A catalog of GAL4 drivers for labeling and manipulating circadian clock neurons in *Drosophila melanogaster*

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Abstract

Daily rhythms of physiology, metabolism, and behavior are orchestrated by a central circadian clock. In mice, this clock is coordinated by the suprachiasmatic nucleus, which consists of 20,000 neurons, making it challenging to characterize individual neurons. In *Drosophila*, the clock is controlled by only 150 clock neurons that distribute across the fly's brain. Here, we describe a comprehensive set of genetic drivers to facilitate individual characterization of *Drosophila* clock neurons. We screened GAL4 lines that were obtained from *Drosophila* stock centers and identified 63 lines that exhibit expression in subsets of central clock neurons. Furthermore, we generated split-GAL4 lines that exhibit specific expression in subsets of clock neurons such as the two DN2 neurons and the six LPN neurons. Together with existing driver lines, these newly identified ones are versatile tools that will facilitate a better understanding of the *Drosophila* central circadian clock.

Understanding circadian neural circuits has been a major challenge in the scientific field of circadian clock research. This is because animal circadian clocks are generally composed of a complex multi-cellular network, which possess multiple input and output pathways. In the fruit fly *Drosophila melanogaster*, approximately 150 brain neurons act as the central pacemaker clock neurons that control behavioral rhythms (Top and Young, 2018; King and Sehgal, 2018). These 150 clock neurons are broadly subdivided into 9 groups: 4 dorsal neuron groups (anterior dorsal neuron 1 (DN1a), posterior dorsal neuron 1 (DN1p), dorsal neuron 2 (DN2), and dorsal neuron 3 (DN3)) and 5 lateral neuron groups (small ventral lateral neuron (s-LNv), large ventral lateral neuron (l-LNv), 5th small ventral lateral neuron (5th s-LNv), dorsal lateral neuron (LNd), and lateral posterior neuron (LPN)). These groups of clock neurons are distinct in their function, morphology, and neurotransmitter content. However, the functions of individual clock neurons and their network properties are still under investigation. The primary techniques for dissecting the function of clock neurons are the GAL4-upstream activating sequence (UAS) system, and analogous gene expression systems such as the LexA and Q-systems, all of which enable expression of the gene of interest in a tissue-specific manner

(Owald et al., 2015). GAL4 lines are engineered to contain the *gal4* gene downstream of a desired gene promoter, enabling tissue specific expression of the GAL4 protein. UAS lines are engineered to contain a gene of interest downstream to the UAS, a specific enhancer to which the GAL4 protein binds in order to activate transcription (Brand and Perrimon, 1993). Thus, the promoter upstream of the *gal4* gene determines in which tissue the gene of interest (downstream of the UAS sequence) is expressed. For example, in circadian rhythm research, the *Pigment-dispersing factor* (*Pdf*)-GAL4 line has significantly contributed to the investigation of the ventral lateral clock neurons (s-LN$_v$ and l-LN$_v$), because of the very specific expression pattern of *Pdf* in those neurons (Renn et al., 1999; Park et al., 2000). To date, several GAL4 lines that express in clock neurons have been generated or identified. However, these GAL4 lines do not cover all types of clock neurons and there is a need for a greater variety of clock neuron GAL4 lines to facilitate further research.

In this study, we screened the databases of GAL4 lines that were established in the Janelia Research Campus and the Vienna Drosophila Resource Center (VDRC), in which there are currently more than 10 thousand GAL4 lines with different gene enhancers (Pfeiffer et al., 2008; Jenett et al.,

2012; Tirian and Dickson, 2017). After screening the databases of Janelia-GAL4

and VT-GAL4 lines (http://flweb.janelia.org/cgi-bin/flew.cgi and

https://stockcenter.vdrc.at/control/main, respectively) we identified

approximately 300 lines as potential GAL4 lines that may exhibit expression in

clock neurons. These GAL4 lines were crossed with a *UAS-Green fluorescent*

protein (*GFP*) line. Their offspring were sampled at zeitgeber time 20 under a 12

hour light: 12 hour dark cycle, and their brains were dissected and processed for

triple immunostaining using antibodies to detect the expression of PAR domain

protein 1 (PDP1), GFP and PDF. As a result, we identified 63 GAL4 lines that

exhibit expression in subsets of clock neurons. Some of these lines showed

specific expression that was restricted to clock neurons, whereas many lines

also exhibited expression in regions not specific to the neuronal clock network

(Fig. 1). Table 1 lists the GAL4 lines that exhibited expression in clock neurons.

We also show images of immunostained brain sections of all GAL4 lines in the

supplemental data (Suppl. Fig. S1-S75), where the GAL4 expression patterns

not only in clock neurons but also in non-clock neurons in the brain can be seen

(for non-clock neurons see also Janelia- and VT-GAL4 databases). While we

were screening the GAL4 lines, some Janelia- and VT-GAL4 lines were already

reported as clock neuron-specific lines (Table 2). Nevertheless, we included

some of these in our list because GFP expression patterns can depend on which

GFP reporter line is used. For example, the *R18H11-GAL4* line has been

previously identified as a GAL4 line specific for DN1 clock neurons (Kunst et al.,

2014). However, we have additionally observed expression in a few LNd

neurons, which may be due to the different GFP line or the sensitivity of the

anti-GFP immunostaining protocol used in this study. In agreement with our

result, at least two LNd neurons are clearly visible in the images of the

R18H11-GAL4 expression pattern presented in the Janelia GAL4 Flylight

database.

Although the *R18H11-GAL4* line exhibits very restricted expression in

subsets of DN1 and LNd neurons, with only few non-clock cells in the brain,

many cells in the ventral nerve cord express GAL4 (from the Flylight project

database). Similarly, while *R15C11* (LNd), *R16C05* (DN1a and LNd), *R51H05*

(DN1p), *R61G12* (s-LNv and l-LNv), *R77H08* (DN3), *R85G07* (LNd), *VT027163*

(s-LNv), *VT055852* (DN1 and LNd), and *VT063307-GAL4* (DN1p) lines exhibit

rather restricted expression patterns in the brain, all express GAL4 in some cells

of the ventral nerve cord. Additionally, all GAL4 lines listed here may have

expression outside of the nervous system, which was not examined in this study.

Therefore, one should consider side effects when those lines are used to

manipulate clock neurons. Several of the 63 GAL4 lines target the same group of

clock neurons. Thus, it is better to use more than one GAL4 line to reduce the

risk of side effects stemming from untargeted cells. Even for non-specific GAL4

lines, clock neuron-specific effectors, such as the *UAS-period* line, can be used

for a *period* rescue experiment (Blanchardon et al., 2001),

UAS-dominant-negative clock and *cycle* lines for a clock disruption (Tanoue et

al., 2004), or *UAS-guide RNA* line for cell-specific CRISPER-Cas9 knockouts (or

UAS-RNAi lines) for genes specifically expressed in clock neurons (Martinek

and Young, 2000; Delventhal et al., 2019; Schlichting et al., 2019).

LNd and DN1p neurons contain heterogeneous neuron types (Johard et

al., 2009; Yoshii et al., 2008). We characterized several GAL4 lines that

expressed in subsets of DN1p or LNd neurons, although not exclusively. Those

lines could be further used to label and manipulate DN1p and LNd neurons.

Moreover, the GAL4 lines identified here cover DN1a, DN2, DN3, and LPN

neurons that are less studied and will be useful for functional analysis of those

clock neurons.

The split-GAL4 system is a technique to refine GAL4 expression in cells by separating the DNA-binding domain (DBD) and the activation domain (AD) of GAL4 and inserting each domain downstream of two different gene promoters (Luan et al., 2006; Pfeiffer et al., 2010; Dionne et al., 2018). Previous studies have already reported split-GAL4 lines for s-LNv, 5th s-LNv and LNd, and DN1p neurons (H. Dionne, G. Rubin and A. Nern, unpublished; Liang et al., 2017; Guo et al., 2017). A large collection of split-GAL4 hemi-drivers is now available in the Bloomington Drosophila Stock Center (donated by G. Rubin and B. Dickson at the Janelia Research Campus). We selected some hemi-drivers and crossed them to generate split-GAL4 lines. Eventually, we created 12 split-GAL4 lines which exhibit expression in DN1a, l-LNv, s-LNv and LPN neurons, amongst others (please refer to Table 1 for the full list). *V013602-AD; R18F07-DBD*, *R43D05-AD; R93B11-DBD* and *VT04317-AD; R93B11-DBD*, *R11B03-AD; R65D05-DBD* lines are specific for the l-LNv, DN1a and LPN populations, respectively, and they fill in the gaps of already reported split-GAL4 lines. Interestingly, *R10H10-AD; R67D09-DBD* line labels three of the four l-LNv neurons, but the other l-LNv split-GAL4 line (*R61G12-AD; R18F07-DBD*) only labels one l-LNv neuron. A recent report demonstrated that one of the l-LNv

neurons has a projection pattern that differs from those of the other three l-LNv

neurons (Schubert et al., 2018), suggesting that the l-LNv neurons comprise two

functionally distinct groups. Similarly, the *R43D05-AD; VT003234-DBD*

split-GAL4 line only labels one of the two DN2 neurons, and it has been reported

that one DN2 neuron exhibits a clock protein expression profile that is distinct

from the other one (Yoshii et al., 2009). We observed non-specific expression,

even in the split-GAL4 lines; however, the level of expression was very low. This

may be because GAL-DBD has weak transcriptional activity on its own (Luan et

al., 2006). Thus, it is still worthwhile to create multiple split-GAL4 lines for a

single clock population to minimize unwanted side effects. Our initial 63 GAL4

lines would provide many combinations of the two enhancers.

In the late 1990s, the discovery of PDF and PDF-positive LNv neurons

caused a breakthrough in our understanding of the circadian clock at a neuronal

level (Helfrich-Förster, 1995; Renn et al., 1999), which led to the functional

analysis of the clock neuron network, such as its division into morning and

evening oscillators (Grima et al., 2004; Stoleru et al., 2004). Recently, the role of

DN1s neurons in sleep has received a lot of attention (Guo et al., 2016; Guo et

al., 2018; Lamaze et al., 2018). Additionally, several recent studies have

performed functional analyses of relatively minor clock neuron groups (Fujiwara et al., 2018; Ni et al., 2019). An understanding of the entire clock circuit remains elusive, but the GAL4 lines presented in this study will provide a new genetic toolkit to facilitate further investigation.

Appendix

The Janelia GAL4 and VT-GAL4 lines were obtained from the Bloomington Drosophila Stock Center (BDSC) and the Vienna Drosophila Resource Center, respectively. The *y w; UAS-GFP S65T* line (BDSC #1522) was used to cross with each GAL4 and split-GAL4 line.

Immunostainings were performed as described previously (Yoshii et al., 2015). The primary antibodies used were chicken anti-GFP (1:1000; Rockland, Limerick, PA, USA), mouse anti-PDF (1:1000; Developmental Studies Hybridoma Bank) (Cyran et al., 2005), and rabbit anti-PDP1 (1:9000) (Cyran et al., 2003). We used the following fluorescent-dye conjugated secondary antibodies at a 1:500 dilution: Alexa Fluor® 488 nm (goat anti-chicken), 647 nm (goat anti-mouse) antibodies (Life technologies, Carlsbad, CA, USA); and goat anti-rabbit Cy3 antibodies (Millipore, Billerica, MA, USA). PDP1 immunostaining

was used to visualize all clock neurons. The s-LNv and l-LNv neurons were characterized by PDF staining, which was also useful for distinguishing the 5th s-LNv and LNd groups that were also located within lateral brain regions. LPN neurons were located posteriorly; therefore, it was easy to distinguish them from other LN groups. DN1 neurons were located in the dorsal brain region. The DN1a group consisted of a pair of neurons that resided within the rim of the dorsal anterior side, compared to the DN1p group. The DN2 group was located deeper within the dorsal brain, compared to the DN1p neurons, and close to the s-LNv dorsal projections, as labeled by PDF staining. DN3 neurons were composed of approximately 30 small cells and were located within the rim of the dorsal brain, but more lateral than the other DN1 group. At least five brains for each strain were used for immunostaining to characterize the number of clock cells stained by anti-GFP antibodies (GAL4 expression) in the first experiment and the clock cells were briefly characterized. In the second experiment, we conducted the same immunostaining, but only for positive strains, to confirm the prior results. Images were taken from at least three different brains using laser scanning confocal microscopes (Olympus FV1200, Olympus, Tokyo, Japan). Finally, we counted the number of clock cells from the scanned brains. When the

GFP immunostaining signals in clock cells were very weak and only slightly stronger than background signals, we added the annotation "weak expression," as seen in Table 1.

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Conflict of Interest Statement

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure legends

Figure 1

Representative GAL4 expression images in the *Drosophila* brain. Each GAL4 line was crossed with a *UAS-GFP* line and their F1 offspring were processed for anti-GFP immunostaining. *VT027163-GAL4* (A) and *VT063307-GAL4* (B) lines exhibit rather specific expression in subsets of clock neurons, whereas the other GAL4 lines exhibit expression in many non-clock neurons (C, D). Arrows indicate clock neurons. Scale bar = 100 µm.