Title: Neurotransmitter and receptor mapping in *Drosophila* circadian clock neurons via T2A-GAL4 screening

Short title: Neurotransmitters and their receptors in *Drosophila* clock neurons

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Abstract

The circadian neuronal network in the brain comprises central pacemaker neurons and associated input and output pathways. These components work together to generate coherent rhythmicity, synchronize with environmental time cues, and convey circadian information to downstream neurons that regulate behaviors such as the sleep/wake cycle. To mediate these functions, neurotransmitters and neuromodulators play essential roles in transmitting and modulating signals between neurons. In *Drosophila melanogaster*, approximately 240 brain neurons function as clock neurons. Previous studies have identified several neurotransmitters and neuromodulators, including the Pigment-dispersing factor (PDF) neuropeptide, along with their corresponding receptors in clock neurons. However, our understanding of the neurotransmitters and receptors involved in the circadian system remains incomplete. In this study, we conducted a T2A-GAL4-based screening for neurotransmitter and receptor genes expressed in clock neurons. We identified two neurotransmitter-related genes and 22 receptor genes. Notably, while previous studies had reported the expression of six neuropeptide receptor genes in large ventrolateral neurons (l-LN_v), we also found that 14 receptor genes—including those for dopamine, serotonin, and γ-aminobutyric acid—are expressed in l-LN_v neurons. These findings suggest that l-LN_v neurons serve as key integrative hubs within the circadian network, receiving diverse external signals.

Keywords: clock neurons, neurotransmitter, T2A-GAL4, immunostaining, Drosophila

Main text

The fruit fly *Drosophila melanogaster* serves as an excellent model organism for investigating the circadian neural network, as it comprises only approximately 240

clock neurons (Reinhard et al., 2024; Helfrich-Förster and Reinhard, 2025). These 240 neurons are classified into nine groups: anterior dorsal neuron 1 (DN_{1a}), posterior dorsal neuron 1 (DN_{1p}), dorsal neuron 2 (DN_2), dorsal neuron 3 (DN_3), lateral posterior neuron (LPN), dorsolateral neuron (LN_d), 5th ventrolateral neuron (5th LN_v), large ventrolateral neurons (l-LN_v), and small ventrolateral neurons (s-LN_v). To investigate the roles of clock neurons, the GAL4-UAS system and its derivatives are powerful genetic tools for manipulating gene expression in a cell-specific manner (Brand and Perrimon, 1993; Lai and Lee, 2006).

In the GAL4-UAS system, GAL4 protein, expressed under the control of a specific gene promoter, activates the expression of a desired gene in a tissue-specific manner by binding to upstream activating sequences (UAS) located upstream of the target gene. This system is widely used in *Drosophila* to visualize gene expression patterns using reporter genes, such as green fluorescent protein (GFP). In the T2A-GAL4 system, GAL4 fused with a T2A self-cleaving peptide sequence is inserted at the C-terminus of the target gene, enabling co-expression of the target protein and GAL4 from a single transcript (Diao and White, 2012). Compared to the conventional GAL4 system, the T2A-GAL4 system more accurately reflects the endogenous expression pattern of the target protein, allowing identification of which clock neurons express the gene of interest. Recently, Reinhard et al. (2024) used the T2A-GAL4 and T2A-LexA systems—the latter being a similar binary expression system—to screen for peptide and receptor genes expressed in *Drosophila* clock neurons and demonstrated peptidergic connections within the circadian neuronal network. However, information on neurotransmitters and their receptors is still lacking. In this study, we screened the remaining T2A-GAL4/LexA lines (Deng et al., 2019; Kondo et al., 2020) that were not included in the analysis by Reinhard et al. (2024).

We first screened 10 T2A-GAL4/LexA lines targeting genes encoding biosynthetic enzymes and transporters (Table S1). Brains from *UAS-GFP/X-T2A-GAL4* (or *LexAop-GFP/X-T2A-LexA*) flies were dissected at Zeitgeber time (ZT) 20 under a 12:12 h light-dark cycle and subsequently immunostained with anti-GFP and anti-PAR-domain protein 1 (PDP1) antibodies. T2A-GAL4/LexA lines that showed GFP expression in clock neurons were further analyzed by triple immunostaining with anti-GFP, anti-TIMELESS (TIM), and anti-Pigment-dispersing factor (PDF) antibodies. Among the 10 lines, the *Dopamine transporter* (*DAT*) and *Tryptophan hydroxylase neuronal* (*Trhn*) - *T2A-GAL4* lines exhibited co-expression of GFP and TIM in clock neurons (Fig. 1). *DAT*, which encodes a dopamine transporter, was expressed in l-LN_v neurons. However, *pale*, the gene encoding tyrosine hydroxylase (dopamine biosynthetic enzyme), was not expressed in clock neurons. Similarly, *Trhn*, which is involved in the serotonin biosynthesis pathway, was expressed in a subset of DN_{1p} neurons, whereas the serotonin transporter (*SerT*) gene was not expressed in clock neurons. These results indicate that genes associated with transporters and biosynthetic enzymes for a particular neurotransmitter are not consistently co-expressed in the same neurons. Using immunostaining with antibodies against tyrosine hydroxylase and serotonin, Hamasaka and Nässel (2006) showed that clock neurons do not use dopamine and serotonin. Consequently, it is possible that the *DAT* and *Trhn* genes possess functions within clock neurons that remain unidentified.

Next, we examined 89 T2A-GAL4/LexA lines targeting receptor genes, including orphan receptors. Of these, 22 lines showed co-expression in clock neurons (Fig. 2; Fig S1). In general, we observed GFP expression patterns that were highly consistent with those reported in previous studies (Deng et al., 2019; Kondo et al., 2020). However, certain GAL4 lines, such as *Dop1R1-RB-T2A-GAL4*, *Nmdar1-T2A-*

GAL4 and *Gyc76C-T2A-GAL4*, exhibited some differences. These variations may be due to the use of a different *UAS-GFP* reporter line than that employed in previous studies, or to differences in imaging settings optimized for specific clock neurons.

LN_v neurons (both l-LN_v and s-LN_v) are known to be responsive to acetylcholine, dopamine, γ-aminobutyric acid (GABA), glutamate, and octopamine (Hamasaka et al., 2007; Shang et al., 2011; Lelito and Shafer, 2012), consistent with our findings that many of the corresponding receptor genes were expressed in clock neurons. Table 1 summarizes the receptor screening results from both this study and Reinhard et al. (2024). Notably, l-LN_v neurons express 14 receptor genes for neurotransmitters and six genes for peptide receptors, suggesting that l-LN_v neurons may function as a central hub in the circadian network, integrating a wide range of external signals. Given that the molecular clock in l-LN_v neurons lacks self-sustainability and requires entrainment by light-dark cycles (Yang and Sehgal, 2001; Shafer and Taghert, 2009; Yoshii et al., 2015), the absence of an autonomous oscillator may enhance their sensitivity to environmental cues. In contrast, DN_2 and 5th LN_v neurons expressed fewer receptor genes than other clock neurons. DN_2 neurons may represent a specialized subgroup involved in temperature entrainment or temperature preference rhythms (Picot et al., 2009; Kanoko et al., 2012), while the 5th LN_v neuron may play a more central role in maintaining autonomous circadian rhythmicity.

Guanylyl cyclase at 76C (*Gyc76C*) encodes a membrane-associated guanylate cyclase (Liu et al., 1995; McNeil et al., 1995) and is expressed in DN_3 clock neurons (Fig. 2). A recent study identified *Gyc76C* as the receptor for ion transport peptide (ITP), which is expressed in the 5th LN_v and one of six LN_d neurons (Johard et al., 2009; Hermann-Luibl et al., 2014; Gera et al., 2024). Consequently, ITP-positive LN neurons may relay circadian signals to DN_3 neurons, as well as to other non-clock

116 *Gyc76C*-positive neurons. It is still unclear whether this signaling is mediated by direct
117 synapses or paracrine mechanisms.
118 While many receptor genes exhibit broad expression across the brain, the
119 *Histamine-gated chloride channel subunit 1* (*HisCl1*), which encodes a histamine-gated
120 chloride channel, is specifically expressed in a subset of DN_{1p} neurons. Histamine is a
121 key neurotransmitter released by *Drosophila* photoreceptor cells and conveys light
122 input signals from the compound eyes (Rieger et al., 2003; Montell, 2012). A previous
123 study has shown that *HisCl1* expression in the eyes is essential for circadian clock
124 entrainment by light (Alejevski et al., 2019). Several lines of evidence suggest a close
125 association between DN_{1p} neurons and photoreception. These neurons express the
126 *glass* gene, a key regulator of photoreceptor differentiation, and are missing in *glass*
127 mutants (Helfrich-Förster et al., 2001; Klarsfeld et al., 2004). A subset also expresses
128 Rh7, a novel rhodopsin (Kistenpfennig et al., 2017). Our finding that *HisCl1* is
129 specifically expressed in DN_{1p} neurons further supports this link. Histamine
130 neurotransmission has also been implicated in the regulation of sleep/wake cycles (Oh
131 et al., 2013) and temperature preference (Hong et al., 2006), both of which are
132 associated with circadian rhythms. DN_{1p} neurons are known to regulate sleep (Kunst et
133 al., 2014; Guo et al., 2016; Lamaze et al., 2017) and temperature entrainment (Yoshii
134 et al., 2010; Chen et al., 2015; Harper et al., 2016; Yadlapalli et al., 2018). Taken
135 together, *HisCl1* expression in DN_{1p} neurons may contribute to circadian regulation,
136 potentially affecting behaviors such as sleep, and entrainment to light and temperature
137 cues.
138 Despite extensive research, it remains unclear how the circadian neuronal
139 network generates coherent and robust rhythms under variable environmental
140 conditions. The combined T2A-GAL4/LexA screening results from this study and

Reinhard et al. (2024) provide a map of neurotransmitters, neuromodulators, and their receptors in *Drosophila* clock neurons. Together with single-cell transcriptomic data and the synaptic connectome (Ma et al., 2021; Shafer et al., 2022; Reinhard et al., 2024), these findings contribute to a growing framework for understanding how circadian networks are organized through synaptic and paracrine signaling.

As a final note, one limitation of this study is that the T2A-GAL4/LexA systems do not provide a complete map of gene expression. Several genes have splicing isoforms that are not covered by the T2A-GAL4/LexA system. In addition, expression patterns may differ between GAL4 and LexA drivers, suggesting that the drivers themselves can influence expression (Deng et al., 2019). Furthermore, the choice of UAS-reporter line and microscope settings may affect the observed images, and weak expression signals could be overlooked. For example, we observed *DAT* expression in l-LN$_v$ neurons, whereas *ple* was not expressed in these neurons. This discrepancy may result from the technical limitations discussed above.

Appendix

Drosophila melanogaster strains used in this study are listed in Supplementary Table 1. T2A-GAL4 and T2A-LexA knock-in lines, generated previously (Deng et al., 2019; Kondo et al., 2020), were obtained from the Bloomington Drosophila Stock Center (BDSC) and Shu Kondo. Flies were reared at 25°C on *Drosophila* medium containing 0.7% agar, 8.0% glucose, 3.3% yeast, 4.0% cornmeal, 2.5% wheat embryo, and 0.25% propionic acid.

Immunostaining was performed as described previously (Sekiguchi et al., 2020). Whole flies were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 0.1% Triton X-100 (PBS-T0.1) for 2.5 h at room temperature (RT). Fixed flies were

washed three times with PBS before dissecting their brains. Samples were washed with PBS-T0.5 three times. The samples were blocked in PBS-T0.5 containing 5% normal donkey serum for 1 hour at RT and subsequently incubated in primary antibodies at 4°C for 48 h. Following six washes with PBS-T0.5, the brains were incubated in secondary antibodies at RT for 3 h. Lastly, the samples were washed six times in PBS-T0.5 and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). At least five brains for each strain were used for immunostaining to briefly characterize the clock neurons stained by chicken anti-GFP antibodies (1:1000; Rockland, Limerick, PA, USA) and rabbit anti-PDP1 antibodies (1:5000; kindly provided by Justin Blau) (Cyran et al., 2003) in the first experiment. In the second experiment, we conducted the same immunostaining with rat anti-TIM (1:3000; kindly provided by Jadwiga Giebultowicz) (Yoshii et al., 2008) and mouse anti-PDF antibodies (1:500; Developmental Studies Hybridoma Bank) (Cyran et al., 2005), but only for positive strains, to confirm the prior results. The following fluorescence-conjugated secondary antibodies were used at 1:1000 dilution: Alexa Fluor® 488 nm (goat anti-chicken), 647 nm (goat anti-mouse) antibodies (Life Technologies, Carlsbad, CA, USA), and Cy3 (goat anti-rabbit and anti-rat) antibodies (Millipore, Billerica, MA, USA). Images were taken from at least three different brains using a laser scanning confocal microscope (Olympus FV3000, Olympus, Tokyo, Japan).

Competing interest statement

The authors declare that there are no competing interests.

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Author contributions

T.Y. conceived and supervised the study. A.F. and A.S. performed the experimental work and analyzed the data. T.Y. wrote the manuscript. All authors read, provided feedback and approved the final manuscript.

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327
328 **Figure legends**
329 **Figure 1**
330 Representative expression patterns of *DAT-T2A-GAL4* (A) and *Trhn-T2A-GAL4* (B) in
331 the *Drosophila* brain. Each GAL4 line was crossed with a *UAS-GFP* reporter line, and
332 their F1 offspring were processed for immunostaining using anti-GFP, anti-TIM, and
333 anti-PDF antibodies. Both *DAT-T2A-GAL4* and *Trhn-T2A-GAL4* lines exhibit GFP
334 expression in specific subsets of clock neurons, as indicated by arrowheads. Scale bar
335 = 100 μm. (C) Schematic diagrams depict simplified expression patterns of each GAL4
336 line in clock neurons.
337

Figure 2

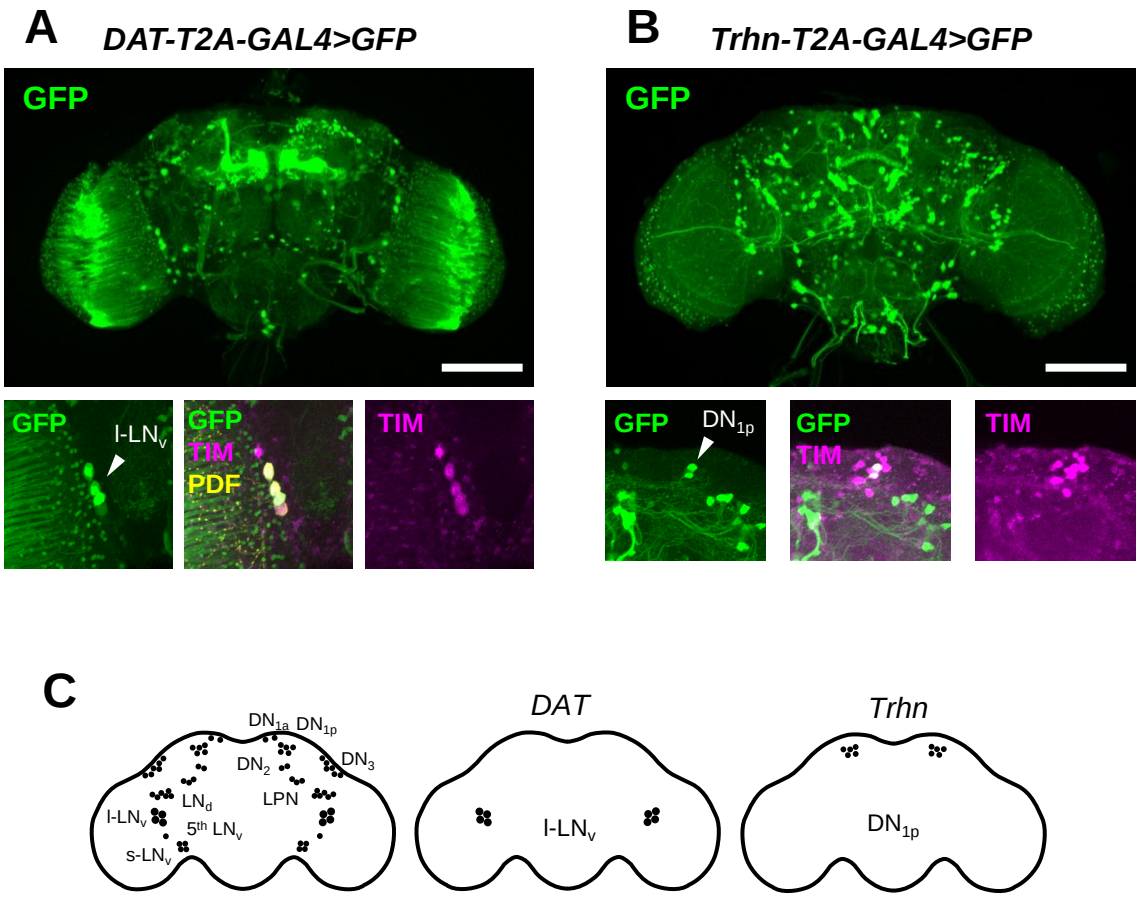
Schematic diagrams illustrate the simplified expression patterns of receptor T2A-GAL4 lines in clock neurons. Representative images for each line are presented in Fig. S1.

Figure S1

Representative expression patterns of T2A-GAL4 lines in the *Drosophila* brain. Each GAL4 line was crossed with a *UAS-GFP* reporter line, and the F1 offspring were processed for immunostaining using anti-GFP, anti-TIM, and anti-PDF antibodies. Arrowheads indicate clock neurons co-labeled with anti-GFP and anti-TIM (or anti-PDF). Scale bar = 100 μm.

Table 1. Receptor gene expression in clock neurons

DN_{1a}	DN_{1p}	DN_2	DN_3	LPN	LN_d	5^{th} LN_v	l-LN_v	s-LN_v
		AstC-R1	*AstC-R1*	*AstC-R1*				
AstC-R2		*AstC-R2*	*AstC-R2*		*AstC-R2*		*AstC-R2*	*AstC-R2*
CCHa1R	*CCHa1R*			*CCHa1R*			*CCHa1R*	*CCHa1R*
			CNMaR					
							DH31R	
			NPFR	*NPFR*	*NPFR*		*NPFR*	*NPFR*
pdfr	*pdfr*	*pdfr*	*pdfr*	*pdfr*	*pdfr*	*pdfr*	*pdfr*	*pdfr*
	sNPFR	*sNPFR*	*sNPFR*	*sNPFR*			*sNPFR*	
5-HT1A	*5-HT1A*		*5-HT1A*					*5-HT1A*
							5-HT2B	
							Dop1R1	
					DopEcR		*DopEcR*	
GABA-B-R1					*GABA-B-R1*		*GABA-B-R1*	
GABA-B-R2			*GABA-B-R2*		*GABA-B-R2*		*GABA-B-R2*	
GluRIA			*GluRIA*		*GluRIA*		*GluRIA*	
	HisCl1							
KaiR1D	*KaiR1D*				*KaiR1D*		*KaiR1D*	*KaiR1D*
mAChR	*mAChR*		*mAChR*		*mAChR*	*mAChR*	*mAChR*	*mAChR*
mGluR	*mGluR*				*mGluR*			*mGluR*
nAChRα2							*nAChRα2*	
nAChRα6			*nAChRα6*		*nAChRα6*			
			nAChRβ2				*nAChRβ2*	
Nmdar1			*Nmdar1*				*Nmdar1*	
Oamb	*Oamb*				*Oamb*		*Oamb*	
							Octβ3R	
			Gyc76C					
			mmt				*mmt*	*mmt*


A
DAT-T2A-GAL4>GFP
GFP
GFP
l-LNv
GFP
TIM
PDF
TIM
B
Trhn-T2A-GAL4>GFP
GFP
GFP
DN1p
GFP
TIM
TIM
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l-LNv
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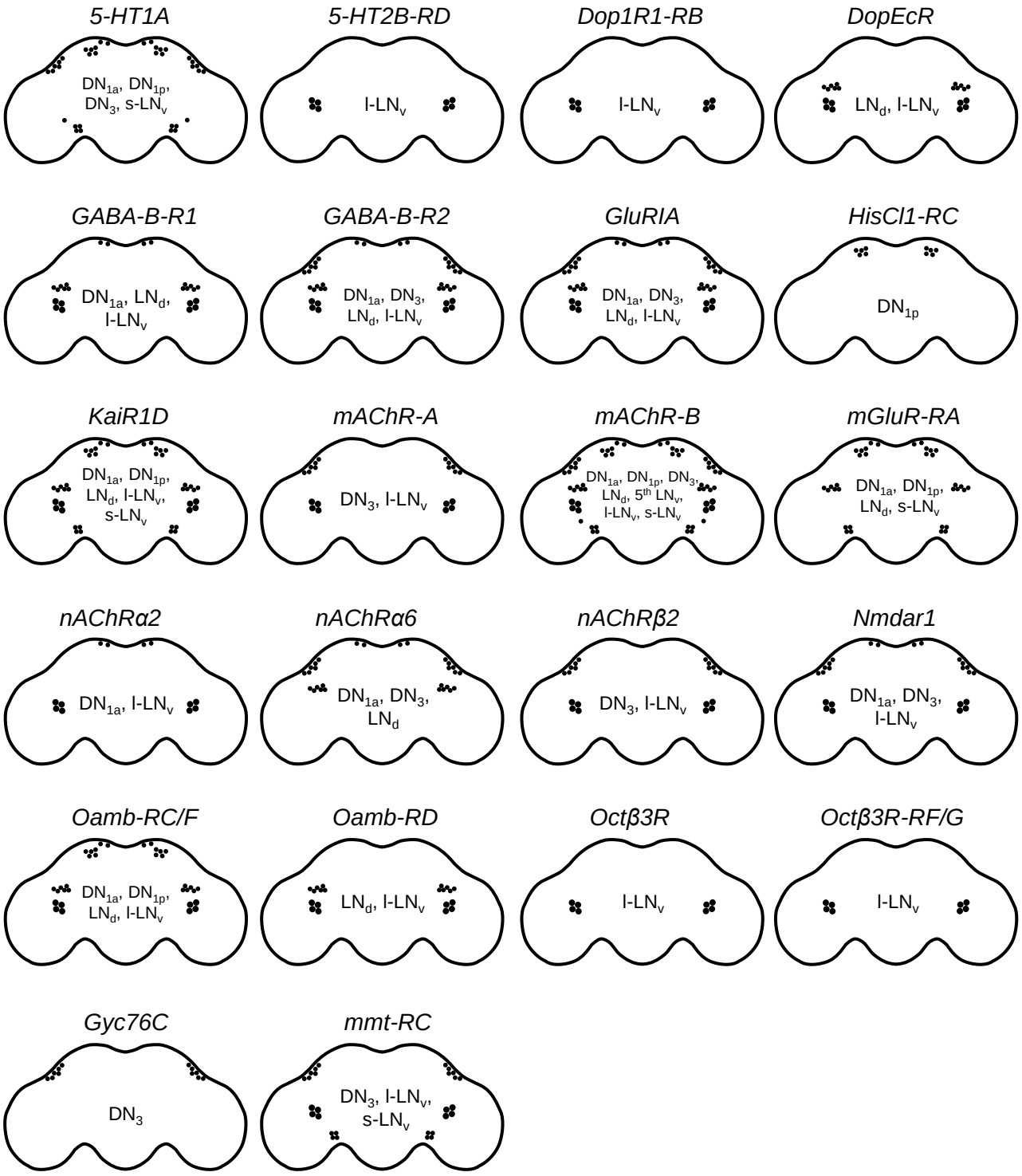
5-HT1A
DN1a, DN1p, DN3, s-LNv
5-HT2B-RD
l-LNv
Dop1R1-RB
l-LNv
DopEcR
LNd, l-LNv
GABA-B-R1
DN1a, LNd, l-LNv
GABA-B-R2
DN1a, DN3, LNd, l-LNv
GluRIA
DN1a, DN3, LNd, l-LNv
HisCl1-RC
DN1p
KaiR1D
DN1a, DN1p, LNd, l-LNv, s-LNv
mAChR-A
DN3, l-LNv
mAChR-B
DN1a, DN1p, DN3, LNd, 5th LNv, l-LNv, s-LNv
mGluR-RA
DN1a, DN1p, LNd, s-LNv
nAChRα2
DN1a, l-LNv
nAChRα6
DN1a, DN3, LNd
nAChRβ2
DN3, l-LNv
Nmdar1
DN1a, DN3, l-LNv
Oamb-RC/F
DN1a, DN1p, LNd, l-LNv
Oamb-RD
LNd, l-LNv
Octβ3R
l-LNv
Octβ3R-RF/G
l-LNv
Gyc76C
DN3
mmt-RC
DN3, l-LNv, s-LNv