Co-transmission of dopamine and GABA in periglomerular cells

Brady J. Maher and Gary L. Westbrook*

Vollum Institute, Oregon Health and Science University

Portland, OR 97239

Running title: Co-transmission of dopamine and GABA

Text pages, 22; Figures, 3

Words in abstract, 226; introduction, 406; discussion, 721

*Address correspondence to:

Gary L. Westbrook

Vollum Institute

Oregon Health and Science University L474

Portland, OR 97239

westbroo@ohsu.edu

Acknowledgements:

We thank John T. Williams for advice on the experiments, Kazuto Kobayashi for the gift of TH-EGFP mice, and AeSoon Bensen for maintenance of the mouse colony. This work was supported by NS26494 (GLW) and fellowship 5-T32-DA-07262 (BJM).

Key words: glomerulus, self-inhibition, co-transmission, olfactory bulb
Abstract

Most central neurons package and release a single transmitter. However co-transmission of fast-acting and modulatory transmitters has been observed in vertebrate and invertebrate systems. Here, we describe a population of periglomerular cells in mouse brain slices (PND14-21) that co-release dopamine and GABA. We made whole-cell recordings from periglomerular cells that expressed EGFP under the control of the tyrosine hyrdoxylase (TH) promoter. Immunolabeling confirmed that EGFP+ periglomerular cells synthesized TH as well as glutamic acid decarboxylase (GAD). Stimulation of ORN afferent input evoked EPSCs in EGFP+ cells that were inhibited by cocaine, which blocks dopamine transport. These effects were reversed by the D2 receptor antagonist sulpiride. Cocaine also increased the paired-pulse ratio of ORN-evoked EPSCs. These results demonstrate that TH+ periglomerular cells spontaneously release dopamine. In addition to dopamine, TH-EGFP+ cells also released GABA. Brief depolarizing voltage-steps in labeled cells evoked a tail current that was completely blocked by the GABA<sub>A</sub> receptor antagonist gabazine and by cadmium, indicative of calcium-dependent self-inhibition in periglomerular cells (Smith and Jahr, 2002). However, similar voltage steps were insufficient to cause D2-receptor mediated inhibition of ORN terminals. Our results indicate that TH+ periglomerular cells are directly activated by ORN input and release both dopamine and GABA. We suggest that concerted activation of multiple periglomerular cells may be required to detect dopamine release under normal physiological conditions.
Introduction

It was once generally accepted that neurons in the central nervous system only release a single neurotransmitter. However, co-transmission of two fast-acting transmitters, or a fast and a modulatory transmitter such as a peptide or monoamine, now have been described in several systems (Nusbaum et al., 2001; Seal and Edwards, 2006). For instance, glutamate can be co-released with acetylcholine in tadpole and mouse spinal neurons (Li et al., 2004; Nishimaru et al., 2005). Glutamate also can be released with dopamine or serotonin in cultured brainstem neurons (Johnson, 1994; Sulzer et al., 1998). The two fast-acting inhibitory transmitters, GABA and glycine are co-released in the spinal cord and auditory brainstem (Jonas et al., 1998; O’Brien et al., 1999; Kotak et al., 1998).

In the olfactory bulb, periglomerular cells are generally considered as GABAergic interneurons, but some periglomerular cells express tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, along with the GABA synthetic enzyme, glutamic acid decarboxylase (GAD; Gall et al., 1987; Kosaka et al., 1985). These TH+ cells are considered to be dopaminergic based on the lack of staining for dopamine-β-hydroxylase (Halasz et al., 1977), but the effects of endogenously released dopamine have not been tested. Within the glomerulus, dopamine and GABA receptors are expressed on afferent nerve terminals. Exogenous applications of dopamine can activate D2 receptors on ORN terminals and inhibit transmitter release (Coronas et al., 1997; Koster et al., 1998).
Evoked release of GABA from periglomerular cells also inhibits ORN terminals by activating GABA$_B$ receptors (Aroniadou-Anderjaska et al., 2000; Bonino et al., 1999; Margeta-Mitrovic et al., 1999; McGann et al., 2005; Murphy et al., 2005), and mitral cells by activating GABA$_A$ receptors (Isaacson and Strowbridge, 1998; Schoppa et al., 1998). Some periglomerular cells modulate their excitability by activating GABA$_A$ autoreceptors (self-inhibition, Smith and Jahr, 2002).

We examined the transmitter phenotype of dopaminergic periglomerular cells using transgenic mice that expressed EGFP under control of the TH promoter (Sawamoto et al., 2001). Whole-cell recordings in brain slices of mice at PND 14-21 revealed that dopamine and GABA were released from TH-EGFP+. Spontaneous dopamine release in the presence of cocaine caused presynaptic inhibition. Step depolarization of a single TH-EGFP+ cell was sufficient to cause GABA-mediated self-inhibition, but not dopamine-mediated presynaptic inhibition.

**Methods**

**Preparation of slices.** All animal protocols were approved by the institutional IACUC and followed NIH guidelines for the ethical treatment of animals. Olfactory bulb slices were prepared from 14-21 day old transgenic DBA/2J mice that expressed EGFP in a subset of periglomerular cells (pTH-GFP, Sawamoto et al., 2001). Only heterozygous mice were used in the experiments. These mice were obtained by breeding a heterozygous animal with a wild type. Mice were deeply

4
anesthetized with isoflurane and then decapitated. Bulbs were rapidly removed and immersed in ice-cold oxygenated (95% O₂ and 5% CO₂) dissection buffer (in mM): 83 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26.2 NaHCO₃, 22 glucose, 72 sucrose, 0.5 CaCl₂, 3.3 MgSO₄. Horizontal slices (290 µM) were cut using a vibrating blade vibrotome (VT1000S; Leica, Bannockburn, IL), incubated in dissection buffer for 30-45 min at 37ºC, and then stored at room temperature.

Slices were visualized using infrared differential interference contrast microscopy (IR/DIC, Zeiss Axioskop) and a CCD camera (XC-ST30, Sony, Japan). Individual periglomerular cells expressing EGFP were visualized with epifluorescent illumination and a 40x Zeiss water immersion (0.75 numerical aperture) objective. All recordings were done at 31-34ºC.

**Electrophysiology.** For all experiments, artificial cerebrospinal fluid (ACSF) was oxygenated (95% O₂ and 5% CO₂) and contained (in mM): 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 3 KCl, and 25 dextrose, 1 MgCl₂, 2 mM CaCl₂, pH 7.3. Patch pipettes were fabricated from borosilicate glass (TW150F-6; WPI, Sarasota, FL) to a resistance of 4-7 MΩ for periglomerular cell somatic recordings. Pipettes were filled (in mM): 125 Kgluconate, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 0.1 EGTA, 10 phosphocreatine, 0.05% biocytin, adjusted to pH 7.3 with KOH. For recording of GABA-mediated self inhibition, Kgluconate was replaced with CsCl; for most of these experiments 10 mM GABA was added to the pipette to allow for stable long-term recording of GABAergic responses (Smith and Jahr, 2002). In some experiments, synaptic currents were blocked with 100 µM D,L-2-amino-5-phosphonopentanoic acid (D,L-AP-5), 10 µM 2,3-
dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX), 5 µM Gabazine (Tocris, Ballwin, MO), and 0.5 µM TTX. Sulpiride, CGP55845 and +baclofen were obtained from Tocris. Cocaine was obtained from NIDA (NIH). Current signals recorded with a Multiclamp 700A amplifier (Axon instruments, Foster City, CA) were filtered at 2 kHz using a built-in Bessel filter and digitized at 10 kHz. Data were acquired using Axograph 4.9 (Axon instruments, Foster City, CA). Data acquisition was terminated when series resistance was >15 MΩ. For voltage clamp recordings mitral cells were held at −60 mV. Mitral cells in current clamp were held at −58 to −65 mV by continuous current injection.

**Immunohistochemistry.** Fixed sections (50 µm) were permeabilized with 0.4% Triton X-100/PBS for 30 min at room temperature (RT) followed by PBS wash (three times, 10 min). The sections were then blocked with 4% normal goat serum for 2 hours, and incubated with chicken polyclonal anti-EGFP antibody (1:5000; Ab16901; Chemicon) in PBS overnight at 4ºC. After PBS washing (three time, 10 min), slices were incubated with goat anti-chicken conjugated with Alexa Fluor 488 (1:5000; Molecular Probes) for two hours at RT. Following PBS wash (three times, 10 min), slices were incubated with either sheep polyclonal anti-TH antibody (1:1000; Ab1542, Chemicon), rabbit polyclonal anti-GAD65/67 (1:1000; Ab1511; Chemicon), or guinea pig polyclonal anti-GABA (1:1000; Ab175; Chemicon) in PBS overnight at 4ºC. After PBS washing (three times, 10 min), slices were incubated with either goat anti-sheep, goat anti-rabbit or goat anti-guinea pig antibodies at 1:5000 in PBS for 2 hours at RT, respectively. These antibodies were conjugated with Alexa Fluor 647 (Molecular Probes). Slices were
washed and mounted on slides with ProLong Antifade (Invitrogen), and imaged on a confocal microscope (Olympus, Melville, NY) with a 20x objective.

**Data analysis and statistics.** All analyses were performed using AxoGraph 4.9 on a Macintosh computer. Estimates of charge for self-inhibition currents were measured by integrating the currents starting at the peak of the response. For all experiments, statistical significance was determined using ANOVA or Student’s t-tests as appropriate (Microsoft EXCEL, Richmond, WA). Averaged data values are reported as mean ± s.e.m.

**Results**

*TH-EGFP positive periglomerular cells*

We used immunolabeling to verify that EGFP+ periglomerular cells expressed TH and GAD, the biosynthethic enzymes necessary for production of dopamine and GABA, respectively. Similar to previous reports with the pTH-GFP mice (Saino-Sato et al., 2004), the cell bodies of EGFP+ cells ringed each glomerulus and were immunoreactive for tyrosine hydroxylase (Fig. 1A-C). As expected, the majority of EGFP+ cells were also immunopositive for TH, although there were a few EGFP+ cells that did not have detectable TH (Fig. 1C). These cells also expressed GAD and GABA, as observed with immunolabeling with an antibody against the two GAD subtypes, GAD65/67 (Fig. 1D-F) as well as GABA (Fig. 1G-I). The subpopulation of EGFP+ cells that had no detectable immunolabeling with TH or GAD/GABA, may represent immature periglomerular cells as EGFP+ cells could be seen in other layers (Baker et al., 2001). These results confirm the co-
expression of TH and GABA in a population of periglomerular cells (Gall et al., 1987; Kosaka et al., 1985), and thus the TH- EGFP+ animals provide a useful tool to examine dopamine and GABA co-transmission in the glomerulus.

**Endogenous release of DA**

Exogenous application of dopamine can inhibit ORN inputs onto mitral, tufted and periglomerular cells by activation of presynaptic D2 receptors (Hsia et al., 1999; Ennis et al., 2001). However, endogenous release of dopamine has never been examined in the olfactory bulb. Similar to other dopaminergic neurons in the brain, dopaminergic periglomerular cells display spontaneous action potential firing (Puopolo et al., 2005; Pignatelli et al., 2005). Therefore, we first looked for spontaneous release of dopamine in the slices. We recorded the evoked EPSC in EGFP+ cells during stimulation of ORN inputs (0.1 Hz), in the presence of the dopamine transporter blocker cocaine (Fig. 2A). Bath application of cocaine (5 µM) strongly inhibited the EPSC (40.4±6.2% of control (n=12; p<0.02) without altering the holding current, and this effect was reversed by the D2 receptor antagonist sulpiride in the 7/12 cells where it was applied (107.9±22.6% of control (n=7; p<0.05; 4 µM, Fig. 2B,D). In the absence of cocaine, sulpiride had no effect on EPSC amplitude (n=6, Fig. 2D), suggesting that there was not a tonic dopamine-mediated inhibition.

. Under control conditions, ORN inputs onto EGFP+ cells were strongly depressing, however cocaine increased the paired-pulse ratio (Fig. 2C, 0.30±0.05 control PPR vs. 0.57±0.10 cocaine PPR, n=12; p<0.02). The effects of
cocaine on EPSC amplitude and PPR were similar to that of the GABA_B agonist baclofen (50 μM) which decreased the EPSC amplitude to 38.4±0.01% of control (n=11). The GABA_B antagonist CGP55845 (10 μM) blocked the effects of baclofen, n=11, not shown), but did not alter basal EPSC amplitude (Fig. 2D). These results suggest that periglomerular cells spontaneously released dopamine, and in the presence of uptake block the available dopamine was sufficient to cause presynaptic inhibition.

In some cells, dopamine release requires bursts of action potentials (Gonan, 1988; Suaud-Chagny et al., 1992; Chergui et al., 1994). We tested whether stimulation of a single periglomerular cell released sufficient dopamine to cause inhibition of the incoming afferent EPSC. However, we did not observe dopamine-mediated inhibition of EPSCs using trains of action potentials or depolarizing voltage steps in voltage clamp (0 mV, 25-100 ms) that preceded the afferent stimulation (n=12, not shown).

**TH-EGFP positive periglomerular cells release GABA**

The activation of GABA_B receptors on ORN nerve terminals (Murphy et al., 2005) as well as self-inhibition by GABA_A receptors (Smith and Jahr, 2002) have only been observed in a subset of periglomerular cells. Because EGFP+ cells express GAD, we tested these cells for GABAergic inhibition and self-inhibition. All EGFP+ cells showed self-inhibition (Fig. 3A). Self-inhibition was present at the onset of whole-cell recording, but was more stable in recordings when GABA was added to the pipette (Smith and Jahr, 2002). However self-inhibition currents
were also present using GABA-free pipette solutions (n=3, data not shown). Brief depolarizing voltage steps (10 ms, +10mV) in chloride-loaded cells produced an inward tail current that was completely blocked by gabazine (5µM, Fig. 3B, 4.5±1.01 pC control vs. 1.14±0.37 pC gabazine, n=7; p<0.004). Self-inhibition reflected vesicular release of GABA because the gabazine-sensitive tail currents were completely blocked by Cd²⁺ (Fig. 2C-D; 3.04±0.72 pC control vs. 0.28±0.18 pC Cd²⁺, n=5; p<0.02). Thus dopaminergic periglomerular cells show Ca²⁺-dependent release of GABA.

Discussion

*Co-transmission in monoamine neurons*

The most common form of co-transmission is co-release of neuropeptides with classical fast-acting neurotransmitters (Hökfelt et al., 2000). However there are now examples of co-localization and co-release of glutamate and GABA with each other as well as other transmitters (reviewed in Seal and Edwards, 2006), including co-localization of dopamine and GABA in Aplysia neurons (Díaz-Ríos and Miller, 2005). Although GABA and glycine may be released from the same vesicles at the same sites (Jonas et al., 1998), co-transmission in other cases may involve discrete groups of vesicles or release sites as seems to be the case with co-release of dopamine and glutamate from ventral midbrain neurons (Sulzer et al., 1998).
In mammalian systems, glutamate has been the only fast-acting transmitter identified thus far that is co-released with dopamine. Cultured dopamine and serotonin neurons release quantal amounts of glutamate, form asymmetric synapses typical of excitatory synapses (Sulzer et al., 1998; Johnson, 1994), and express vesicular glutamate transporter 3 (VGLUT3). Serotonin neurons \textit{in vivo} also express VGLUT3 (Fremeau et al., 2004; Schafer et al., 2002; Gras et al., 2002). Although VGLUTs have not been detected in dopamine neurons \textit{in vivo}, specific stimulation of dopamine neurons in the VTA evokes a CNQX-sensitive current in the nucleus accumbens and medial prefrontal cortex (Chuhma et al., 2004; Lavin et al., 2005).

Our results provide functional evidence that dopaminergic neurons can also co-release GABA. Our evidence is supported by prior anatomical data showing colocalization of the synthesizing enzymes, TH and GAD (Gall et al., 1985; Kosaka et al., 1987). In fact, Gall et al. (1985) reported that virtually all TH+ immunoreactive cells in the glomerulus also were GABA-immunoreactive. Because TH is the first enzyme in dopamine biosynthesis and is a reliable marker for dopaminergic neurons, this suggests that all dopaminergic periglomerular cells also release GABA. Although we were unable to demonstrate evoked dopamine release from individual EGFP+ cells, there are no centrifugal terminals containing dopamine (Halasz et al., 1977). Thus the source of DA transmission in the olfactory bulb must be TH+ periglomerular cells. It is possible that not all TH+ cells at the developmental state we studied (PND 14-21) are capable of robust dopamine release. TH+ periglomerular cells
originate from stem cells in the lateral ventricular zone and migrate along the rostral migratory stream into the olfactory bulb (Hack et al., 2005). However, TH expression does not occur until the cells incorporate into a glomerulus where TH levels are upregulated by afferent input (McLean and Shipley, 1988; Saino-Saito et al., 2004).

**Functional Consequences**

Periglomerular cells function as interneurons to modulate both afferent input and the excitability of mitral cells, the principal cells in the glomerulus. Co-transmission of a modulatory transmitter dopamine with GABA expands the modulatory capability of periglomerular cells. Perhaps the most likely is that release of the dopamine requires either strong stimulation or the activation of multiple dopaminergic neurons to affect ORN terminals. GABA release has been detected following individual stimulation of periglomerular cells (Murphy et al., 2005). However, we were unable to observe dopaminergic or GABAergic presynaptic inhibition after depolarization of individual TH+ periglomerular cells. This result may indicate that there are discrete functional groups of periglomerular cells. Postsynaptically, GABA rapidly depolarizes periglomerular cells at resting membrane potentials, reducing input resistance and shunting excitatory conductances. This results in self-inhibition (Smith and Jahr, 2002) and lateral inhibition (Murphy et al., 2005). Whether dopamine also plays an intraglomerular function remains to be determined.
GABA and dopamine can both act on ORN terminals to reduce transmitter release. This is likely to be important to sensory processing given the convergence of thousands of axons onto a single glomerulus and the high release probability of ORN terminals (Shepherd, 1972; Murphy et al., 2004). Although TH+ periglomerular cells spontaneously fire rhythmic action potentials (Pignatelli et al., 2005; Puopolo et al., 2005), the effect of dopamine in our experiments was only apparent in the presence of a transport blocker. However, ORN input directly activates dopaminergic periglomerular cells, thus convergence of many inputs may be required to evoke dopamine release as has been observed in other systems (Gonon, 1988; Suaud-Chagny et al., 1992; Chergui et al., 1994; Beckstead et al., 2004).
References


Coronas V, Srivastava LK, Liang JJ, Jourdan F and Moyse E. Identification and localization of dopamine receptor subtypes in rat olfactory mucosa


Kosaka T, Hataguchi Y, Hama K, Nagatsu I and Wu JY. Coexistence of immunoreactivities for glutamate decarboxylase and tyrosine hydroxylase in some neurons in the periglomerular region of the rat main olfactory bulb: possible coexistence of gamma-aminobutyric acid (GABA) and dopamine. *Brain Res* 343: 166-171, 1985.
Koster NL, Norman AB, Richtand NM, Nickell WT, Puche AC, Pixley SK and Shipley MT. Olfactory receptor neurons express D2 dopamine receptors. 


Schoppa NE, Kinzie JM, Sahara Y, Segerson TP and Westbrook GL.


Suaud-Chagny MF, Chergui K, Chouvet G and Gonon F. Relationship between dopamine release in the rat nucleus accumbens and the

**Sulzer D, Joyce MP, Lin L, Geldwert D, Haber SN, Hattori T and Rayport S.**

**Figure Legends**

**Figure 1.** Brain slice of the mouse olfactory bulb were labeled with anti-EGFP (green, A) and anti-TH (red, B) antibodies. The images show labeling of cell bodies surrounding individual glomeruli and fibers labeled in the central neuropil of the glomerular layer. The merged image in C shows that the TH-EGFP labeled cells also expressed endogenous TH. Cells labeled with anti-EGFP (green, D) also expressed GAD and GABA as show by labeling with anti-GAD 65/67 or anti-GABA, respectively (red, E, H). The merged images are shown in F and I. The white dashed lines outline individual glomeruli. Scale bars were 50 µM (A-F) and 25 µM (G-I).

**Figure 2.** A. The dopamine uptake blocker cocaine (5 µM) reduced the amplitude of EPSCs evoked by stimulation of the olfactory nerve layer. The D2 receptor antagonist, sulpiride (4 µM) reversed the effect of cocaine. The EPSCs are representative traces from control, cocaine and sulpiride application periods. B. The inhibition for each EPSC is shown with the average highlighted in bold. Reversal of the effect of cocaine was tested in 7 of the 12 cells. C,D. Although cocaine reduced the amplitude of the EPSC, it enhanced the paired pulse ratio, similar to the known effects of the GABAB agonist, baclofen. The D2 receptor antagonist sulpiride or the GABAB antagonist CGP55845 had no effect on EPSC amplitudes in the absence of cocaine or baclofen, respectively. AP5 (100 µM) and gabazine (5 µM) were included to block NMDA and GABA_A receptors, respectively. Holding potential was -60 mV.
**Figure 3.** A. Under voltage clamp in an EGFP+ periglomerular cell, a 10 ms voltage step from -60 mV to +10 mV evoked an inward tail current that was completely blocked by the GABA<sub>A</sub> receptor antagonist, gabazine (5 µM). B. The plot shows the block by gabazine of the integral of the inward tail current. The average is shown in bold. C,D. The inward current was also completely blocked by cadmium (200 µM), consistent with calcium-dependent GABA release evoked by the depolarizing voltage step. The bath contained AP5, NBQX, and TTX; whole-cell pipettes contained CsCl.
Figure 1. Brain slice of the mouse olfactory bulb were labeled with anti-EGFP (green, A) and anti-TH (red, B) antibodies. The images show labeling of cell bodies surrounding individual glomeruli and fibers labeled in the central neuropil of the glomerular layer. The merged image in C shows that the TH-EGFP labeled cells also expressed endogenous TH. Cells labeled with anti-EGFP (green, D) also expressed GAD and GABA as show by labeling with anti-GAD 65/67 or anti-GABA, respectively (red, E, H). The merged images are shown in F and I. The white dashed lines outline individual glomeruli. Scale bars were 50 µM (A-F) and 25 µM (G-I).
Figure 2. A. The dopamine uptake blocker cocaine (5 μM) reduced the amplitude of EPSCs evoked by stimulation of the olfactory nerve layer. The D2 receptor antagonist, sulpiride (4 μM) reversed the effect of cocaine. The EPSCs are representative traces from control, cocaine and sulpiride application periods. B. The inhibition for each EPSC is shown with the average highlighted in bold. Reversal of the effect of cocaine was tested in 7 of the 12 cells. C,D. Although cocaine reduced the amplitude of the EPSC, it enhanced the paired pulse ratio, similar to the known effects of the GABAB agonist, baclofen. The D2 receptor antagonist sulpiride or the GABAB antagonist CGP55845 had no effect on EPSC amplitudes in the absence of cocaine or baclofen, respectively. AP5 (100 μM) and gabazine (5 μM) were included to block NMDA and GABAA receptors, respectively. Holding potential was -60 mV.

188x145mm (300 x 300 DPI)
Figure 3. A. Under voltage clamp in an EGFP+ periglomerular cell, a 10 ms voltage step from -60 mV to +10 mV evoked an inward tail current that was completely blocked by the GABAA receptor antagonist, gabazine (5 μM). B. The plot shows the block by gabazine of the integral of the inward tail current. The average is shown in bold. C,D. The inward current was also completely blocked by cadmium (200 μM), consistent with calcium-dependent GABA release evoked by the depolarizing voltage step. The bath contained AP5, NBQX, and TTX; whole-cell pipettes contained CsCl.