Expression of the foraging gene in adult Drosophila melanogaster

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ABSTRACT

The foraging gene in Drosophila melanogaster, which encodes a cGMP-dependent protein kinase, is a highly conserved, complex gene with multiple pleiotropic behavioral and physiological functions in both the larval and adult fly. Adult foraging expression is less well characterized than in the larva. We characterized foraging expression in the brain, gastric system, and reproductive systems using a T2A-Gal4 gene-trap allele. In the brain, foraging expression appears to be restricted to multiple sub-types of glia. This glial-specific cellular localization of foraging was supported by single-cell transcriptomic atlases of the adult brain. Foraging is extensively expressed in most cell types in the gastric and reproductive systems. We then mapped multiple cis-regulatory elements responsible for parts of the observed expression patterns by a nested cloned promoter-Gal4 analysis. The mapped cis-regulatory elements were consistently modular when comparing the larval and adult expression patterns. These new data using the T2A-Gal4 gene-trap and cloned foraging promoter fusion GAL4’s are discussed with respect to previous work using an anti-FOR antibody, which we show here to be non-specific. Future studies of foraging’s function will consider roles for glial subtypes and peripheral tissues (gastric and reproductive systems) in foraging’s pleiotropic behavioral and physiological effects.

Introduction

Genes that influence behavior are often pleiotropic, expressed throughout development and in many tissues (Hall, 1994). Investigating the temporal and spatial expression of pleiotropic genes can aid in the interpretation of their many functions. The Drosophila melanogaster foraging (for) gene, which codes for a cGMP-dependent protein kinase (PKG), has long been a model system for studies of gene-environment interaction and pleiotropy in the field of behavior genetics (Allen, Anreiter, Neville, & Sokolowski, 2017; de Belle, Hilliker, & Sokolowski, 1997; reviewed in Anreiter & Sokolowski, 2019). Foraging is a complex gene with a modular structure that includes four promoters (for0, for0, for1, for2), which produce approximately 20 RNA transcripts that code for nine distinct protein isoforms (Allen et al., 2017). The different isoforms of foraging have varied expression levels depending on the developmental stage and tissue being assayed (Allen et al., 2017; Anreiter, Kramer, & Sokolowski, 2017; Brown et al., 2014; Dason et al., 2020; Leader, Krause, Pandit, Davies, & Dow, 2018), but little is known about the cell-specific expression of foraging.

Some indication of the tissue-specific requirement for a suite of feeding-related and physiological phenotypes has been described in the larva (Allen, Anreiter, Vesterberg, Douglas, & Sokolowski, 2018; Dason, Allen, Vasquez, & Sokolowski, 2019, Dason & Sokolowski, 2021). We previously generated a full genetic deletion of the foraging gene, for0, and found that these foraging null larvae had reduced locomotion on food, reduced food intake, and increased triglyceride content (Allen et al., 2017). We were then able to rescue these attributes using different cloned foraging regulatory regions, which drove expression in discrete and restricted patterns in third instar larvae (Allen et al., 2018). Specifically, the decrease in larval locomotion on food of the for0 mutant was rescued by UAS-for0-RNAi, for0-RNAi, for0-Gal4 expressed in neurons of the CNS and enteroendocrine cells (EE cells) of the gut. Triglyceride levels were rescued by for0-Gal4, which expressed in many cell types such as perineurial glia, visceral muscle, trachea, and fat cells (among others). Finally, food intake was rescued by for0-Gal4, which expressed in the developing optic lobes, hindgut, and spiracles (Allen et al., 2018). Similar tissue-specific requirements of foraging were found at the larval neuromuscular junction where the for0 null mutant phenotype of nerve terminal overgrowth was rescued by glial expression, and increased neurotransmission was rescued by neuronal expression (Dason et al., 2019). Hence, these discrete and restricted expression patterns were successfully used to dissect some of the pleiotropic behavioral and physiological functions of foraging during the larval stage.

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In addition to its larval phenotypes, the foraging gene has been implicated in many adult behavioral phenotypes. These include post-feeding locomotion (Pereira & Sokolowski, 1993), sucrose responsiveness (Belay et al., 2007; Scheiner, Sokolowski, & Erber, 2004), learning and memory (Kaun et al., 2007; Kohn et al., 2013; Kuntz, Poeck, Sokolowski, & Strauss, 2012; Mery, Belay, So, Sokolowski, & Kawecki, 2007; Reaume, Sokolowski, & Mery, 2011; Wang et al., 2008), habituation (Edsion, Belay, Sokolowski, & Heberlein, 2012; Engel, Xie, Sokolowski, & Wu, 2000; Scheiner et al., 2004), social behavior (Donlea et al., 2012; Foucaud et al., 2013; Alwash et al., 2021), sleep (Donlea et al., 2012), starvation resistance (Anreiter et al., 2017; Donlea et al., 2012), aggression (Wang & Sokolowski, 2017), and stress tolerance (Dawson-Scully, Armstrong, Kent, Robertson, & Sokolowski, 2007; Dawson-Scully et al., 2010). Many of these phenotypes show parallels between the larva and the adult developmental stages. For instance, foraging affects food search behavior in both larvae and adults (Anreiter et al., 2017; Hughson et al., 2018; Kent, Daskalchuk, Cook, Sokolowski, & Greenspan, 2009; Pereira & Sokolowski, 1993; Sokolowski & Riedl, 1999). However, the behavioral patterns seen for food intake are reversed when comparing larvae and adults. Larvae carrying the forpR null allele show decreased food intake relative to controls, and this can be rescued by a full transgenic complement of the locus as well with a tissue-specific expression of a foraging cDNA (Allen et al., 2017, 2018). In contrast, food deprived adults with forpR foraging transcripts ubiquitously knocked-down with RNAi consume more sucrose drops than controls (Anreiter et al., 2017). This suggests the possibility of differential regulation of the foraging gene for specific phenotypes at the larval and adult developmental stages. In the larva, foraging’s pleiotropy is in part due to foraging’s four promoters driving distinct expression patterns of subsets of transcripts, each associated with different phenotypes. We wondered whether foraging exhibited promoter-specific expression patterns in the adult and if they paralleled those observed in larvae.

Here we characterize the adult expression of foraging in the brain, gastric system, and reproductive systems, using: a) a transcriptional and translational trap T2A-Gal4 allele (Lee et al., 2018), b) published data from single-cell transcriptomic analyses (Davie et al., 2018; Hung et al., 2020), c) foraging promoter Gal4 fusions, and d) by revisiting a previously published anti-FOR antibody. We further refine and map regions containing cis-regulator elements using a series of nested promoter Gal4 fusions and compare the modular patterns of expression of the larval and adult fly.

Materials and methods

Fly strains and rearing

Flies were reared in 40 ml vials with 10 ml of food with a 12L:12D photocycle at 25 ± 1°C. The food recipe has previously been described (Allen et al., 2017). The following fly strains were used in this study: forpR/Cyo, [Act-GFP] (Allen et al., 2018), [forpR-Gal4]attP2 (Allen et al., 2018), [forpR2-Gal4]attP2 (Allen et al., 2018), [forpR3-Gal4]attP2 (Allen et al., 2018), {forpR4-Gal4}attP2 (Allen et al., 2018), y+w; T[GFPR3x3p3.Clar]=CRIMIC.TG4.2[forCR00867-TG4.2] (Bloomington Drosophila Stock Center # 79329; Lee et al., 2018), w++; P[UAS-mCD8::GFP]/CyO (denoted as mCD8::GFP), w118; P[UAS-Stinger2] (Bloomington Drosophila Stock Center # 84277, denoted as nls::GFP; Barolo, Carver, & Posakony, 2000), w++; UAS-myr-GFP-V5-P2A-H2B-mCherry-HA/TM3, Ser (denoted as UAS-Watermelon; Chang, Keegan, Prazak, & Dubnau, 2019).

Construction of nested Gal4s

Construction of the forpR-Gal4s was previously described (Allen et al., 2018). A similar strategy was used to generate the nested forpR3-Gal4 constructs. The Nor1 fragment, containing the Gal4 sequence, of pMARTINI-Gal4 (Billeter & Goodwin, 2004) was cloned into the Nor1 digested pStinger-attB vector, replacing the GFP. This insulated Gal4 vector with attB was then digested with KpnI and end filled with Klenow (cat # M0210S, New England Biolabs). Nested regions of foraging were amplified by PCR from the larger forpR-Gal4 vectors and cloned into the end filled insulated Gal4 vector. All forpR3-Gal4s constructs were injected into the P{CarryP}attP2 landing site (Groth, Fish, Nusse, & Calos, 2004) by Genetic Services Inc. (Cambridge, MA, USA). Successful integration was confirmed with PCR.

The primers used for the nested forpR3-Gal4s are as follows:

- forpR1.1F: 5’-TCGAATAACCCACCCATTCA-3’,
- forpR1.2F: 5’-CAGCAATCATTATTGGCCT3-’,
- forpR1.3F: 5’-CTTTCTCCAGTCTATAT-3’,
- forpR1.4F: 5’-CAGTATATCATGTCGAT-3’,
- forpR1.5R: 5’-ACAGTCTGATAAACCAGCC-3’,
- forpR2.1F: 5’-CTAAGGTTTCTCCGCAGCA-3’,
- forpR2.2F: 5’-ACAAGGAATGGAACCCGGAAC-3’,
- forpR2.3R: 5’-CACAACCCAGTTGAACAC-3’,
- forpR3.1F: 5’-ATACCCCTCATCAGAAGGC-3’,
- forpR3.2F: 5’-TCCCAACGAGTTTTCTTTT-3’,
- forpR3.3F: 5’-CAGGGGAATGATAACCCGA-3’,
- forpR3.4F: 5’-GCACATTAGAACCCTAGGGA-3’,
- forpR3.5R: 5’-GGGATCTGGTCAATTCTG-3’,
- forpR4.1F: 5’-CCCTACTCTATAAACTCCGAC-3’,
- forpR4.2F: 5’-AGTTGGGATCTGGGTTT-3’,
- forpR4.3F: 5’-TTCCTGCTTCCACAGACAC-3’,
- forpR4.5R: 5’-CGAATTTAAACATCACTAGAC-3’.

Recombineering BAC(forRES-Gal4) allele

We generated a transgenic copy of the entire foraging locus, replacing the common coding region with a Gal4 coding sequence, using recombineering. We used the BAC containing the entire 35 kb foraging locus (previously described in Allen et al., 2017) and replaced the common coding region of foraging with a premature stop codon, followed by an Internal-Ribosomal-Entry-Site (RES) and codon optimized Gal4 coding sequence. The Gal4 coding sequence replaced...
the foraging common coding sequence, but still utilized the endogenous foraging 3′UTR.

To generate this BAC{forIRES-Gal4} allele we needed to clone a donor construct with Gal4 sequence flanked with homology arms to foraging. Restriction sites added to primers are in parentheses and their sequences are italicized and separated by a hyphen. A D. melanogaster codon optimized Gal4 sequence was PCR amplified, with primers dmGal4-F (Ascl) 5′-GGCCCAGC-ATGGAAGCTGCTA GTATG-3′ and dmGal4-R (BsfI) 5′-CTCTGAGG-CTACT CTCTTTTGGTCTGG-3′, from the pBPGAL4.1Uw vector (Pfeiffer et al., 2010; addgene # 26226) and into the pSC-A-amp/kan vector from the StrataClone PCR Cloning Kit (Agilent, cat # 240205). The Spel–NotI fragment containing an FRT-kan-FRT cassette from the pGCN21 vector (Lee et al., 2001) was cloned into the Spel and NotI sites of the pSC-dmGal4 vector. An Internal-Ribosomal-Entry-Site (IRES) was PCR amplified from the Ubx locus (as in Halfon et al., 2002) with the primers Stop-ires-F (HindIII) 5′-AAGCTT-CTAGACTAGCTCTGAGCAGAAGTTGCAATTT GCTAAAACC-3′ and Stop-Ires-R (Ascl) 5′-GGCCCAGCC- GATTCTTACCGCGACGCG-3′ and cloned into the HindIII and Ascl sites of the pSC-dmGal4-FRT-kan-FRT vector. An all 6 frame stop codon cassette added to the forward primer is underlined and separated by a hyphen. A left homology arm corresponding to foraging specific sequence was PCR amplified with L-comGal4-F (KpnI) 5′-GGTACC-GCTCCGACCCAGAACC-3′ and L-comGal4-R (HindIII) 5′-AAGCTT-CTCTGAGGAAACCTCCACG-3′ and cloned into the KpnI and HindIII sites of the pSC-IRES-dmGal4-FRT-kan-FRT vector. A right homology arm corresponding to foraging specific sequence was PCR amplified with R-comGal4-F (BglII) 5′-AGATCT-GGAGAACATCGA ACCGTTTC-3′ and R-comGal4-R (NotI) 5′-GGCCGCGCG- GCATAAAATCGGTTGCT-3′ and cloned in the BglII and NotI sites of the pSC-LHA-IRES-dmGal4-FRT-kan-FRT vector.

The KpnI–NotI fragment from the pSC-LHA-IRES-dmGal4-FRT-kan-FRT vector was transformed into EL250 E. coli strain (Lee et al., 2001) which already contained a bacterial artificial chromosome (BAC) containing the 35 kb foraging locus (previously described in Allen et al., 2017). Recombinered BACs were selected by kanamycin resistance. The FRT-kan-FRT was then removed by arabinoside induction. Proper integration Gal4 sequence and replacement of the foraging common coding region was verified with PCR, restriction digest, and Sanger sequencing. qC31 integration was used to integrate the BAC into the VK00013 landing site on the third chromosome (Venken, He, Hoskins, & Bellen, 2006). Transgenesis was performed by BestGene Inc. Primer design, in-silico cloning, and analysis of Sanger sequencing reactions were all performed in the Geneious 8 software package (Kearse et al., 2012).

**Immunohistochemistry**

Adult and larval samples were dissected in 1 × PBS and then fixed in 4% paraformaldehyde in 1 × PBS for 40 min. The tissues were then rinsed twice and then washed 4 × for 45 min each in 0.3% Triton X in 1 × PBS (PBT). The tissues were blocked in 10% normal goat serum (NGS, Jackson ImmunoResearch Laboratories) in PBT for 18–36 h at 4°C. Primary antibody incubations were conducted in blocking solution and incubated for 36–48 h at 4°C. The tissues were then rinsed twice and washed 4 × for 45 min. each in PBT. Secondary antibody, in blocking solution, were incubated for 36–48 h at 4°C. Tissues were washed as described above for the primary antibody. Tissues were then cleared in 70% glycerol in PBS for 18–24 h at 4°C. Tissues were mounted on slides in Vectorshiel (cat # H-1000–10, Vector Laboratories). Tissues were imaged using a Zeiss Axioscope epifluorescence microscope as well as a Zeiss LSM 510 and Leica SP5 confocal microscopes. Images were analysed using Fiji (Schindelin et al., 2012). The following antibodies were used at the following concentrations: guinea pig anti-FOR (1:200, Belay et al., 2007), mouse anti-Btp (1:50, Developmental Studies Hybridoma Bank), chicken anti-GFP (1:400, cat # A10262, Thermo Fisher Scientific), rabbit anti-GFP (1:200, cat # A-11122, Thermo Fisher Scientific), rat anti-mCherry (1:400, cat # M11217, Thermo Fisher Scientific), Alexa Fluor 546 Phalloidin (1:400, cat # A22283, Thermo Fisher Scientific), goat antichicken Alexa Fluor 488 (1:400, cat # A-11039, Thermo Fisher Scientific), goat antirabbit Alexa Fluor 488 (1:400, cat # A-11008, Thermo Fisher Scientific), goat antimouse Alexa Fluor 488 (1:400, cat # A-11001, Thermo Fisher Scientific), goat antimouse Alexa Fluor 633 (1:400, cat # A-21052, Thermo Fisher Scientific), goat antirat Alexa Fluor 546 (1:400, cat #A-11081, Thermo Fisher Scientific), goat antirat Alexa Fluor 633 (1:400, cat # A-21094, Thermo Fisher Scientific), goat antigalactina pig Alexa Fluor 633 (1:400, cat # A-21105, Thermo Fisher Scientific).

**Single-cell RNA-Seq data and analysis**

The single-cell transcriptomic atlas of the adult brain was previously published (Davie et al., 2018) and the data were acquired from Scope (http://scope.aertslab.org/#/e0816194-aea3-48d8-af80-569baf58be35/Aerts_Fly_AdultBrain_Filtered_57k.loom/gene). These cells were reprocessed as previously described (Allen et al., 2020). Briefly, the data were processed in R (R Core Team, 2020) with Seurat v2.3.4 (Satija, Farrell, Gennert, Schier, & Regev, 2015). Data were normalized and scaled with ‘NormalizeData’ and ‘ScaleData’ functions. A principal component analysis was run, with the ‘RunPCA’ function, on the variably expressed genes (as deduced by ‘FindVariableGenes’). A 2-dimensional t-distributed stochastic neighbor embedding (t-SNE; Van Der Maaten, Courville, Fergus, & Manning, 2014) was performed, using ‘RunTSNE’, on the first 82 principal components for visualization. Groups of cells with similar expression patterns were deduced with the ‘FindClusters’ function. The expression patterns of repo and foraging were visualized with the ‘FeaturePlot’ function. The per cluster average scaled expression was calculated with the
'AverageExpression' function and plotted using the pheatmap package (Kolde, 2019).

The single-cell transcriptomic atlas data of the adult midgut were previously published (Hung et al., 2020) and the data were acquired from Gene Expression Omnibus (accession no. GSE120537). The data were processed with Seurat v3.2.2 (Stuart et al., 2019) as described in the original publication (Hung et al., 2020). Briefly, the data were normalized and scaled with 'NormalizeData' and 'ScaleData' functions. Variably expressed genes were identified with the 'FindVariableGenes' function. The different replicates were integrated with the 'FindIntegrationAnchors' and 'IntegrateData' functions. A principal component analysis was run, with the 'RunPCA' function. A 2-dimensional Uniform Manifold Approximation and Projection (UMAP; McInnes, Healy, & Melville, 2018) was performed, using 'RunUMAP', for the first 30 principal components for visualization. Groups of cells with similar expression patterns were deduced with the 'FindClusters' function. The per cluster average scaled expression was calculated with the 'AverageExpression' function and plotted using the pheatmap package (Kolde, 2019).

**Results**

**Foraging expression in the adult brain**

To infer the expression patterns and regulation of foraging in the adult, we took advantage of a novel T2A-based Gal4 line and cloned promoter Gal4 fusion lines from multiple regions of the locus. T2A-based Gal4 alleles are designed to reliably capture endogenous gene expression due to being a transcriptional trap, as well as a translational trap. In the present study, we used the previously generated forCR00867-TG4.2 CRIMIC allele (Lee et al., 2018), which is integrated downstream of the first common coding exon (Figure 1(A)), allowing the splice acceptor sequence to capture the endogenous transcription from foraging. forCR00867-TG4.2 is a homozygous lethal allele of foraging, dying as a pharate adult late in pupal development and does not complement the pupal lethality of the for0 null allele (Allen et al., 2017; Anreiter et al., 2021). Heterozygous forCR00867-TG4.2 driven UAS-forRNAi induces a consistent late pupal lethality (Anreiter et al., 2021). We found that forCR00867-TG4.2 drove expression in many cells with morphology consistent with multiple glial subtypes in the adult brain (Figure 1(B–D)). Most prominently, forCR00867-TG4.2 expressed in the surface glia (Figure 1(C), arrow). Although we did not perform co-localization with a perineural marker, the surface glial pattern observed using the forCR00867-TG4.2 was consistent with perineural glia as evident from the number of cells (Figure 1(C′)); Asasaki, Lai, Ito, & Lee, 2008). Previous studies found that foraging was among the top 50 most enriched genes when comparing surface glia transcripomes to neuronal transcripomes, and a protein trap allele P[P{PTT-GB}forC092956 also expressed in perineural glia (DeSalvo et al., 2014). Surface glia function as the blood–brain barrier in the larval and adult fly and regulate the transport of hormones, nutrients, and metabolites between the hemolymph and the brain (Bainton et al., 2005; Laughlin, De Ruyter Van Steveninck, & Anderson, 1998; Limmer, Weiler, Volkenhoff, Babatz, & Klämbt, 2014; Schwabe, Bainton, Fetter, Heberlein, & Gaul, 2005; Stork et al., 2008). Functions for glial subtypes and neuron–glia interactions in behavioral phenotypes have received relatively little attention (Artiushin & Sehgal, 2020; Bittern et al., 2020).

Multiple distinct glial subtypes are found in the fly brain, all of which play distinct roles (Kremer, Jung, Batelli, Rubin, & Gaul, 2017; Yildirim, Petri, Kottmeier, & Klämbt, 2019). forCR00867-TG4.2 drove expression in many of these subtypes. The next strongest expression in glial subtypes was found in the outer optic chiasm glia (Figure 1(C), arrowhead) and the inner optic chiasm glia (Figure 1(D), arrowhead). The outer optic chiasm glia have a fine, wispy structure found between the lamina and medulla (Kremer et al., 2017). The inner chiasm glia fill the space between the medulla and the lobula and lobula plate (Kremer et al., 2017). Weaker expression was seen in the neuropil- and tract-ensheathing glia (Figure S1(A,B)) and in the cortex glia (Figure S1(C–C″)).

We also observed expression in the tracheal system surrounding and innervating the brain (data not shown). The forCR00867-TG4.2 allele drove expression in both the large air sacs and the fine branching trachea in the brain. foraging expression in larval trachea has previously been found (Leader et al., 2018).

To map the regulatory elements responsible for the observed expression, we next examined expression from the forPR-Gal4 constructs (Figure 1(E)). The cloned regions encompassed 2–5kb upstream of each transcription start site (TSS) to 200bp downstream of each TSS. These regions were cloned into a Gal4 vector and inserted into the attP2 landing site (described in Allen et al., 2018). The forPR-Gal4 regulatory regions together cover 15 kb of the 35 kb foraging locus. As was previously characterized in the larval CNS (Allen et al., 2018), each forPR-Gal4 line showed different expression patterns in the adult brain (Figure 1(E′–E″′)). In adults, forPR1-Gal4 drove expression in only a few neurons in the brain (Figure 1(E′)). We saw the most robust expression in a pair of neurons with arborcs connecting the suboesophageal zone to the medulla. None of these neurons were evident in the expression patterns observed with the forCR00867-TG4.2 allele. forPR2-Gal4 expressed in the trachea and air sacs surrounding and innervating into the adult brain, but there was no observable neuronal or glial expression (Figure 1(E″)). forPR3-Gal4 drove expression in the surface glia (Figure 1(E″″), Figure S1(D)). This expression is consistent with perineurial glia as was seen in forCR00867-TG4.2. forPR4-Gal4 expressed in the outer and inner chiasm glia of the optic lobes (Figure 1(E″″′), Figure S1(F)).

The forPR-Gal4s do not express in all of the cells observed in forCR00867-TG4.2 but mostly comprise a subset of the brain expression pattern observed in forCR00867-TG4.2. As less than half of the foraging locus is covered by these forPR-Gal4s lines, the necessary CREs for the remaining expression may lie in the un-cloned regions. Nevertheless, we found parallels between the expression patterns observed using the forCR00867-TG4.2 gene trap and cloned forPR-Gal4s.
In recent years, the advent of massively parallel single-cell transcriptomics has led to the unprecedented characterization of gene expression of individual cell types. Multiple data sets characterize the transcriptomic profiles of different subsets of the adult CNS (Allen et al., 2020; Croset, Treiber, & Waddell, 2018; Davie et al., 2018; Gao et al., 2015; Konstantinides et al., 2018; Li et al., 2017). We took advantage of a whole brain data set (Davie et al., 2018) to explore foraging expression at the single-cell resolution. Individual cells are grouped based on the similarity of their gene expression profiles. For this experiment, we took advantage of a whole brain data set (Davie et al., 2018) to explore foraging expression at the single-cell resolution. Individual cells are grouped based on the similarity of their gene expression profiles.
expression profiles, and different colors represent distinct cell types (see "Methods", Figure 2(A)). Using the established glial marker gene repo, we can identify the glial clusters (Figure 2(B)). foraging expression was for the most part restricted to repo positive clusters, but expression was also seen in hemocytes and photoreceptors (Figure 2(C,D)). Looking at the average scaled expression for multiple neuronal, glial, and glial sub-type markers across each cluster of cells (colors in Figure 2(A)), it is clear that foraging is specifically enriched in glia with highest enrichment in perineurial glia (Figure 2(D), red arrow).

The for\textsuperscript{CR00867-TG4.2} allele (Figure 1(B–D)) and the single-cell transcriptomics data (Figure 2(A–D)) found no detectable neuronal expression in the adult fly brain. We were surprised not to detect neuronal expression in the adult fly brain because functional studies that manipulate foraging levels in neurons are known to alter several behavioral phenotypes (reviewed in Anreiter and Sokolowski 2019 and discussed further below). Consequently, we used two more Gal4 alleles, the for\textsuperscript{MB01791-TG4.1} (Diao et al., 2015; Lee et al., 2018) and BAC[for\textsuperscript{RES-Gal4}] to investigate foraging expression in the adult brain. The for\textsuperscript{MB01791-TG4.1} allele also drove expression in the surface glia, and similar to the CRIMIC allele did not have any detectable neuronal expression (Figure S1(G–G')). Both the for\textsuperscript{MB01791-TG4.1} and for\textsuperscript{CR00867-TG4.2} alleles rely on a splice acceptor and T2A sequence to trap the endogenous transcription and translation of foraging. We also implemented an independent strategy to generate a recombinered bacterial artificial chromosome (BAC), containing the entire foraging locus, with the coding sequence common to all isoforms replaced with an Internal-Ribosomal-Entry-Sequence and Gal4 coding sequence. An un-mutated copy of this BAC was previously shown to have similar expression, as measured by RT-qPCR and western blot, to that of wild-type flies, and was sufficient to rescue the for\textsuperscript{0} null mutant in many of foraging's larval associated phenotypes (Allen et al., 2017; Dason et al., 2019). BAC[for\textsuperscript{RES-Gal4}] allele drove expression in the surface glia and again had no detectable neuronal expression (Figure S1(H–H')). We provide a more detailed expression analysis using the for\textsuperscript{CR00867-TG4.2} allele as its expression was stronger than the for\textsuperscript{MB01791-TG4.1} and BAC[for\textsuperscript{RES-Gal4}] alleles.

Previous studies characterized the expression of foraging proteins in the adult brain using an anti-FOR antibody (Belay et al., 2007; Mery et al., 2007). A 40 amino acid sequence from the C-terminus shared by all predicted FOR isoforms was used to generate the polyclonal antibody used in this study (Figure 3(A)). Alignments showed that the full C-terminal segment used to make this antibody was not encoded by any other sequences in the D. melanogaster genome (Belay et al., 2007). At the time of publishing Belay et al. (2007), we did not possess a complete genetic deletion of foraging. Consequently, we used a partial deletion of foraging called Df(2L)ED243 to confirm the effectiveness of this anti-FOR antibody. This deletion removed 2 of the 4 TSSs of foraging spanning from 50bp downstream of for\textsuperscript{Pr1} to

![Figure 2](image-url)
65 bp upstream of for<sup>pr-e4</sup> (Ryner et al., 1996). In Belay et al. (2007), the specificity of the anti-FOR antibody in immunohistochemical analyses was evaluated using the larval proventriculus instead of adult tissue because homozygous Df(2L)ED243 do not survive to adulthood. Immunoreactivity in the larval proventriculus was absent in Df(2L)ED243 homozygous larvae compared to the control. Localized signal in the adult brain was also missing when tissues were incubated with the preabsorbed antibody. Belay et al. (2007) concluded that the anti-FOR antiseraum detects endogenous FOR.

However, we recently published a full 35 kb genetic null of the foraging gene, for<sup>0</sup> (Allen et al., 2017). The stage of lethality of the for<sup>0</sup> null mutant is late pupal lethal (Allen et al., 2017; Anreiter et al., 2021). This new allele allowed us to re-examine the efficacy of the anti-FOR staining using late stage for<sup>0</sup> null mutant pupal brains (4 days post-pupariation). Anti-FOR antibody staining in control pupae showed the same expression patterns previously reported in adult heads (Figure 3(B); Belay et al., 2007; Mery et al., 2007). All of the previously reported primary anti-FOR clusters were seen in the pupal brain, optic lobe cluster, medulla cluster, ellipsoid body cluster, frontal cluster, dorsal posterior cluster, and the mushroom bodies. However, when we examined the for<sup>0</sup> pupal brains, all of these clusters remained (Figure 3(C), except for part of the medulla cluster (Figure 3(B,C), arrows). Specifically, immunoreactivity in the for<sup>0</sup> null brains was seen in the ellipsoid body cluster (Figure 3(D–D‘)), the dorsal posterior cluster (Figure 3(E)), the mushroom bodies (Figure 3(F)), the frontal cluster (Figure 3(G)), the optic lobe cluster (Figure 3(H), arrow), and medulla cluster 1 (Figure 3(H), arrowhead), but was lacking from medulla cluster 2 (Figure 3(H), double-headed arrow). The medulla cluster 2 was previously reported to be ELAV negative (Belay et al., 2007), has a similar characteristic structure to that of the outer optic chiasm glia (Kremer et al., 2017), and is consistent with that seen in the for<sup>CR00867-TG4.2</sup> and for<sup>pr-e4</sup>, Gal4<sub>e</sub> expression patterns (Figure 1(C,E’’’’)). This finding suggests that in the adult brain, the anti-FOR antibody labels bone fide FOR expression in the outer optic chiasm glia but also has significant non-specific immunoreactivity suggesting that the majority of the previously described primary expression patterns in the adult brain is not FOR. Previous functional studies that showed significant effects on adult behaviors when manipulating foraging expression in the mushroom bodies and ellipsoid body are discussed below.

It is notable that the anti-FOR antibody displays high specificity to FOR proteins in western blot analyses of control and for<sup>0</sup> mutant larvae and that the for<sup>0</sup> null mutants did not display any immunoreactivity (Allen et al., 2017; Dason et al., 2019). We confirmed that there were no strong matches other than FOR along the whole 40 amino acid sequence (Belay et al., 2007), with a BLASTp search. However, low-affinity targets may be of concern when non-specificity of an antibody is found (Fritschy, 2008), and we did find smaller stretches of significant similarity with Protein kinase A catalytic subunits Pka-C1 and Pka-C3 (data not shown). Both Pka-C1 and Pka-C3 have been shown to be expressed in the Kenyon cells of mushroom bodies (Croset et al., 2018; Davie et al., 2018; Davis et al., 2020; Skoulakis, Kalderon, & Davis, 1993). Further purification of this antibody may ameliorate its performance in the context of whole mount immunohistochemistry, for instance, pre-absorbing the antibody with protein extracted from for<sup>0</sup> mutants. The anti-FOR antibody specificity and utility works well to detect FOR proteins on westerns but not in immunohistochemical analysis. The context specificity of the utility of antibodies is a common problem (Baker, 2015).

**Foraging expression in the adult gastric system**

The gastric system plays a crucial and central role in feeding-related phenotypes. Not only is it essential for the digestion of food, absorption of nutrients, and excretion of waste, but also it provides feedback to the brain via hormone signaling that can affect behavior (reviewed in Miguel-Aliaga, Jasper, & Lemaître, 2018). The foraging gene influences many feeding-related phenotypes suggesting that foraging may have functions in the adult gastric system. We found that the for<sup>CR00867-TG4.2</sup> allele expressed throughout the gastric system (Figure 4(A–F)). Expression was evident in all major compartments (foregut and crop, cardia, midgut, hindgut, and Malpighian tubules) of the gastric system and all major cell types.

Expression was found in the esophagus, foregut (crop duct), and crop (Figure 4(A,B)). The crop provides a central role in nutrient sensing and digestion in the adult fly (Hadjieconomou et al., 2020). for<sup>CR00867-TG4.2</sup> expressed in the enterocytes (ECs; Figure 4(A,B)) and enteroendocrine cells (EE cells; Figure 4(C)) of the midgut. The EE cells can be identified by their morphology and by co-expression with Brp (Figure 4(C’)); Zeng, Lin, & Hou, 2013). The ECs and EE are important for absorption and digestion of ingested nutrients. Moving from the anterior to the posterior, digestion shifts from larger macromolecules to smaller monosaccharides. We also observed expression in the intestinal stem cells of the midgut (Figure 4(D)). The ISC are important for midgut homeostasis (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006; reviewed in Miguel-Aliaga et al., 2018).

Expression was found in the visceral muscle throughout the entire length of the gut, from the foregut to the hindgut (Figure 4(E)). The visceral muscle is required to push these nutrients along via peristalsis. for<sup>CR00867-TG4.2</sup> was expressed throughout the Malpighian tubules (Figure 4(B), arrow; Figure 4(F)). The tubules are vital for ion balance in the fly’s hemolymph. There was strong expression in the hindgut proliferation zone of the pylorus, and in the epithelial cells throughout the rest of the hindgut and ampulla (Figure 4(A)). Much of the hindgut is important for ion absorption, and the ampulla is crucial for water balance (Lemaître & Miguel-Aliaga, 2013). for<sup>CR00867-TG4.2</sup> also drove expression in the salivary glands (Figure 4(B), arrowhead) and the trachea innervating the gut (Figure 4(C), arrow).

We next turned to our for<sup>pr-Gal4</sup> driver lines to parse this expression pattern and map its regulatory elements
As for the larva, we found a modular pattern of expression in the adult. We found no detectable expression from forpr1-Gal4 in the gastric system. forpr2-Gal4 expression was found in the epithelia of the foregut, crop, and cardia (Figure 4(H)). forpr2-Gal4 expression was also seen in the midgut ISCs (Figure 4(H′)) as typified by co-expression with DELTA (Figure S2(A); Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006) and in the EE precursor cells (pre-EE) as typified by co-expression with Perrimon, 2006; Ohlstein & Spradling, 2006) and in the EE (Suzuki & Ohlstein, 2020). The forpr3-Gal4 drove expression in a narrow band of ECs in the mid-region of the midgut, a few anterior EEs, visceral gut muscle, and the principal cells of the transitional segment of the Malpighian tubules (Figure 4(I–I′)). forpr4-Gal4 drove expression in the epithelia of the hindgut and in the rectal ampulla (Figure 4(J–J′)). forpr5-Gal4 drove expression throughout the salivary glands (Figure 4(I′′)), and forpr4-Gal4 was restricted to the salivary duct of the salivary gland (Figure 4(J′′)). Overall, the expression seen in the forpr-Gal4 lines is a subset of the expression found using the forcr00867-TG4.2 allele.

foraging transcripts have previously been detected throughout the gastric system in dissected bulk RNA-Seq experiments (Buchon et al., 2013; Leader et al., 2018), and sorted cell types from the midgut (Dutta et al., 2015). foraging transcripts were detected in all major cell types (ISC, EB, EE, EC, and VM) in all major segments (cardia and R1, R2, R3, R4, and R5) of the midgut (Dutta et al., 2015). To further explore and validate foraging’s expression in the gastric system, we turned to the adult midgut’s single-cell transcriptomic atlas (Figure 5A; Hung et al., 2020). Cell types were identified using known and established markers (as described in Hung et al., 2020). foraging expression was seen in all the midgut cell types (Figure 5(B)), with its expression most enriched in the ISC/EB cluster (Figure 5(C)).
**Foraging expression in the adult reproductive systems**

*for*~CR00867-TG4.2~ was expressed in most cell types throughout the female and male reproductive system. Expression was seen in the epithelia of the uterus, common oviduct, lateral oviducts (Figure 6(A,B)). Expression was observed in the ovarioles and epithelial sheath surrounding the ovarioles (Figure 6(B)). The most robust expression was seen in the spermatheca (Figure 6(A), arrowhead; Figure S2(C)) and fat cells associated with the spermatheca (Figure 6(A), arrow).
foraging is also known to have high levels of expression in the spermatheca, from microarray and RNA-Seq experiments on dissected tissue (Chintapalli, Wang, & Dow, 2007; Leader et al., 2018). forpr\textsubscript{C00867-TG4.2} is also expressed in the follicle cells of the developing eggs (Figure 6(B), arrowhead). The maternal loading of foraging in the developing eggs and the early embryo has been well characterized (Graveley et al., 2011; Jambor et al., 2015; Koencke et al., 2016; Tomancak et al., 2002).

forpr\textsubscript{C00867-TG4.2} is expressed throughout the male reproductive system, with strongest expression in the anterior ejaculatory duct (Figure 6(C), white arrow), secondary cells of the accessory gland (Figure 6(C,D), white arrowhead), and ejaculatory bulb (Figure 6(E)). The male accessory glands produce many proteins required for fertility, and their secretions have been shown to have substantial effects on female post-mating behaviors (Wolfnier, 1997). The ejaculatory bulb functions to pump the ejaculate and contributes to glandular secretions. Extensive expression was seen in the rest of the reproductive system, including the testes, seminal vesicle, and primary cells of the accessory gland (Figure 6(C,D)). The seminal vesicles are primarily a storage organ for mature sperm prior to copulation, and they also produce glandular secretions for the seminal fluid (Ram & Wolfnier, 2007). Expression was also seen in the smooth muscle throughout the female and male reproductive systems.

Once again, we return to the forpr\textsuperscript{Gal4} lines to parse these expression patterns and map its regulatory elements. We saw expression of forpr\textsubscript{1}-Gal4, but not forpr\textsubscript{1-2}, forpr\textsubscript{2}, and forpr\textsubscript{2-3}, in the female reproductive system. forpr\textsubscript{2-3-Gal4} expressed in the spermatheca (Figure 6(H)) and the follicle cells of the developing eggs (Figure 6(H')). forpr\textsubscript{2-3-Gal4} also expressed in a small segment of the common oviduct (Figure 6(H')). forpr\textsubscript{1-2}, forpr\textsubscript{2}, and forpr\textsubscript{4-Gal4} all drove expression in the seminal vesicle and secondary cells of the accessory glands, albeit to varying extents (Figures 6(H', I, G), respectively). forpr\textsubscript{2-Gal4} had the added inclusion of a small ring of cells at the base of the vas deferens where it joins with the ejaculatory duct (data not shown). forpr\textsubscript{4-Gal4} drove expression in the ejaculatory bulb of the male reproductive system (Figure 6(I')). forpr\textsubscript{3-Gal4} had no observed expression in the male reproductive system.

Mapping foraging’s cis-regulatory elements (CREs)

To further map and refine the CREs within the foraging gene, we generated a series of 13 nested forpr\textsuperscript{Gal4} driver lines covering regions within the four original forpr\textsubscript{Gal4}s (Figure 7(A), bars below locus). These regions were cloned into the same insulated vector used for the original forpr\textsuperscript{Gal4}s and were integrated into attP2 with pC31 integration (see “Methods”). By comparing the expression patterns seen between these different lines, we could narrow the putative CREs in the foraging locus (Figure 7(B), above the locus). For instance, in the adult fly, forpr\textsubscript{3-Gal4} and forpr\textsubscript{3A1-Gal4} did not differ in their expression patterns, so we did not map any element to the first forpr\textsubscript{3} region. In contrast, forpr\textsubscript{3A2-Gal4} had all the expression of forpr\textsubscript{3A1-Gal4}, except for the perineurial glia expression. This allowed us to map the perineurial CRE to this second segment of the forpr\textsubscript{3} region. All nested forpr\textsuperscript{Gal4}s exhibited either the same or a subset of the expression patterns seen in their respective larger constructs.

Many genes have highly conserved promoter structure and show consistent expression across development (Graveley et al., 2011; Hoskins et al., 2011). Is this trend also true for foraging? The forpr\textsubscript{1-4-Gal4} lines were previously characterized for their expression in the larval CNS and gastric system (Allen et al., 2018). Examining the expression of the newly generated nested lines allows us to compare the regulation between the larva and adult fly. As was seen in the adult, the expression in the larval of the nested Gal4 lines were either the same or a subset of the expression seen in the larger forpr\textsuperscript{Gal4} lines. A number of these refined CREs drove comparable expression in the adult (Figure 7(B), above the locus) and the larva (Figure 7(B), below the locus). For example, the first region from forpr\textsubscript{1-Gal4} drove expression in neurons. The first region from forpr\textsubscript{2-Gal4} drove expression in the larval and adult intestinal stem cells (“Adult midgut precursor cells” and “Midgut ISC”, respectively). The perineurial CRE and the CRE driving expression in the salivary gland, EE cells, ECs, and Malpighian tubules mapped in the forpr\textsubscript{3-Gal4s} also drove consistent expression in the larva and adult. The CRE driving expression in the epithelia of the hindgut and rectal ampulla from forpr\textsubscript{3-Gal4s} was also shared. These data provide evidence that foraging has conserved promoter structure exhibiting consistencies in expression between the larval and adult stage of development.

Discussion

Relatively little is known about the precise spatial- and cell-specific expression of foraging, a highly conserved gene with a complex gene structure (reviewed in Anreiter & Sokolowski, 2019). Here we set out to characterize foraging gene expression in the adult brain, gastric system, and reproductive systems of D. melanogaster. We employed a multimodal approach by combining foraging gene-trap T2A-Gal4, promoter Gal4 fusions, and the mining of existing single-cell transcriptomic data sets. The advantages and limitations of these techniques are described below.

Trojan/CRIMIC style insertion alleles are unparalleled in their expression fidelity due to their function as both a transcriptional and translational trap of the locus (Diao et al., 2015; Lee et al., 2018). Unlike insertion alleles made with older technologies, Trojan/CRIMIC insertion alleles significantly limit offsite effects on the readout of a gene’s expression. Consequently, it is more likely that the forpr\textsuperscript{C00867-TG4.2} allele, inserted into the foraging locus, captures the expression of all known foraging isoforms and therefore, should represent the full foraging expression pattern (Figure 1(A)).

Promoter fusion reporters have been widely used to characterize the spatial expression pattern of genes in D. melanogaster. However, the position where they are integrated
into the fly’s genome can significantly alter the level and patterns of expression that are seen (Kellum & Schedl, 1991; Markstein, Pitsouli, Villalta, Celniker, & Perrimon, 2008; Spradling & Rubin, 1983). Problems with position effects can be reduced using gypsy insulators (Barolo et al., 2000; Billeter & Goodwin, 2004; Gdula, Gerasimova, & Corces, 1996; Markstein et al., 1999; Merli, Bergstrom, Cygan, & Blackman, 1996; Ohtsuki, Levine, & Cai, 1998). Our lines exploited these strategies when constructing our forpr-Gal4 transgenic constructs. The lack of shared expression between the largest of each forpr-Gal4 constructs suggests a lack of position effects from our forpr-Gal4 insertions or expression driven by the empty Gal4 vector itself. An enhancer’s ability to induce expression can also be dependent on the core promoter element it is paired with (Lehman et al., 1999; Merli, Bergstrom, Cygan, & Blackman, 1996; Ohtsuki, Levine, & Cai, 1998). Our lines exploited foraging’s native core promoters and TSS from the locus. Together, these strategies should increase the likelihood that the expression we found in our forpr-Gal4 experiments faithfully recapitulates foraging expression.

Massively parallel single-cell transcriptomic experiments provide unprecedented cellular resolution of gene expression but are not without their caveats. For instance, droplet-based methods suffer from a number of factors, such as drop-out events where only a subset of the transcriptome from a cell is captured (Kim, Zhou, & Chen, 2020; Qiu, 2020), doublet and multiple formation where multiple cells are co-encapsulated (Bernstein et al., 2020; McGinnis, Murrow, & Gartner, 2019; Wolock, Lopez, & Klein, 2019), and contamination of ambient RNA generated during the dissection and dissociation (Yang et al., 2020; Young & Behjati, 2018). Sufficient sampling of each given cell type along with the above cited bioinformatic approaches can reduce these issues.

Overall, we found foraging expressed in multiple glia subtypes in the brain with the strongest expression seen in the perineurial glia. This was supported by the T2A-Gal4 alleles forCR00867-TG4.2 (Figure 1(B-D)) and forM01791-TG4.1 (Figure S1(G)), the BAC{forRES-Gal4} recombineered allele (Figure S1(H)), the forpr3-Gal4 cloned promoter Gal4 transgene (Figure 1(E′′)); Figure S1(D–E)), single-cell RNA-Seq data sets (Figure 2(A–D); Davie et al., 2018; Croset et al., 2018), and bulk RNA-Seq data on sorted cells (DeSalvo et al., 2014). No clear neuronal expression was seen in the brain in either gene-trap allele, or the single-cell transcriptomic data, however, a few neurons were labelled with forpr1-Gal4 (Figure 1(E)). We also show that the previously characterized anti-FOR immunoreactivity in adult brain neurons (Belay et al., 2007; Mery et al., 2007) is non-specific and does not reflect bone fide FOR expression (Figure 3). Finally, we also found that the foraging gene expressed extensively throughout the gastric and reproductive systems (Figures 4–6) and the expression in these tissues and cells was corroborated with data from microarray, bulk RNA-Seq, and single-cell RNA-Seq based transcriptomic studies (Brown et al., 2014; Buchon et al., 2013; Chintapalli et al., 2007; Dutta et al., 2015; Hung et al., 2020; Leader et al., 2018). Each of these results are discussed in more detail below.

Foraging in the adult brain

Glia are intertwined with neuronal physiology and behavior. Glia influence the development and function of the synapse, neurotransmitter release, ion homeostasis and immune function throughout an organism’s life (reviewed in Artiushin & Sehgal, 2020). The number of studies of the role of glia in behavior is growing rapidly but is still much more limited than their neuronal counterparts. Glia serve important roles in behavior (reviewed in Freeman, 2015; Zwarts, Van Eijs, & Callaerts, 2015). For example, in D. melanogaster, glia function in embryonic motility and adult locomotion (Lehmann & Cierotzki, 2010; Pereanu et al., 2007) and in male courtship behavior (Grosjean, Grillet, Augustin, Ferveur, & Featherstone, 2008). A subset of glia called astrocytes function in sleep and circadian rhythms of locomotion and their cellular correlates (Artiushin & Sehgal, 2020).
**Figure 6.** (A–E) forCR00867-TG4.2 CRIMIC allele driving UAS-Watermelon in female and male reproductive systems with membrane bound GFP in green (A–E), nuclear mCherry in magenta (A′–E′), membrane and nuclear merged with F-actin in blue (A″–E″). (A–A′) Maximal projections of expression in the female reproductive system; uterus, spermatheca (white arrowhead), and common oviduct (white double arrowhead) of the female reproductive system. Expression was also seen in the spermatheca associated fat (white arrow) and smooth muscle. (B–B′) Maximal projections of expression in the female reproductive system; ovarioles and lateral oviducts. Expression was seen in the common and lateral oviducts, epithelial sheath surrounding the ovariole, and follicle cells (arrowhead). (C–C′) Maximal projections of expression in the male reproductive system. Expression was seen throughout; ejaculatory duct (white arrow), seminal vesicles, testis, and accessory glands (white arrowhead). Other tissues are also present (Malpighian tubules – yellow arrowhead). (D–D′) Magnification of the accessory gland. Primary cells indicated with white arrow, and secondary cells indicated with white arrowhead. Other tissues are also present (Malpighian tubules – yellow arrowhead, trachea – yellow arrow). (E–E′) Magnification of the ejaculatory bulb. Lower ejaculatory duct (white arrow) and fat tissue (white arrowhead) are indicated. (F) Schematic of the foraging locus depicting regions of cloned forpr-Gal4s. G. forpr1-Gal4 driven GFP expression in the male reproductive system. (H) forpr2-Gal4 driven expression in the spermatheca. (H′) forpr2-Gal4 driven expression in the ovaries. (H″) forpr2-Gal4 driven expression in the oviduct. (H‴) forpr3-Gal4 driven expression in the male reproductive system. (I) forpr4-Gal4 driven expression in the male reproductive system. (I′) forpr4-Gal4 driven expression in the ejaculatory bulb. Scale bars = 50 μm. [Please refer to the online version for colors.]
At the larval neuromuscular junction (NMJ) glial-specific expression of foraging decreases neurotransmitter release by negatively regulating nerve terminal growth (Dason et al., 2019; Dason & Sokolowski, 2021). The for0 null mutant has increased nerve terminal growth which can be rescued by glial-specific, and not neuronal-specific (pre-synaptic) or muscle-specific (post-synaptic) expression of foraging (Dason et al., 2019). Furthermore, glial-specific knockdown of foraging phenocopies the nerve terminal over-growth and increased neurotransmitter release phenotypes seen in the for0 null mutant (Dason & Sokolowski, 2021). Knockdown of foraging in presynaptic neurons of the NMJ impairs synaptic vesicle endocytosis, whereas knockdown of foraging in glia does not. Overall, D. melanogaster foraging can alter neurotransmitter release at the NMJ by regulating both synaptic structure and function. Similar functions are likely also at play in the adult nervous system. In mammalian systems, PKG has been implicated in Ca2+ mobilization and influx in glia (Willmott, Wong, & Strong, 2000), and in translocation to the nucleus to regulate gene expression in glia (Gudi, Hong, Vaandrager, Lohmann, & Pilz, 1999). In D. melanogaster, the perineurial and sub-perineurial glia function as the blood–brain barrier and are important for regulating the transport and exchange of nutrients, metabolites, and hormones between the brain and the hemolymph (Stork et al., 2008). foraging may affect behaviors, such as learning and memory, in the fly by regulating the Ca2+ environment surrounding neuronal cell bodies via its cortex glial expression. Cortex glia, in the fly, have been shown to have Ca2+ oscillations which when disrupted can alter behavior (Melom & Littleton, 2013). Alternatively, foraging may alter the metabolic environment of the brain by mediating nutrient and waste transport via its perineurial glia expression. Further experiments are required to test this hypothesis.

Although we found strong evidence that foraging is expressed in glia of the adult brain, we found no detectable neuronal expression in the adult brain using the for0CR0867-TG4.2 allele (Figure 1(B–D)) and the single-cell transcriptomics data (Figure 2(A–D)). In contrast, foraging is expressed in some peripheral neurons and central neurons at earlier stages of development. foraging was detected in adult photoreceptor neurons (Davie et al., 2018; Davis et al., 2020; Figure 2(D)), the 3rd instar larval neuromuscular junction (Dason et al., 2019), and in the VNC along the midline of late-stage embryos (Peng et al., 2016). Many studies have demonstrated a role for PKG in mammalian nervous systems (reviewed in Feil, Hofmann, & Kleppisch, 2005; Hofmann, Feil, Kleppisch, & Schlossmann, 2006). For example, cerebellum specific conditional knockout of PKG in the mouse impairs long-term depression affecting motor learning (Feil et al., 2003), and a nociceptor specific knockout in the dorsal root ganglia abolishes long-term potentiation affecting pain sensitivity (Luo et al., 2012). Prkgl1, foraging’s mouse homologue, is readily detected in many subtypes of neurons and glia in the brain of both the developing mouse embryo and adolescent mouse, using single-cell RNA-Seq (La Manno et al., 2020; Zeisel et al., 2018). The readily detectable neuronal expression in the mouse may suggest some differences in regulation between the fly and the mouse. The expression was strongest in the neuroblasts of the developing embryo (La Manno et al., 2020) and the enteric and sensory neurons of the peripheral nervous system in the adolescent mouse (Zeisel et al., 2018). Taken together, it is possible that foraging, in D. melanogaster, may play a stronger role in the developing nervous system and the developed peripheral nervous system then it does in the adult central brain.

We also found that our previously published anti-FOR antibody (Belay et al., 2007) is not an effective tool for immunohistochemical analyses of FOR in the adult brain. Problems with the unreliability of antibodies have been well discussed in the literature (Egelhofer et al., 2011; Fritschy, 2008; Michel, Wieland, & Tsujimoto, 2009; Rhodes & Trimmer, 2006; Saper & Sawaouchen, 2003). We re-evaluated the efficacy of immunohistochemistry of a previously published (Belay et al., 2007) anti-FOR antibody using a complete null allele of foraging (for0; Allen et al., 2017). We found that the anti-FOR antibody labels bone fide FOR expression in cells with morphology consistent with the outer optic chiasm glia. However, the anti-FOR antibody had extensive nonspecific immunoreactivity in neurons, including mushroom and ellipsoid bodies. We concluded that the anti-FOR antibody known to be a valuable tool on westerns (Allen et al., 2017; Dason et al., 2019) is not effective for immunohistochemical analyses of FOR in the adult brain. Data from single-cell RNA-Seq supported the findings of glial expression, but no neuronal expression, seen in the for0CR0867-TG4.2 allele. We cannot, however, distinguish between a very low expression of foraging or no expression of foraging outside of glia in the adult brain. This is discussed further, below.

Previous studies suggest that foraging functions in neurons to affect adult behaviors, including various forms of adult learning and memory (Kuntz et al., 2012; Mery et al., 2007; Wang et al., 2008), olfactory startle habituation (Eddison et al., 2012), movement in an open field (Burns et al., 2012) and sleep (Donlea et al., 2012). Many of these studies relied on targeting a UAS-fordNA pan-neuronally, or to specific subsets of neurons from multiple regions of the brain, including antennal lobe, mushroom bodies, and central complex. These studies, which use a UAS-fordNA, clearly show that FOR protein can alter multiple behaviors when expressed in these neuronal populations. What is not clear is whether foraging is naturally expressed in the cells in these above studies. Two of the above studies, however, took advantage of UAS-forRNAi (Donlea et al., 2012; Kuntz et al., 2012). Donlea et al. (2012) showed that driving UAS-forRNAi in the α/β Kenyon cells, with the 30Y enhancer trap, altered short term memory when compared with controls. Kuntz et al. (2012) altered visual orientation memory by driving UAS-forRNAi in Kenyon cells and ring neurons, with the lilli189 enhancer trap. This strengthens the case for bone fide foraging expression in these respective cells, although, RNAi can have off-target effects (Ma et al., 2006; Moffat et al., 2007).
Many of these studies relied on the previously published anti-FOR pattern in the adult brain (Belay et al., 2007; Mery et al., 2007) which we show in this study to be non-specific (Figure 3). It is important to note that non-specific binding of the anti-FOR antibody does not necessarily mean that foraging is not expressed in neurons such as the Kenyon cells of the mushroom bodies or the ring neurons of the central complex. One of the above studies showed that driving a UAS-for^{DNA} in the R3 ellipsoid body ring neurons affects working memory in a visual orientation paradigm (Kuntz et al., 2012). In this case, a number of Gal4 enhancer trap lines were used, targeting subsets of the ellipsoid body ring neurons, and mushroom body Kenyon cells. One such enhancer trap was lilli^{189Y} (previously referred to as for^{189Y}) that was initially thought to be inserted in foraging (Osborne et al., 1997) but was subsequently shown to be inserted in lilliputian (Sokolowski 2007 FlyBase update FBrf0198702; Wang et al., 2008). Since lilli^{189Y} is not inserted in foraging, we cannot assume that it would necessarily recapitulate foraging expression.

Another of the above studies used enhancer traps in foraging to infer the expression of foraging in the adult brain (Eddison et al., 2012). Eddison et al. (2012) used two enhancer trap lines, for^{11,247} and for^{2014}, which were inserted in the foraging gene at its most 5’ end near the for^{TSS}. At the time, parts of the expression of for^{11,247} were validated with the anti-FOR antibody. The enhancer traps in foraging and the promoter fusions from foraging, used in the present study, are equally likely to reflect actual foraging expression and may represent distinct subsets of expression in the adult CNS. However, it is also possible that these 5’ insertions may trap enhancers of the neighbouring gene, Drgx, which has been shown to contain regulatory elements that drive expression in the antennal lobe, mushroom bodies, as well as other parts of the brain.
as the ellipsoid body (Jenett et al., 2012). The possibility of crosstalk with DrgX enhancers may also exist for our forpr1-Gal4 transgene, as this cloned region lies between foraging and DrgX.

There is an apparent discrepancy between the lack of detectable foraging expression in adult brain neurons found in the present study and published papers showing that transgenically expressing foraging, with a UAS-forDNA, in adult brain neurons alters behavioral phenotypes. Some possible reasons for this are as follows: 1) foraging is expressed in these tissues, but at levels too low to be detected with the methods used in the present study. 2) foraging is only conditionally expressed in these neuronal populations depending on certain environmental conditions. 3) foraging is expressed in these neurons, but only during development. 4) These previous studies using UAS-forDNA represent ectopic expression, driving FOR protein where it is not naturally expressed. We discuss these possibilities below.

To attempt to address the sensitivity issue of single-cell RNA-Seq, we looked for foraging expression in bulk RNA-Seq studies, which are more sensitive, on sorted neuronal cell populations. Three separate studies, in which multiple sub-populations of Kenyon cells were assayed, failed to detect foraging in Kenyon cells at levels higher than that of repo, or other glial-specific or non-neuronal markers (Crocker, Guan, Murphy, & Murthy, 2016; Davis et al., 2020; Shih, Davis, Henry, & Dubnau, 2019). Davis et al. (2020) did however find foraging expression in photoreceptor neurons in the eye of the fly, which was also seen in single-cell transcriptomic data (Davie et al., 2018; Figure 2(D)). Mammalian PKG has previously been shown to be involved in neurite plasticity in rod and cone cells (Zhang, Beuve, & Townes-Anderson, 2005).

We currently do not know whether foraging is conditionally expressed in adult brain neurons in different environmental contexts, such as different nutritional states or different learning paradigms. foraging expression has previously been shown to be up regulated by food deprivation in whole adult heads (Kent et al., 2009). Although, the head is more than just neurons, and so fat cells, trachea, and glia in the head capsule are also candidate cell types for this observed effect. Further experiments specifically assaying adult brain neurons in differing environmental conditions are required to explicitly test any potential condition expression of foraging.

We currently do not have any direct measures of foraging expression in neurons during different developmental stages. However, two of the above-mentioned studies (Kuntz et al., 2012; Wang et al., 2008) did employ the temperature sensitive GAL4 repressor GAL80s (McGuire et al., 2003) to restrict expression of the UAS-forDNA to post-eclosion adults. In both cases, the authors found that adult restricted expression was sufficient to modulate their respective phenotypes, working memory in a visual orientation paradigm (Kuntz et al., 2012) and visual pattern memory (Wang et al., 2008). In these cases, the developmental possibility is, at least in part, ruled out. Again, further experiments are required to assess the potential role for foraging in neurons of developing larvae and pupae.

Finally, if in fact, foraging is not naturally expressed in the adult brain neurons where UAS-forDNA was expressed in previous studies, then the UAS-forDNA manipulations constitute ectopic expression. In this case, FOR protein may still be interacting with bone fide PKG targets, which may typically co-express in other cell types. The observed effects may then represent neomorphic-like phenotypes. Alternatively, at the biochemical level there can be crosstalk between the cAMP and cGMP systems, whether by PKG activation by cAMP (Lin, Liu, Chow, & Lue, 2002; Lin, Liu, Tu, Chow, & Lue, 2001; Lincoln & Cornwell, 1991; Ruiz-Velasco, Zhong, Hum, & Keef, 1998; White, Kryman, El-Mowafy, Han, & Carrier, 2000), or PKG phosphorylating a PKA target due to similar phosphorylation target recognition sites (Douglass et al., 2012), or even by PKG phosphorylating shared target proteins (Döppler & Storz, 2013; Huang, Tsai, Chen, Wu, & Chen, 2007). More research is needed into the precise cellular localization and expression of foraging to decipher how its expression influences each of the gene’s pleiotropic phenotypes.

**Foraging in the gastric and reproductive systems**

There are many potential roles for foraging in the gastric and reproductive systems. The visceral muscle of the gut is required to push nutrients along via peristalsis. Mice with foraging’s orthologue knocked out in smooth muscle show increased gut passage times relative to controls (Weber et al., 2007). foraging has been implicated fecal excretion rate in adults (Urquhart-Cronish & Sokolowski, 2014) and the visceral muscle is a candidate tissue to mediating this function. foraging has also been implicated in the rate of glucose absorption in larvae (Kaun et al., 2007). Simple sugars are absorbed by the enterocytes of the midgut (reviewed in Miguel-Aliaga et al., 2018) and so are strong candidate cells for foraging’s glucose absorption phenotype. foraging has also previously been shown to affect adult Malpighian tubule secretion rate (MacPherson, Broderick, et al., 2004; MacPherson, Lohmann, & Davies, 2004) and the expression in the principal cells is consistent with this phenotype.

As for the reproductive systems, differences in the number of eggs laid by flies reared in differing nutritional conditions during development (Burns et al., 2012), as well as oviposition site selection (McConnell & Fitzpatrick, 2017) has previously been associated with foraging. The expression seen in the spermatheca and visceral muscle of the reproductive system represent candidate cells for mediating this difference. The spermatheca are essential for long term storage of sperm (Gilbert, 1981), and perturbations in these cells can cause a decrease in egg-laying over time (Schnakenberg, Matias, & Siegal, 2011). foraging has also been shown to play a role in developmental death and cell clearance in the epithelial follicle cells of the D. melanogaster ovary under starvation conditions (Lebo et al., 2021). The maternal loading of foraging in the developing eggs and the early embryo is also well known (Graveley et al., 2011; Jambor et al., 2015; Koenecke et al., 2016; Tomancak et al., 2021).
Foraging’s complexity and the mapping of CREs

Three genes, foraging, Pkg21D, and CG4839 code for a cGMP-dependent protein kinase (PKG) in D. melanogaster (Thurmond et al., 2019). foraging is by far the most complex locus. Pkg21D is less than 5 kb with 1 TSS, 1 transcript and 1 protein isoform, while CG4839 is less than 12 kb with 1 TSS, 2 transcripts, and 1 protein isoform (Thurmond et al., 2019). In contrast, foraging is a 35 kb locus with 4 TSSs, more than 20 transcripts, and potentially more than 9 protein isoforms (Allen et al., 2019). Like Pkg21D and CG4839, the spa promoters of foraging are primarily expressed in hindgut and Malpighian tubules, and CG4839 is primarily expressed in the midgut, salivary gland, and reproductive tissues (Leader et al., 2018). Neither appear to be detected in the adult brain (Davie et al., 2018; Leader et al., 2018). foraging orthologues play multiple roles in behavioral and physiological phenotypes in multiple taxa (reviewed in Anreiter & Sokolowski, 2019; reviewed in Reaume & Sokolowski, 2009).

A clue to foraging’s complexity may reside in the structure of foraging’s promoters and the mapped CREs. Core promoters are described by two different shapes; “narrow” (a.k.a. “peaked”, “focused”, “sharp”) promoters with transcription always initiating at a within a narrow range of a few bases, and “broad” (a.k.a. “dispersed”) promoters with transcription initiating over a larger range of bases (Haberle & Stark, 2018). Narrow promoters tend to have transcription initiating over a larger range of bases than broad promoters (Thurmond et al., 2019; Hoskins et al., 2011; Schor, et al., 2017). Three of foraging’s promoters, forpr1, forpr2, and forpr4, are narrow promoters and one, forpr3, is broad (Allen et al., 2017; Hoskins et al., 2011). Based on the forpr-Gal4 expression patterns reported here, the CRE driving the secondary cells of the male accessory glands mapped very close to the TSS of forpr1, forpr2, and forpr4. This closely linked accessory gland CRE and the fact that forpr1, forpr2, and forpr4 are all narrow promoters may suggest a common evolutionary origin of these TSSs.

Promoter analyses has been successfully used previously to identify CREs and deduce isoform-specific expression and function in many genes (Arredondo et al., 2001; Billeter & Goodwin, 2004; Brenner, Thomas, Becker, & Atkinson, 1996; Lehman et al., 1999; Okada et al., 2001; Park et al., 2000). Consequently, we used a promoter analysis strategy to map multiple CREs along the foraging locus responsible for a subset of the expression seen in the T2A-Gal4 gene trap line. The expression seen in the forpr-Gal4s may be only a subset of that seen in the forpr-CR00867-TG4.2 allele because the regions cloned into the forpr-Gal4s encompass only 15 kb of the 35 kb locus, suggesting that some CREs were missed and/or because each forpr-Gal4 was inserted outside the context of the foraging locus at a common site on the third chromosomes (see “Methods”).

Conserved expression and regulation across development

We can draw parallels between the regulation, expression, and function of genes across development from larva to adult despite the differences in life-history traits of these developmental stages. Many genes show consistent expression across development (Graveley et al., 2011). Additionally, promoter structure can be highly conserved between developmental stages (Hoskins et al., 2011). Like foraging, the CREs of slowpoke, pigment-dispersing factor, and paramyosin drive consistent expression across development (Arredondo et al., 2001; Brenner & Atkinson, 1996; Park et al., 2000). The expression levels and spatial distribution of foraging are similar between tissues of the third instar larva and adult fly, as deduced from microarray and RNA-Seq on dissected tissues (Chintapalli et al., 2007; Leader et al., 2018). Here we show that many but not all the CREs within the foraging locus also drive consistent expression between these two stages. The parallels in the expression of foraging at these two developmental stages mirror many of the parallels seen in the larval and adult phenotypic traits.

Conclusion

Our characterized expression patterns of foraging provide novel candidate tissues and cell types to explore foraging’s pleiotropic influences on physiology and behavior. We provide exciting new data on foraging expression in multiple subsets of glia in the adult brain, paving the way towards functional studies that address the importance of glial subtypes in each of foraging’s suite of pleiotropic behavioral phenotypes.

Acknowledgments

The authors thank the past and present members of the Sokolowski Lab for years of helpful discussions. The authors thank Andreas Prokop for the adult Drosophila illustration and Josh Dubnau for the UAS-Watermelon line. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. The authors thank Stephen F. Goodwin for comments on the manuscript and support for the work conducted while at the Centre for Neural Circuits and Behaviour. The authors thank the two anonymous reviewers for their comments and review of the manuscript.

Disclosure statement

The authors report no conflict of interest.

Funding

This research was supported by grants from the Natural Sciences and Engineering Council of Canada (NSERC), the Canadian Institutes for Health Research (CIHR) and NIDDK grant 5R01DK70141-2 to M.B.S.
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