were detected by estimating a punctum size of 0.5 μm and then using the 'Quality' filter. Detection threshold was kept within an unbiased consistent range (1000 to 2700) that allowed adaptation to variability between staining experiments and mice. Dendritic traces were performed using the 'Filaments' function, and the total length and maximum length of dendrites were calculated accordingly. Puncta density was estimated by dividing the total number of puncta by the total length of dendrites.

For co-localisation analysis, the 3-dimensional centroid coordinates for the synaptophysin-mRuby puncta detected in Imaris were exported and analysed using a custom-written script in Matlab. This determined, at the y- and z-axis centroid co-ordinates of each mRuby punctum, the vGAT fluorescence intensity averaged over a 1 μm window centred on the local maximum of the mRuby distribution's x-position. If this mRuby-centred vGAT fluorescence intensity was greater than 1 standard deviation higher than local background vGAT fluorescence (taken from a 1 μm window immediately outside the x-axis border of the mRuby punctum), the punctum was considered to be 'co-localised.' The overall percentage of co-localised puncta was then determined for each cell.

For soma size measurements, 3D stacks were first transformed into maximum intensity projections. We used the polygon drawing tool on Fiji (Schindelin et al., 2012 [DOI](#)) to draw the soma and obtained the area measurements using the 'measure' function.

Illustration

All images in the manuscript displaying examples of immunostaining were taken as high-resolution snapshots on ImarisViewer (version 10.2.0). Image stitching in **Figure 3** [DOI](#) was performed using maximum intensity projections of individual stacks that were assembled using the ImageJ plugin MosaicJ (Thevenaz and Unser, 2007 [DOI](#)). Orthogonal views in **Figure 1F** [DOI](#) were created in Fiji (Schindelin et al., 2012 [DOI](#)). Schematics, drawings, and figures layouts were designed using Inkscape (version 1.3.2).

Acute slice preparation

DAT-tdTomato mice at P21-35 were decapitated under isoflurane anaesthesia, and the olfactory bulb was removed and transferred into ice-cold slicing medium containing (in mM): 240 sucrose, 5 KCl, 1.25 Na₂HPO₄, 2 MgSO₄, 1 CaCl₂, 26 NaHCO₃ and 10 D-Glucose (all reagents from Sigma-Aldrich), bubbled with 95% O₂ and 5% CO₂. Horizontal slices (300 µm) of the olfactory bulb were cut using a vibratome (VT1000S, Leica and 7000 smz-2, Campden Instruments) and maintained in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.25 Na₂HPO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃ and 15 D-Glucose, bubbled with 95% O₂ and 5% CO₂. Slices were kept for 30 minutes in a water bath at 34 °C and then left to recover for 30 minutes at room temperature (RT).

Acute slice electrophysiology

Individual slices were placed in a chamber mounted on a FN1 Fixed Stage Nikon microscope and held in place with a stainless-steel slice anchor. Slices were continuously superfused with ACSF heated to physiologically relevant temperature (34 ± 1°C) using an in-line solution heater (TC-344B, Warner Instruments). The ACSF contained, at all times, the NMDA receptor blocker DL-2-Amino-5-phosphonopentanoic acid at 50 µM (APV; A5282, Sigma-Aldrich), the AMPA receptor blocker 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline at 10 µM (NBQX; N183, Sigma-Aldrich). The sodium channel blocking tetrodotoxin citrate (TTX; T-550, Alomone and 1069, Tocris) was used at 10 µM for all experiments excluding action potential recordings. Block of GABA-A receptors was achieved by the application of SR-95531 gabazine (1262, Tocris) for 5 minutes in the bath at 10 µM and confirmed by the absence of IPSCs. D1-like and D2-like receptors were blocked by applying SKF 83566 hydrobromide for 5 minutes in the bath at 10 µM (1586, Tocris) and sulpiride at 10 µM (S8010, Sigma-Aldrich), respectively. Calcium channels were blocked by applying cadmium chloride (202908, Sigma) at 200 µM for 5 minutes in the bath. The gap junction blocker carbenoxolone disodium at 100 µM (3096, Tocris) and the NKCC1 blocker bumetanide at 20 µM (3108, Tocris) were applied in the bath from the beginning and kept in the ACSF for at least 20-40 minutes. We adopted this strategy due to two key considerations: (1) carbenoxolone induced instability in the membrane properties of the neurons during the initial minutes of application, and (2) sufficient time was required for chloride transporters to reach a steady intracellular chloride concentration ([Cl⁻]) in response to bumetanide.

Recording pipettes were pulled from borosilicate glass (1B150F-4, World Precision Instruments) with a vertical puller (PC-10, Narishige). Some were tip fire-polished with a microforge (MF-830, Narishige). All auto-evoked inhibition (AEI) recordings were performed using Caesium Chloride intracellular solution (in mM: 125 CsCl, 10 HEPES, 0.1 EGTA, 4 MgATP, 0.3 Na₃GTP, 10 phosphocreatine, 10 GABA (280-290 mOsm and pHed to ~7.3 with CsOH), and pipette resistances of 2-4 MΩ.

Auto-evoked inhibition recordings in non-injected mice

DAT-tdTomato neurons were visualised with a 40x water immersion objective and tdTomato fluorescence was revealed by LED excitation (CoolLED pE-100) with appropriate excitation and emission filters. Recordings were performed using a HEKA EPC 10/2 amplifier coupled to PatchMaster acquisition software (HEKA Elektronik). Signals were digitised and sampled at 20 kHz (50 µs interval sample) unless otherwise stated, and Bessel filtered at 10 kHz (filter 1) and 2.9 kHz (filter 2).

Axon-bearing and anaxonic DA neurons were identified based on their action potential waveform. For that, cells were held at -60 mV in current clamp with 100% bridge balance. 10 ms somatic current injections of Δ+5 pA steps were applied until threshold was reached and an action potential occurred. 10 ms duration current injections were sampled at high temporal resolution 200 kHz (5 µs sample interval) and the recordings were smoothed prior to differentiation using a

20 point (100 μ s) sliding filter. The first derivative of the smoothed trace was used for dV/dt and phase plane plot analyses. Neurons were then visually determined as axon-bearing or anaxonic based on their biphasic or monophasic phase plane plot profiles, respectively (Galliano et al., 2018 [DOI](#)).

Spontaneous IPSCs were recorded as inward events at -80 mV for 1 minute while 1μ M TTX was added to the ACSF. Sodium channel block was confirmed by running an IV protocol and observing the block of the large, inward sodium current.

AEI recordings were sampled at 50 kHz (20 μ s sample interval), with slow capacitance compensated. After membrane rupture, a test series was performed. Series resistance (R_s), calculated from the peak current, was assessed continuously and recordings were excluded if they exceeded 30 M Ω at any point or changed more 30% during any recording. AEI protocols were performed in voltage-clamp from a holding potential of -80 mV, unless otherwise stated. Neurons were depolarised to 0 mV for 10 or 100 ms, before returning to -80 mV. Paired recordings were two 10 ms duration voltage steps with 50 ms inter-stimulus interval. All protocols were repeated 3 times, with 30 seconds in between each sweep, and an average response calculated. Due to the presence of passive currents, sweeps were analysed from 10 ms after the end of the voltage step for 500 ms.

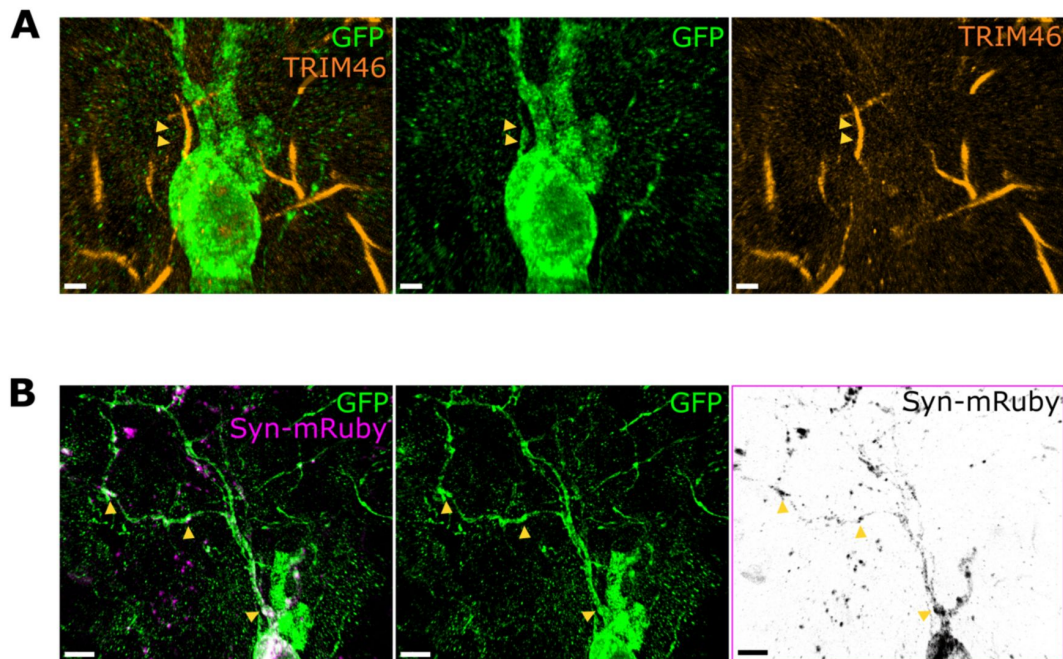
Gabazine and cadmium-sensitive AEI currents were measured as the difference between the pre- and the post-treatment averaged traces. The final charge was calculated as the area under the curve of the subtracted response.

We confirmed no change in the AEI response over time by repeating the 10 ms voltage step protocol 3 times, with 5 minutes in between each protocol. No significant change in the charge of the AEI response was found during the experiment (mean $\pm$ SEM: timepoint 1, 25 ± 7.99 pC; timepoint 2, 27.29 ± 9.57 pC; timepoint 3, 29.29 ± 11.22 pC; $n = 5$, $N = 4$ mice; one-way repeated measures ANOVA, $p = 0.44$). We also ruled out the effect of somatic depolarisation on the AEI response by holding the DA neurons either at -50 mV or -80 mV for approximately 30 seconds before the start of the recordings. Neurons were then depolarised to 0 mV for 10 ms and then brought to -80 mV. The AEI response was not different between holding at -50 mV or -80 mV (mean $\pm$ SEM: -50 mV response, 25.45 ± 4.53 pC; -80 mV response, 25.3 ± 7.3 pC; $n = 5$, $N = 3$ mice, paired t -test, $p = 0.96$). These results suggest that our experimental design produces a reliable and stable response to study auto-evoked inhibition in this neuronal population.

Data analysis

For RNAseq data plotting, we made use of a single cell RNAseq dataset derived from whole olfactory bulb, subsetting and plotting only the dopaminergic neurons (Brann et al., 2020 [DOI](#)). Violin plots depicting the expression of *Th* (tyrosine hydroxylase), *Syp* (synaptophysin) and *Drd1* and *Drd2* (dopamine receptors 1 and 2), in the cell cluster corresponding to the dopaminergic neurons, were generated with the R package Seurat.

Overall, data analysis, statistical analysis and plotting were carried out using GraphPad (San Diego, CA), R and custom-written Matlab (Mathworks) scripts. Data are reported as mean $\pm$ SEM unless otherwise stated. 'N' represents the number of mice and 'n' is the number of cells. Details of individual statistical tests can be found in the results section and in figure legends. Data sets were tested for normality using the D'Agostino and Pearson tests where the datasets were sufficiently large, and Shapiro-Wilk test if not. Statistical tests were determined considering the distribution of the data (normal or non-normal) and sample variance (equal or unequal). All tests were two-tailed, with $\alpha = 0.05$.



Supplementary figure 1.

A single example of an axon-bearing DA neuron with potential dendritic release sites.

(A) Snapshot of a 3D confocal stack of a DA neuron with GFP (green) and TRIM46 (orange) labelling, revealing the axon-bearing identity of the neuron. Yellow arrowheads point to the GFP+/TRIM46+ co-localised zone. Scalebars, 2 μm . **(B)** Snapshot of a 3D confocal stack showing synaptophysin-mRuby label (magenta, black) in the dendrites of the axon-bearing neuron from (A) (green). The levels of synaptophysin-mRuby look dramatically higher and with a less defined puncta profile than in all anaxonic DA cells – note levels of somatic expression compared to [Figure 2B](#). Yellow arrowheads point to examples of detected puncta. Scalebars, 5 μm .

Data availability statement

The full raw dataset supporting this article will be openly available upon manuscript acceptance in a peer-reviewed journal at the King's College London research repository (reserved DOI: 10.18742/27731157).

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Additional information

Author contributions

DJB and MSG designed experiments; AD-R, DJB, YL, MC, CA and MCF performed experiments; AD-R, DJB, YL, ML and MSG analysed data, AD-R and MSG wrote the paper.

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Reviewer #1 (Public review):

Summary:

Dorrego-Rivas et al. investigated two different DA neurons and their neurotransmitter release properties in the main olfactory bulb. They found that the two different DA neurons in mostly glomerular layers have different morphologies as well as electrophysiological properties. The anaxonic DA neurons are able to self-inhibit but the axon-bearing ones are not. The findings are interesting and important to increase the understanding both of the synaptic transmissions in the main olfactory bulb and the DA neuron diversity. However, there are some major questions that the authors need to address to support their conclusions.

(1) It is known that there are two types of DA neurons in the glomerular layer with different diameters and capacitances (Kosaka and Kosaka, 2008; Pignatelli et al., 2005; Angela Pignatelli and Ottorino Belluzzi, 2017). In this manuscript, the authors need to articulate better which layer the imaging and ephys recordings took place, all glomerular layers or with an exception. Meanwhile, they have to report the electrophysiological properties of their recordings, including capacitances, input resistance, etc.

(2) It is understandable that recording the DA neurons in the glomerular layer is not easy. However, the authors still need to increase their n's and repeat the experiments at least three times to make their conclusion more solid. For example (but not limited to), Fig 3B, n=2 cells from 1 mouse. Fig.4G, the recording only has 3 cells.

(3) The statistics also use pseudoreplicates. It might be better to present the biology replicates, too.

(4) In Figure 4D, the authors report the values in the manuscript. It is recommended to make a bar graph to be more intuitive.

(5) In Figure 4F and G, although the data with three cells suggest no phenotype, the kinetics looked different. So, the authors might need to explore that aside from increasing the n.

(6) Similarly, for Figure 4I and J, L and M, it is better to present and analyze it like F and G, instead of showing only the after-antagonist effect.

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Reviewer #2 (Public review):

Summary:

This study provides novel insights into the neurotransmitter release mechanisms employed by two distinct subclasses of dopaminergic neurons in the olfactory bulb (OB). The findings suggest that anaxonic neurons primarily release neurotransmitters through their dendrites, whereas axon-bearing neurons predominantly release neurotransmitters via their axons. Furthermore, the study reveals that anaxonic neurons exhibit self-inhibitory behavior, indicating that closely related neuronal subclasses may possess specialized roles in sensory processing.

Strengths:

This study introduces a novel and significant concept, demonstrating that two closely related neuron subclasses can exhibit distinct patterns of neurotransmitter release. Therefore, this finding establishes a valuable framework for future investigations into the functional diversity of neuronal subclasses and their contributions to sensory processing. Furthermore, these findings offer fundamental insights into the neural circuitry of the olfactory bulb, enhancing our understanding of sensory information processing within this critical brain region.

Weaknesses:

While this study offers novel insights, it is hindered by several limitations. The experimental approaches sometimes lack comprehensive justification and often rely on citations without providing adequate explanatory context. The small sample sizes (n values) compromise the statistical reliability and generalizability of the findings. Furthermore, the reliance on synaptophysin-based presynaptic structures raises concerns regarding whether these structures represent functional synapses. These shortcomings highlight the need for further

refinement and additional data to substantiate the study's central conclusions. Addressing these concerns would improve the rigor and impact of the study's findings while ensuring the validity of its conclusions.

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