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Metamorphosis of Tangential Visual System Neurons in *Drosophila*

Paul H. Taghert,[1] Marie E. Roberts, Susan C. P. Renn, and Pamela S. Jacobs

Department of Anatomy and Neurobiology, Box #8108, Washington University School of Medicine, Saint Louis, Missouri 63110

To learn about construction of the adult nervous system, we studied the differentiation of imaginal neurons in the *Drosophila* visual system. OL2-A and OL3 are tangential neurons that display *dFMRFa* neuropeptide gene expression in adults but not in larvae. The two large OL2-A neurons are generated near the end of the embryonic period and already show morphological differentiation at the start of metamorphosis. The numerous small OL3 neurons are generated postembryonically and first detected later in metamorphosis. The onset of *dFMRFa* transcription coincides with that of neuropeptide accumulation in OL2-A neurons, but it precedes peptide accumulation in the OL3 neurons by days. Altering each of the five conserved sequences within the minimal 256-bp OL *dFMRFa* enhancer affected *in vivo* OL transcriptional activity in two cases: alteration of a TAAT element greatly diminished and alteration of a 9-bp tandem repeat completely abolished OL2-A/OL3 reporter activity. A 46-bp concatamer containing the TAAT element, tested separately, was not active in OL neurons. We propose a model of neuronal differentiation at metamorphosis that features developmental differences between classes of imaginal neurons.

***Key Words: Drosophila*; metamorphosis; tangential neuron; neuropeptide; FMRFamide.**

INTRODUCTION

Metamorphosis represents the delayed development of adult features due to the interposition of juvenile characteristics (Riddiford, 1993; Truman and Riddiford, 1999). In holometabolous insects, metamorphic changes in the nervous system include the modification of differentiated larval neurons, the addition of newly born (imaginal) neurons, and the formation of new synaptic connections and circuits (Truman *et al.*, 1993). The mechanisms by which larval neurons acquire new adult properties and the mechanisms which shape the properties of imaginal neurons are largely unexplored.

Postembryonic developmental events are analogous to those occurring in the embryo. However, they present novel considerations with respect to timing—what mechanisms delay cell differentiation until appropriate developmental stages? These developmental processes are critical as newly differentiated cells must be integrated within an already functioning nervous system (Witten and Truman, 1991; Loi and Tublitz, 1993; Kent and Levine, 1993; Levine *et al.*, 1995; Schubiger *et al.*, 1998). Thus metamorphic neural differentiation is orchestrated by a cascade of steroid hormones and hormone-response genes (Riddiford, 1993; Thummel, 1997), which ensures that premature differentiation does not interfere with the completion of larval development.

Metamorphic development in the visual system of the insect brain has received substantial investigation. The majority of that work has focused on the differentiation of retinal cell types (e.g., Karim *et al.*, 1996) and on the connectivity of the retina to the central brain (e.g., Martin, 1995; Schmucker and Gaul, 1997; Huang and Kunes, 1998). Retinal innervation is required for the proper development of the optic lobe brain centers, which include the lamina, medulla, and lobula (Meinertzhagen, 1973; Meyerowitz and Kankel, 1978; Fischbach, 1983; Fischbach and Technau, 1984; Nässel *et al.*, 1987; Steller *et al.*, 1987; Selleck *et al.*, 1992). This study analyzes the metamorphic development of central neurons that make broad tangential projections within neuropil of the optic lobes (Strausfeld, 1976; Fischbach and Dittrich, 1989). Such neurons are often identified by immunoreactivity to serotonin or to peptide transmit-

[1] To whom correspondence should be addressed. E-mail: taghertp@pcg.wustl.edu.

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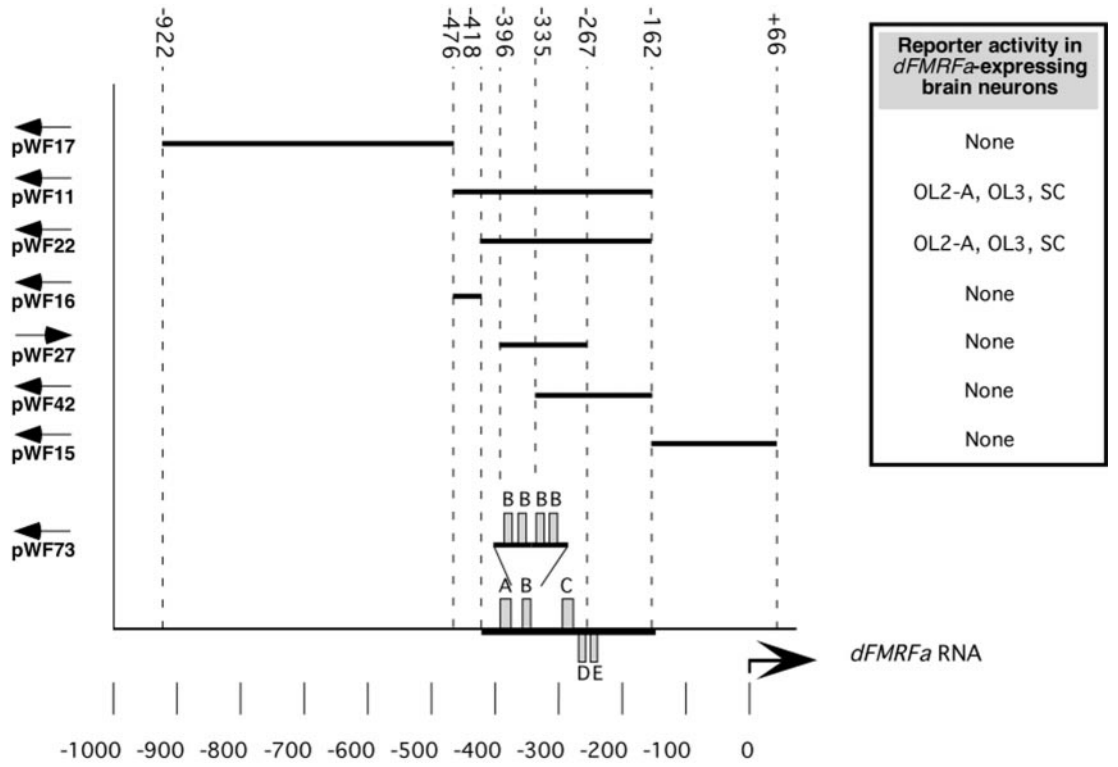


FIG. 1. Schematic of several fragments within the first ~1 kb of the 5′ upstream region of the *dFMRFa* gene that were tested for transcriptional activity in the adult brain. Numbers refer to basepairs before the *dFMRFa* transcription start site (open arrow). Solid arrows atop construct names (at the left) indicate the orientation of the test fragment with respect to the heterologous promoter. Descriptions to the right indicate incidence of reporter activity in *dFMRFa*-expressing neurons of the adult brain; some lines were also active in the segmental nerve cord (e.g., pWF17, Benveniste and Taghert, 1999). The smallest fragment active in OL2/OL3 neurons is the 256-bp pWF22, also represented by the raised dark bar along the ordinate. The five gray boxes (A–E) represent evolutionarily conserved regions of the promoter (Taghert and Schneider, 1990); also see Fig. 5.

ters like FMRFa and pigment-dispersing hormone (β-PDH; Nässel *et al.*, 1988, 1991; Homberg *et al.*, 1991; Helfrich-Förster, 1997; Meinertzhagen and Pyza, 1999). Several aspects of tangential neuron development have been described. Schedules of amine (Ohlsson and Nässel, 1987; Ohlsson, 1998) and neuropeptide (Ohlsson *et al.*, 1989; Helfrich-Förster, 1997) transmitter differentiation have been defined in *Drosophila* and in larger flies. Likewise, several studies have examined afferent influences on tangential neuron survival (Fischbach and Technau, 1984; Nässel *et al.*, 1987; Helfrich-Förster and Homberg, 1993; discussed by Meinertzhagen and Hanson, 1993).

In the adult *Drosophila* CNS, there are several cell types expressing *dFMRFa* that do not do so in larval stages (O'Brien *et al.*, 1991; Schneider *et al.*, 1993b); the larval status of these imaginal neurons is not yet defined. In the brain, these include 2 neurons of the lateral protocerebrum (SP4), 5–10 neurons that lie at the boundary of the tritocerebrum and subesophageal neuromeres (SC), and two groups of visual system neurons lying between the central brain and the medulla (OL1 and OL2). We have focused on mechanisms underlying the *dFMRFa* transmitter phenotype by OL neurons. In particular, we have considered *dFMRFa* transcriptional regulation, because this property defines the cellular expression pattern and because its mechanisms appear highly cell-type-specific (cf. Schneider *et al.*, 1993a; Benveniste *et al.*, 1998; Benveniste and Taghert, 1999). These data address the mechanisms by which imaginal neurons differentiate in the brain of adult *Drosophila*.

MATERIALS AND METHODS

Fly stocks. Animals were raised at 25°C on a standard cornmeal–agar diet. The stock used for analysis of wild-type animals was $y\ w^{67c23}$.

DNA constructions. All constructs were tested in the P[*hs43-lacZ*]CaSpeR vector (Schneider *et al.*, 1993a). All fragments except pWF3 are described in Fig. 1. pWF3 contains ~8 kb of *dFMRFa* attached to *lacZ* as a translational fusion (Schneider *et al.*, 1993a). All constructs in Fig. 1, except pWF27 and 42, originated as restriction fragments from pBluescript subclones of genomic DNA.

pWF27 and 42 fragments were generated by polymerase chain reaction (PCR) using oligonucleotides that introduced restriction sites compatible with P[*hs43-lacZ*]. Previous experiments determined that, within the ~8-kb pWF3 fragment, a 330-bp fragment represented by pWF11 was both necessary and sufficient for transcriptional activity in neurons called OL2 and that it was not active in other *dFMRFa* neurons (Schneider *et al.,* 1993a). Mutant variations of pWF22 were generated by PCR (Ho *et al.,* 1989) using designed oligonucleotides; mutated sequences are described in Fig. 5. All constructs were confirmed by sequence analysis in pBluescript and then transferred as *Bam*HI–*Xho*I fragments to P[*hs43-lacZ*]. pWF22 and all its mutant derivatives were tested in the same position within P[*hs43-lacZ*] and all but one (pWF27) had a similar (opposite) orientation relative to the heterologous promoter.

A concatamer of the 46-bp fragment—[5′-GTGCTTCTT-GGCTAATAAGTCAGCATGAATGCGGTCTGTGTT, 5′ end is at bp (−)361 (Schneider and Taghert, 1990)]—was made by ligating double-stranded, annealed oligonucleotide with *Bgl*II and *Bam*HI ends to *Bgl*II-cut pBluescript. A subclone containing four copies of the oligonucleotide, all in similar orientation, was identified by restriction and sequencing, then transferred to the *Bam*HI and *Xho*I sites of P[*hs43-lacZ*]. The final orientation of the concatamer matched that of the majority of other constructs.

Germ-line transformations. The methods were described by Schneider *et al.* (1993a) and by Benveniste and Taghert (1999). We injected embryos of the stock *yw;;Ki, P[Δ 2-3] 99B.* Progeny were backcrossed to $y\ w^{67c23}$ or to balancer stocks as necessary. Homozygous stocks were analyzed by Southern blot to determine uniqueness and insertion copy number. The incidence of ectopic expression in lines bearing pWF22-6 and its mutated variants was high. Typically, at least half the lines tested for any given DNA displayed reproducible patterns of ectopic expression. In a number of lines, all cells of the CNS expressed reporter at a low to moderate level (data not shown). Similarities in patterns of ectopic expression were noted frequently and are evident in images from representative lines (Fig. 7).

Detection of dFMRFa expression. *dFMRFa*-specific gene expression was scored with two independent histochemical stains: (i) CT antibodies (1:2000) were generated to a synthetic peptide matching the final 19 amino acids of the predicted pro-dFMRF precursor; that peptide is not predicted to encode any amidated peptides likely to interact with anti-FMRFa antibodies (Chin *et al.,* 1990; Benveniste *et al.,* 1999). (ii) β-Gal antibodies (1:1000; Promega, Madison WI) were used to detect expression of *dFMRFa–lacZ* reporter fusion transgenes (Schneider *et al.,* 1993a). There is good general correspondence between the patterns of neurons stained using these two methods (Benveniste and Taghert, 1999). Transformant reporter activity was analyzed by immunostaining whole-mount brains dissected from male pharate or newly emerged adult brains. Developmental analyses were performed on animals of either sex; each developmental stage was scored in at least five specimens. For pupal staging, animals were selected at pupariation, transferred to agar–apple juice plates, and dissected at times indicated. Under these conditions, head eversion occurred at ~12 h and eclosion at ~110 h.

Tissues were fixed for 1 h at room temperature (RT) in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, pH 7.5) and washed in PBS containing 0.3% Triton X-100 for 1 h. They were preincubated for 1 h at RT in 5% normal goat serum and 5% bovine serum albumin (PNBTx), then incubated in primary antibodies overnight at 4°C diluted in PNBTx. The fixative for developmental analyses and for double peptide immunostains (Fig. 8) also contained 7% (v/v) saturated picric acid, which improved the peptide immunohistochemistry and was compatible with β-gal immunohistochemistry. A rabbit antiserum against β-PDH (Dirckson *et al.,* 1987) was used at 1:2000. Double immunofluorescent staining of adult brains with two rabbit antisera (CT and β-PDH, Fig. 8) was performed sequentially. Secondary antibodies (anti-rabbit IgG and anti-mouse IgG; The Jackson Laboratory, Bar Harbor, ME; conjugated to Cy3 or FITC) were used at 1:500, and incubations proceeded for 2 h at RT. Tissues were cleared in glycerol and photographed with an Olympus laser scanning confocal microscope and Fluoview software. Use of interference filters and manipulation of the relative powers of the lasers were employed to minimize bleedthrough in double antibody labeling experiments. In addition, separate scans were acquired for FITC (at 466 nm) and rhodamine or Cy3 labels (at 586 nm), then later assembled with Adobe PhotoShop. Image acquisition and modification were performed in parallel for cohorts of photographs that compared genotypes.

Scoring cell phenotypes. We scored at least six male pharate adult or early adult brains; genotypes were unknown to the person scoring. A three-point scoring system [high (2), moderate (1), undetectable (0)] was used to rank expression and to average it across specimens from one line and between lines of the same genotype. We did not quantify the number of expressing cells per identified group within a tissue.

5-Bromo-2′-deoxyuridine (BUdR) incorporation and staining. Animals were fed BUdR (Sigma; St Louis, MO) at concentrations of 0.1 to 0.2 mg/ml food (Truman and Bate, 1988). Following fixation in 4% paraformaldehyde, tissues were stained with a rabbit anti-β-galactosidase antibody (1:500), followed by a FITC anti-rabbit secondary antibody. DNA was then denatured by incubation in 0.2 N HCl in PBS for 30 min at RT, followed by another round of primary and secondary antibodies [1:20 anti-BUdR (Becton–Dickinson) and Cy3 anti-mouse IgG].

RESULTS

A Subset of FMRFa-Positive Visual System Neurons Express dFMRFa

The neurons described in this report are generically called OL because of their proximity to the optic lobes. They lie on the anterior adult brain surface, at the lateral border of the central brain (Fig. 2). Along the dorsal to ventral aspect of this region, several neurons display immunostaining with antibodies to the molluscan tetrapeptide FMRFa (White *et al.,* 1986; Nässel, 1993). Here we show that only a subset of these neurons express the *dFMRFa* gene (O'Brien *et al.,* 1991; Schneider *et al.,* 1993a,b). The remaining FMRFa-immunoreactive cells express structurally related peptides derived from unrelated genes. Among the many FMRFa-positive OL neurons, *dFMRFa* OL neurons include three cell types: 2 large cells called OL2-A, a moderately sized cell called OL1-A, and two packets of small neurons (≤50 neurons per packet) collectively called OL3 (Fig. 2). This assignment is based on double-staining experiments with *dFMRFa-lacZ* reporter lines (e.g., Figs. 2A–2C) and with use of antibodies specific for the dFMRF precursor (e.g., Figs. 2D–2F). The complete set of FMRFa-positive OL neurons is schematized in Fig. 2G. All OL neurons are

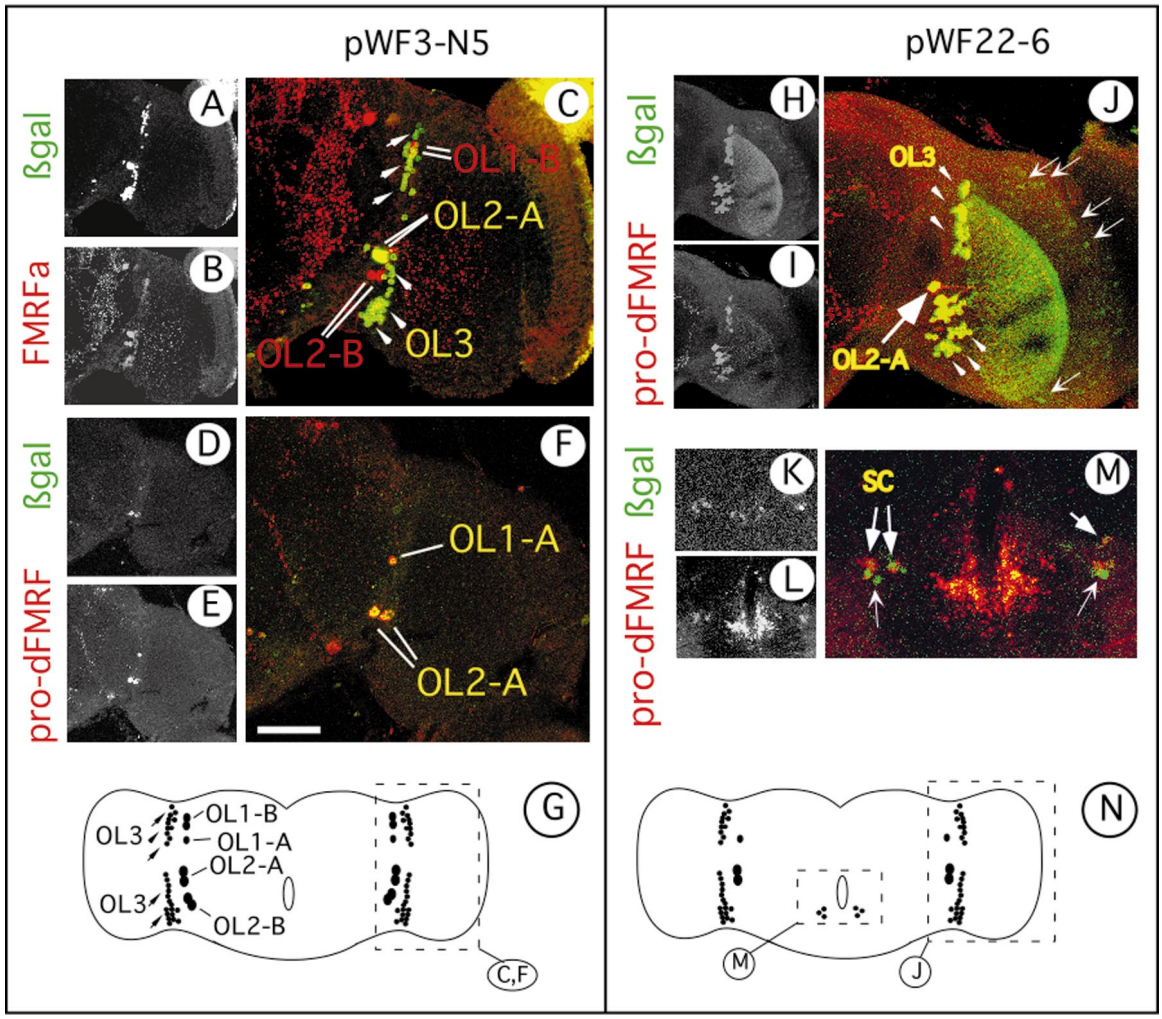


FIG. 2. Definition of the *dFMRFa* OL neurons and the *dFMRFa* OL minimal enhancer fragment. Whole-mount immunofluorescent staining of brains from transgenic animals: pWF3-N5—an 8-kb fragment of *dFMRFa* fused to *lacZ* (left); pWF22-6—a 256-bp fragment fused to *hsp43lacZ*. The images are confocal scans representing 50-μm projections through the anterior aspect of the adult brain. β-Gal immunosignals (βgal) are shown in green; neuropeptide immunosignals (FMRFa and pro-dFMRF) are shown in red. Double-stained OL cells appear yellow. Schematic in (G) summarizes positions and names of all FMRFa-positive neurons as shown in (C). Schematic in (N) summarizes positions of the *dFMRFa* neurons active in pWF22-6 (from J and M). (A–C) There are a large number of FMRFa-positive OL neurons, but only the OL2-A, OL1-A, and OL3 are double stained. OL1-A is not clearly identified in the specimen shown in (C). OL2-A neurons have irregular cell bodies, show granular immunostaining, and are larger than OL2-B, which are round and display smooth immunostaining. OL1-B and OL2-B cell numbers varied between 2 and 3. Other (non-OL) cells are stained with both or either antibody, but these are not identified here. (D–F) Antibodies to the pro-dFMRF precursor double label the OL2-A and OL1-A; it can also double label OL3 neurons, but not in this specimen. (H–M) Activity of the 256-bp minimal OL enhancer of *dFMRFa* in the OL2-A/OL3 and SC *dFMRFa* neurons. (H–J) pWF22-6 provides a complete stain for the 2 OL2-A cells and the two groups of 30–50 small OL3 neurons (arrowheads). Scattered thin arrows in the medulla (to the right) indicate β-gal-positive cells that may be similar to so-called OL4 cells (Nässel, 1993); they were not peptide-positive. The reporter stain fills axons of OL2-A and OL3 cells which project centrifugally and form a broad tangential field in proximal medulla. (K–M) Staining of SC neurons: 3–6 small, weakly stained β-gal-positive cells that lie on either side of the esophageal foramen. There is partial overlap of the dFMRFa-precursor-positive group (L) and the β-gal-positive group (K). In the composite (M), the red channel was enhanced to illustrate the otherwise weak neuropeptide staining of SC cells. The very strong neuropeptide staining in processes from other cells (below and around the esophageal foramen (L)) bled though in the composite; such signals were not present in specimens stained only for β-gal. The schematic in (N) indicates the relative positions of (J) and (M). Scale, 50 μm (C), (F), (J), and (M).

TABLE 1
Reporter Activity in Transformant Lines

Construct	Element mutated[a]	No. of lines	OL2-A activity	OL3 activity
17	. . . –	3	0/3	0/3
16	. . . –	3	0/3	0/3
11	. . . –	5	5/5	2/5
22	. . . –	11	8/11	7/11
27	. . . –	2	0/2	0/2
42	. . . –	4	0/4	0/4
15	. . . –	2	0/2	0/2
22MA	. . . A	4	4/4	4/4
22MB	. . . B	8	2/8	0/8
22MC	. . . C	8	0/8	0/8
22MD	. . . D	3	3/3	0/3
22ME	. . . E	3	2/3	3/3
22MA/D	. . . A/D	2	2/2	1/2
22MD/E	. . . D/E	2	2/2	1/2
22MA/E	. . . A/E	3	3/3	2/3
73	—	3	0/3	0/3

Note. Line 11 was described by Schneider *et al.* (1993). Line 17 was described by Benveniste and Taghert (1999).

[a] Letters A–E refer to conserved *dFMRFa* sequence elements indicated in Figs. 2 and 4; mutated sequences were tested within the fragment marked No. 22 in Fig. 3.

adult-specific with respect to FMRFa expression (i.e., not seen in feeding stage larvae): All differentiation markers currently available that permit identification of OL2-A and OL3 fail to identify them during prior larval stages.

The 256-bp dFMRFa Enhancer Drives Reporter Expression in OL2-A/OL3 Neurons

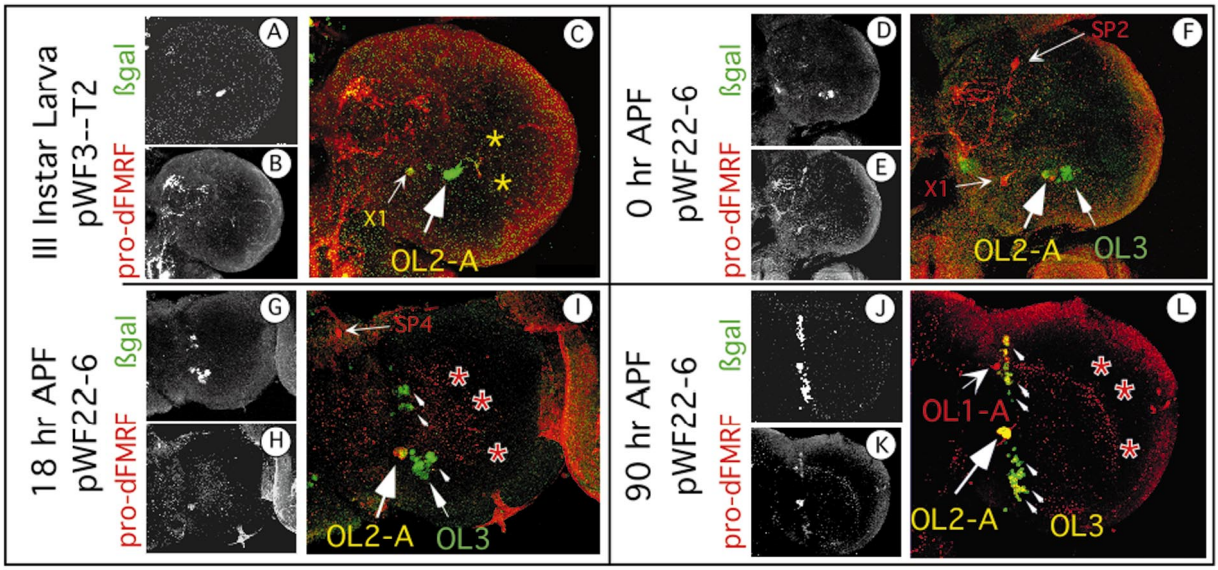
dFMRFa OL neuron-specific activity was localized to a 256-bp fragment (pWF22), which was dubbed the OL minimal enhancer (Figs. 1 and 2H–2J). It was active in a high proportion of independent lines, when tested in relation to a heterologous promoter and in opposite orientation (Table 1). A contiguous ~90-bp fragment (pWF16, representing the remainder of pWF11) contains conserved sequences (Taghert and Schneider, 1990) but was not active in this assay. Likewise, minimal enhancer fragments smaller than 256 bp were not active (e.g., pWF27, pWF42). A concatenated subfragment of the minimal enhancer (four copies of the 44-bp region from −361 to −320, including conserved domain B) was also not active in OL neurons nor in other CNS cells (data not shown).

OL2-A reporter activity in the pWF22 lines was as strong as that in the pWF3 lines, which contained a much larger amount of *dFMRFa* regulatory sequence. Several pWF22 lines also displayed weak-to-strong activity for OL3 neurons as well (Table 1). In addition to routine labeling of the *dFMRFa* OL2-A and OL3 cells, pWF22-6 animals also expressed the β-gal reporter in several unidentified cells distributed within the medulla (Fig. 2J). Anti-FMRFa antibodies stain many small neurons (called OL4) in this area of the optic lobes in various insects, including larger flies such as *Calliphora* (Nässel, 1993), but not in our preparations. The strongest pWF22 lines also showed weak expression in SC neurons of the dorsal brain surface near the esophageal foramen (Figs. 2K–2M): SC neurons (like OL neurons) also differentiate at metamorphosis (O'Brien *et al.*, 1991). SC reporter activity was never displayed by all pro-dFMRF-positive neurons in the "SC cell" region, and not all β-gal-positive cells in the SC cell region were pro-dFMRF-positive (Fig, 2M). The *dFMRFa* staining pattern of the pWF22-6 OL minimal enhancer fragment is schematized in Fig. 2N. In summary, these results indicate that a small region of *dFMRFa* regulatory sequences is capable of producing robust and accurate expression in one of the many cell types that normally express this gene (OL2-A) and also contributes to weaker activity in other cell types (OL3 and SC). Because SC reporter activity was especially weak, we concentrated on OL2-A and OL3 neurons.

OL Neurons Are Metamorphic

The OL2-A neurons begin *FMRF-lacZ* expression at the wandering stage of the third larval instar (Schneider *et al.*, 1993a). OL2-A reporter activity was first evident in wandering larvae ($n > 20$; Figs. 3A and 3C) and OL2-A distal processes were also stained faintly by pro-dFMRF antibodies (Fig. 3B). In favorable preparations, the neurons displayed a complex axonal arbor in the incipient optic lobe neuropil (Figs. 3B and 3C). At 0 h APF (after puparium formation), weak neuropeptide expression in OL2-A cells was matched by weak β-gal expression ($n = 5$; Figs. 3D–3F) and both markers subsequently increased in intensity. OL2-A neurons remained pro-dFMRF-positive thereafter.

OL3 neurons differentiated more slowly and never showed pro-dFMRF immunostaining before ~70–90% of adult development. The extent of OL3 pro-dFMRF staining in adults ranged from zero to moderate staining of ~50 cells per cluster. OL3 reporter staining often preceded and far exceeded staining for pro-dFMRF; it first appeared in late larvae or early pupariating animals. We never observed *dFMRFa* reporter activity in putative OL3 cells prior to the third larval instar. In large *dFMRFa* reporters (e.g., pWF3-T2), a small number of putative OL3 neurons were visible on the dorsal surface of the brain in wandering larvae (Schneider *et al.*, 1993a). These neurons resembled OL3 cells, although they were anomalously far (tens of micrometers) from OL2-As. In animals approaching pupariation, the putative OL3 cells appeared to migrate ventrally toward OL2-A neurons. By 0 h APF, OL3 reporter expression was unambiguous in a small number of neurons occupying a position adjacent to OL2-A neurons (Figs. 3D and 3F). By 18–30 h APF, there were 35 to 50 OL3 β-gal-positive cells per brain hemisphere ($n = 5$; Figs. 3G and 3I), and they often remained strong in adults (Figs. 2H, 2J, 4J, and 4L).



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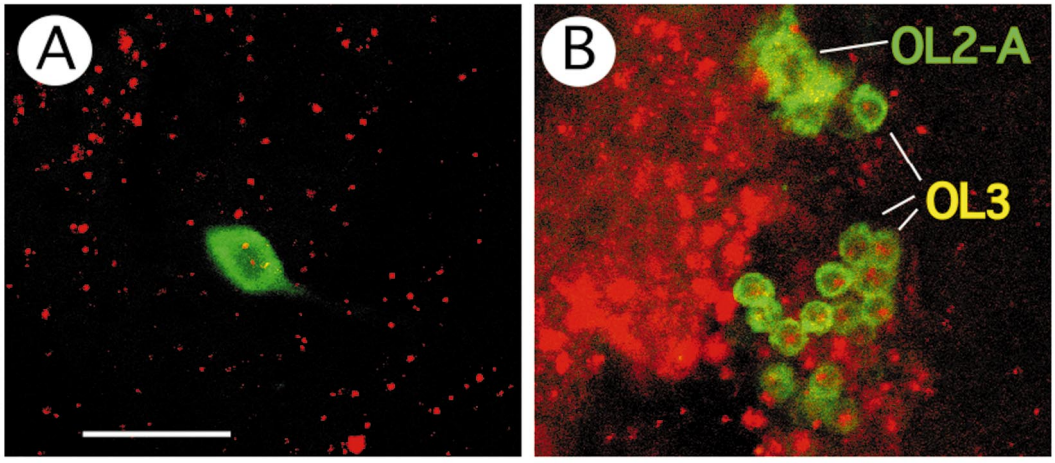

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FIG. 3. Differentiation of pro-dFMRF precursor protein and *dFMRFa*-reporter gene expression in OL2-A and OL3 neurons during metamorphosis. The images show double immunolabeling for β-gal (green) and for pro-dFMRF (red) in two different *dFMRFa-lacZ* reporter lines. (A–C) Initial differentiation at the wandering larval stage seen in a 30-μm projection: in pWF3-T2, β-gal is present in the majority of larval *dFMRFa* cells (e.g., X1, SP2), including the incipient OL2-A cells. These neurons express pro-dFMRF immunostaining coincidentally at distal processes only (yellow asterisks), revealing a small rudimentary OL2-A arbor. Little OL3 differentiation is evident. (D–F) At 0 APF, a 34-μm projection in pWF22-6 animals reveals OL2-A neurons stained by both anti-β-gal and anti-pro-dFMRF; other pro-dFMRF-positive neurons (e.g., X1, SP2) are not β-gal-positive in the pWF22-6 reporter line. A few OL3s of the ventral cluster have begun to express β-gal (green), but are not pro-dFMRF-positive. Some ectopic β-gal-positive neurons are stained toward the midline of the brain hemisphere. (G–I) At 18 APF, dorsal OL3 cells (arrowheads) begin expressing β-gal but still lack pro-dFMRF-immunostaining (a 36-μm projection). (J–L) At 90 APF, both OL2-A and OL3 cells are strongly stained by both markers (a 66-μm projection). Red asterisks indicate pro-dFMRF-positive OL2-A axons in the developing optic lobe neuropil.

FIG. 4. Birthdate analysis of OL neurons in the pWF3-T2 transgenic line. (A) Double immunolabeling of a wandering larval brain showing a single OL2-A cell body (*dFMRFa-lacZ:* green) that is weakly stained for BUdR-like immunoreactivity (red); most OL2-A neurons were not BUdR-positive at all. (B) Double immunolabeling of a P8 pupal brain showing an OL2-A neuron and several OL3 cells all expressing *dFMRFa-lacZ* (green); most OL3 neurons contain moderate levels of BUdR-like immunoreactivity in their nuclei, while the OL2-A neurons are not BUdR-positive. Both images represent single confocal scans; scale, 10 μm.

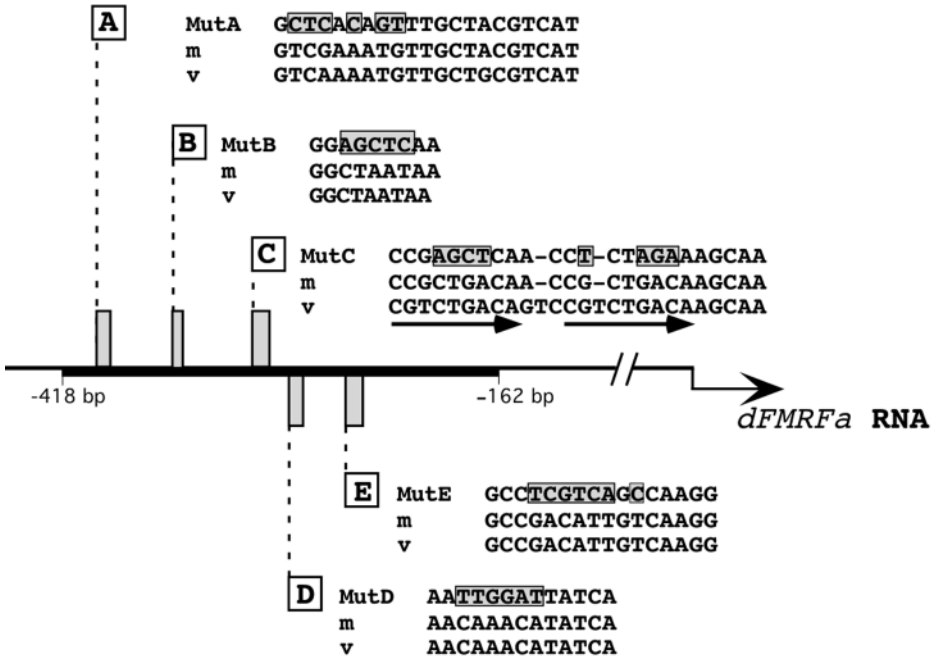

FIG. 5. Positions and sequences of conserved elements within the OL minimal enhancer that were mutated and tested *in vivo*. Numbers refer to basepairs upstream of the *dFMRFa* transcription start site (bent arrow). Black bar between −418 and −162 represents the OL minimal enhancer. Small gray boxes represent short sequence elements (9–24 bp) A through E. Each element is conserved in the comparable position of the *dFMRFa* gene in the related species *D. virilis* (Taghert and Schneider, 1990). For each element, the wild-type *melanogaster* (m) and *virilis* (v) sequences are presented below the mutated *melanogaster* sequences (Mut) that were tested. Shaded boxes highlight mutated sequences.

OL Neuron Birthdating

To determine their relative birthdates, we examined *dFMRFa* OL neurons in the pWF3-T2 reporter line following administration of the nucleotide analog BUdR by feeding throughout the larval stage (Truman and Bate, 1988). Strong BUdR signals completely stained the nucleus, especially at its margins; moderate to weak labeling (as seen for here for OL neurons) consisted of one to three large spots within the nucleus. When studied at the wandering larval stage, a β-gal-expressing OL2-A neuron was infrequently labeled (in two of eight brain hemispheres), and such staining was very weak (Fig. 4A). In each of six brain hemispheres studied at the P8 pupal stage, about 60% of β-gal-expressing OL3 neurons were weakly to strongly labeled (Fig. 4B). These observations are consistent with the hypothesis that most OL3 neurons are generated postembryonically and that OL2-A neurons are generated at the end of embryogenesis or during the first larval instar.

Structure–Function Analysis of the dFMRFa OL Minimal Enhancer

The 256-bp minimal *dFMRFa* OL enhancer contains several domains that have retained sequence similarity with a comparable region of the *dFMRFa* gene in *Drosophila virilis* (Taghert and Schneider, 1990). Figure 5 illustrates five OL domains (called A through E) which were chosen for analysis because they display at least 90% similarity over a minimum of 9 bases. Domain C was an exception; it was included because it includes a 9-bp tandem repeat, separated by 3 or 5 bp in each species. Within a species, the repeat sequences of domain C are perfectly conserved; between species, they diverge at 3 of 9 bases. The domain C repeat includes the sequence CTGACA, which bears similarity to the canonical AP1 binding site (C/GTGACTC/AA; Angel *et al.*, 1987; Lee *et al.*, 1987). Domains A–E display conservation in their order and orientations with respect to the transcription start site. The spacing between these domains is not a conserved feature (Taghert and Schneider, 1990).

We altered 4 to 8 bp within each of the five conserved domains and tested each mutated domain within the context of the 256-bp enhancer fragment (Figs. 6 and 7). In certain cases, we also tested pairs of mutated domains. Altering sequences within domains B and C produced a clear diminution of OL activity *in vivo*. The TAAT sequence within domain B appeared necessary for full activity; six of eight lines showed no activity (Figs. 6 and 7B). Several negative mutant B lines nevertheless exhibited ectopic reporter activity in either glial and/or neuronal cells

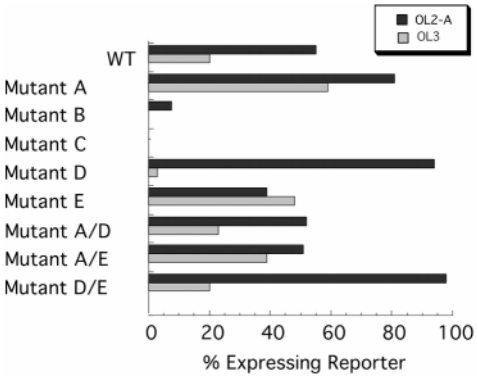

FIG. 6. Incidence of reporter expression in OL2-A and OL3 neurons in transgenic animals bearing the pWF22 enhancer fragment (or mutant variants). The positions and sequences of mutated domains are indicated in Fig. 5. At least six specimens from each line were examined. See Table 1 for numbers of independent lines tested and Materials and Methods for scoring details.

of the brain (Fig. 7B). In the two pWF22-MB mutant lines that retained OL activity (Table 1), there was weak β-gal expression in OL2-A and none in OL3 (data not shown). Domain C mutations were tested in eight independent lines and none displayed OL reporter activity (Figs. 6 and 7C); however, many retained ectopic activity in cell types that appeared similar to those seen in transformant lines bearing the wild-type fragment (Fig. 7C).

Altering domains A, D, or E produced inconsistent changes in the incidence of OL reporter expression. Alterations of domains A and E individually increased OL3 incidence two- to threefold, while alteration of domain D increased OL2-A, but lowered OL3, incidence (Figs. 6, 7A, 7D, and 7E). We next asked whether these changes were reproduced, or made more pronounced, when pairs of domains were mutated. The A:D and A:E double-mutant fragments resembled the activity of the wild-type molecule; the D:E double-mutant fragment had high OL2-A incidence—resembling the single D mutant, but different

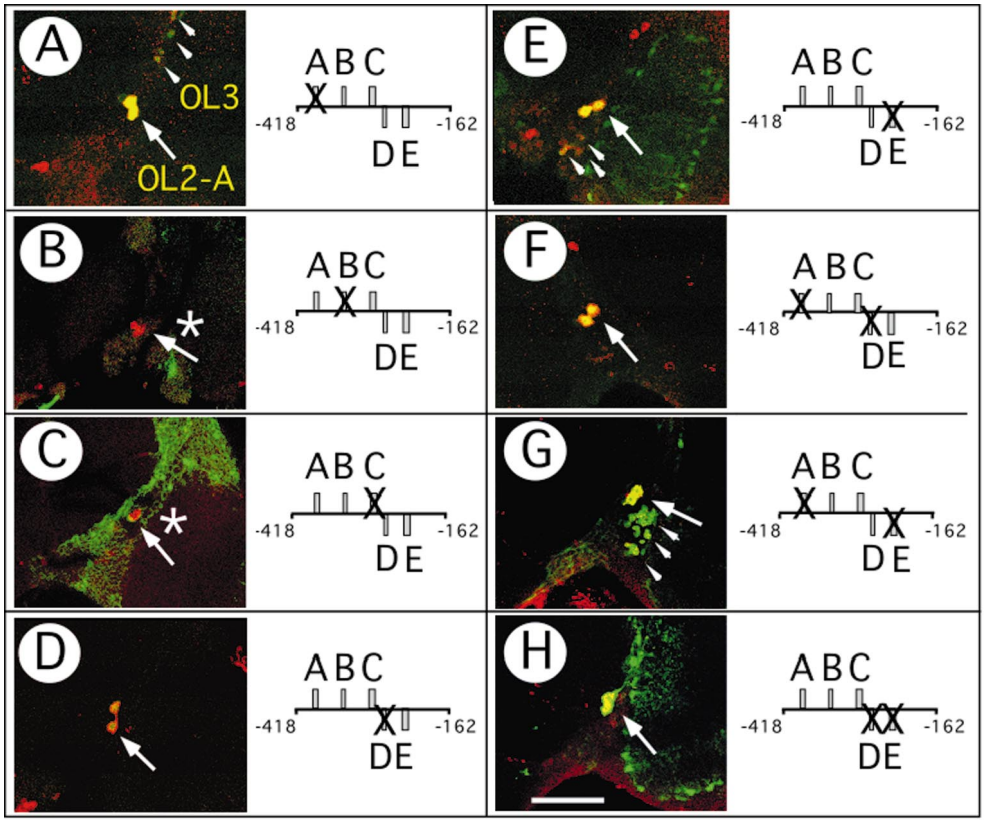

FIG. 7. *In vivo* assay of the transcriptional activity of the *dFMRFa* OL minimal enhancer containing single or pair-wise mutations in conserved elements. The photographs represent single confocal scans of double-immunostained specimens from representative single insertion lines bearing test fragments. The site containing mutated sequence is indicated by an "X" in the schematic to the side of each photograph (see Fig. 5 for sequence details). In each example, the green channel illustrates anti-β-gal immunostaining and the red channel indicates the immunostaining for the CT epitope of the pro-dFMRFa precursor. The large arrows indicate the OL2-A cell bodies and the small arrowheads indicate OL3 cell bodies. Arrows with asterisks indicate OL2-A neurons that were stained by pro-dFMRF antibodies but were not β-gal-positive (B and C). Scale, 50 μm.

from the A:D double-mutant fragment (Figs. 6, 7F, 7G, and 7H).

Differential Gene Regulation of OL2-A versus OL3 Neurons

With only one exception, all reporter constructs that were active in OL2-A were also active in OL3 in at least some of the independent lines studied. The exception was domain E mutant line pWFM22-12A1, in which pro-dFMRF-positive OL3 neurons consistently and strongly expressed reporter, but the pro-dFMRF-positive OL2-A neurons never expressed reporter (Figs. 8D–8F). Other independent lines bearing the same mutation reported in both OL2-A and OL3 cells (e.g., Fig. 7E). Together these observations indicate a strong position effect that influences the pattern of expression in line pWFM22-12A1. They also feature an instance in which OL2-A and OL3 neurons diverged in their capacity to express a common transgene.

Morphology of the OL2-A/OL3 Neurons

We used the pWF22-6 line to describe the morphology of OL2-A and OL3 neurons because it gave the strongest and most complete staining of OL processes throughout the optic lobe (Fig. 2). The morphology of OL2-A cells corresponds in many ways to the FMRFa-positive, MeRF1 amacrine neurons of the visual system in the blowfly *Musca* (Nässel *et al.*, 1988). Both are tangential neurons which ramify along the axis perpendicular to that of retinotopic projections (Meinertzhagen and Pyza, 1999). In *Drosophila*, OL2-A axons ramify broadly within the most proximal two or three layers of the medulla (Figs. 8A–8C). They do not send recurrent projections into the central brain. The smaller OL3 neurons appear to follow the direction of the OL2-A neurons and produce a similar terminal field. The morphology of a single OL2-A or a single OL3 neuron could not be determined from these preparations.

Immunostaining for the CT epitope of pro-dFMRF and for pWF22-6 β-gal marked overlapping terminal fields. However, the reporter-stained terminal fields were wider and more distal (Figs. 8A–8C). Because the pro-dFMRF antibody primarily stains OL2-A and not OL3 neuronal processes (due to weaker OL3 pro-dFMRF expression), the difference suggested that OL3 neurons produce a wider terminal field than do OL2-A or that other β-gal-immunostained cells of the optic lobes were contributing to the width of the reporter-stained terminal field. Support for the first explanation came from examination of pWFM22-12A1 animals (Figs. 8D–8G). This was the only line to show β-gal expression in OL3 and not in OL2-A neurons: here OL3 processes could be traced to the medulla independent of OL2-A processes. Counterstaining with the pro-dFMRF antibodies (to visualize OL2-A processes) revealed OL3 projections occupying a domain that is slightly wider and more distal than OL2-A processes (Fig. 8G). Four to five large β-PDH-expressing neurons have cell body positions in the same location as *dFMRFa* OL2-A/OL3 neurons and similarly extend centrifugal axons that form tangential terminal fields (Helfrich-Förster and Homberg, 1993; Nässel *et al.*, 1993). They are called large LN_v (lateral neuron–ventral) and are candidate pacemakers regulating circadian behaviors (reviewed by Kaneko, 1998). LN_v neurons are distinct from all *dFMRFa* OL cells here described. The terminal fields of LN_v neurons do not overlap those of OL2-A and OL3 neurons: the β-PDH axon terminals occupy more distal layers of the medulla (Figs. 8H and 8I).

DISCUSSION

To analyze CNS development at metamorphosis we studied the specification of cellular phenotypes by tangential imaginal neurons of the *Drosophila* CNS. The neurons chosen for study included representatives of the two classes of imaginal neurons that develop new cellular properties at metamorphosis. The first class includes neurons which are born during embryonic stages (e.g., OL2-A) and which may have a differentiated state in larval stages (e.g., Truman and Reiss, 1976). The second class is represented by OL3 neurons which are born postembryonically and which differentiate for the first time at metamorphosis. The results we have described contribute to two related issues in development. The schedules of cellular differentiation provide the basis with which to define the signals that differentially regulate these neuronal classes at metamorphosis. The structure/function analysis of the OL *dFMRFa* cell enhancer provides essential information with which to define the developmental sequence underlying a cell type-specific transcriptional mechanism. We incorporate these results into a model in order to discuss these issues and to help frame future experiments.

OL2-A/OL3 Neurons—Their Developmental Histories and Identities

The two classes of metamorphic neurons are defined by their times of generation. We interpreted DNA replication experiments to indicate that OL2-A neurons are born just prior to, or at, the time when animals first saw the BUdR. Very weak or undetectable staining suggested OL2-A are born just before the labeling period (the start of feeding) or just after it ends. However, OL2-A neurons were already differentiating by the end of feeding (Fig. 3C), hence these imaginal neurons are born at the end of embryogenesis or during the first few postembryonic hours. In contrast, the routine labeling of OL3 neurons following larval exposure indicated that these neurons are generated postembryonically. The absence of BUdR labeling in a minority of OL3 neurons is most likely explained by their generation after the cessation of feeding, during periods of wandering and/or pupariation. Neurons occupying similar positions and common neurotransmitter phenotypes are often related by lineage (Taghert and Goodman, 1984; Huff *et al.*, 1989;

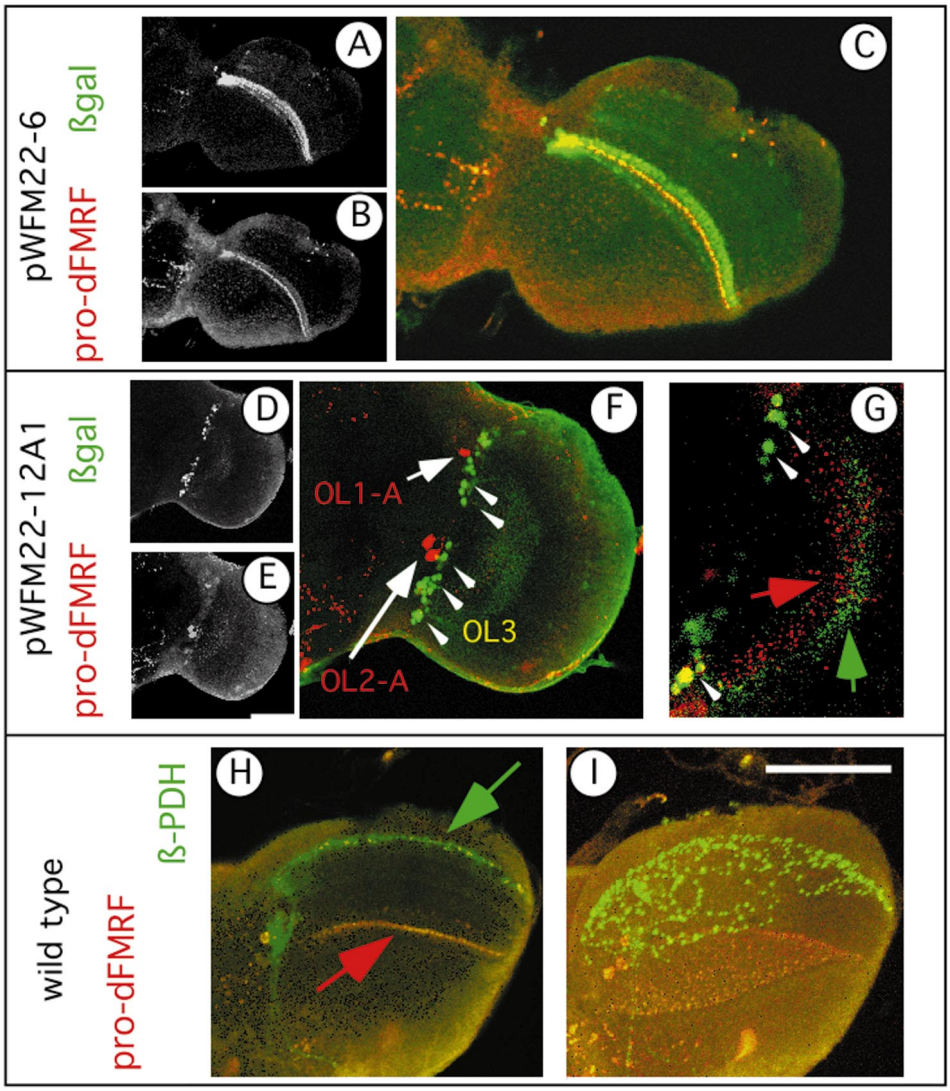

FIG. 8. OL2-A/OL3 form tangential terminal fields across specific proximal layers of the medulla. (A and B) 10-μm confocal images of the medulla stained by β-gal and pro-dFMRF antibodies, respectively, in the pWF22-6 line: note the wider β-gal projection evident in the overlapping image (C). The middle (D–G) illustrates the relative positions of OL2-A and OL3 terminal fields as studied in the pWFM22-12A1 line, which is the only line that displays reporter expression in OL3 but not OL2-A neurons. (D and E) 52-μm projections stained by β-gal and pro-dFMRF antibodies, respectively; (F) is the overlap of the two. Note that several of the β-gal-positive OL3 cell bodies are also stained by pro-dFMRF (E). (G) A single scan: anti-pro-dFMRF antibodies primarily stain the OL2-A processes (red), while anti-β-gal primarily stains OL3 processes (green). The OL2-A field (red arrow) is narrower, more proximal, and only partially overlapping with that of the OL3 projection (green arrow). The bottom illustrates scans in the optic lobe of a wild-type adult brain stained with pro-dFMRF antibodies (red) and β-PDH antibodies (green). A single scan (H) and a 116-μm projection (I) indicate the similarities and relative positions of the β-PDH processes in the distal medulla (green arrow) and dFMRFa processes (OL2-A) in proximal layers of the medulla (red arrow). In (H) and (I), anterior is to the top; in all other, dorsal is to the top. The two primary antibodies used in (H) and (I) were both made in rabbits: this could generate cross-reactivity between secondary antibodies and is a likely reason for the yellow aspect to the signals in the red channel (see Materials and Methods). Scale, 100 μm for (C), (F), (H), and (I); 66 μm for (G).

Lundell and Hirsh, 1997). A potential lineal relationship between OL2-A and OL3 neurons cannot be deduced from the present results and awaits direct lineage analysis.

OL2-A and OL3 neurons are similar by position, axonal trajectory, expression of the *dFMRFa* transmitter, and a shared requirement for the same minimal enhancer within *dFMRFa*. Yet, in addition to their distinctive birthdates, OL2-A and OL3 clearly represent distinct cell types. While

both cell types delay differentiation until metamorphosis, OL2-A initiate dFMRFa neuropeptide expression at the start, and OL3 cells delay until the end. This difference indicates that mechanisms underlying delayed metamorphic development are similar but not uniform among all neurons differentiating at that time. Ohlsson and Nässel (1987) and Ohlsson *et al.* (1989) previously showed that visual system neurons sharing similar transmitter peptidergic phenotypes could display different schedules of differentiation.

Even in the context of highly similar *dFMRFa* transcriptional mechanisms, one observation underscored the retention of at least some critical differences between OL2-A and OL3 neurons. In animals bearing the pWFM22-12A1 reporter, only OL3 neurons were β-gal-positive, while the OL2-A neurons (still present and otherwise normal) never were. The difference likely derives from the interaction of the test DNA with neighboring sequences around that specific insertion site. From this result, we infer the existence of one or more differences in the properties or combination of factors that regulate *dFMRFa* regulatory sequences in OL2-A versus in OL3 cells.

Developmental Control of dFMRFa Transcription

The activity of the OL minimal enhancer faithfully mimicked the spatial and temporal features of OL *dFMRFa* expression. No manipulation of the OL enhancer (either truncation or sequence alteration) produced premature *dFMRFa* reporter expression by OL2-A or OL3 neurons prior to the wandering larval stage. That specific result supports a model of OL cell differentiation in which production of the adult phenotype results from the activation of a positive transcriptional regulator(s) at the wandering stage. We term this event the triggering or acquisition of "transcriptional competence," and we hypothesize that it represents a series of steps permitting *dFMRFa-lacZ* expression in both cell types (Fig. 9). Differentiated OL2-A neurons of pre-wandering stages (lacking *dFMRFa* expression but displaying a rudimentary axonal arbor) may have a physiological function in the larval optic center (Meinertzhagen, 1973; Busto *et al.*, 1999), or they may be required to pioneer or help organize the adult system (Tix *et al.*, 1989). The earliest expression of *dFMRFa* by OL2-A neurons occurred within hours of the ecdysteroid pulse (Riddiford, 1993; cf. Booker and Truman, 1987). OL3 neurons followed shortly thereafter. This timing suggests a potential role of the steroid in triggering or accelerating transcriptional competence within *dFMRFa* OL neurons. Because we did not find canonical ecdysteroid binding sites within the *dFMRFa* regulatory regions (comparable to those found in other *Drosophila* neuropeptide genes, e.g., Park *et al.*, 1999), we suspect that the putative ecdysteroid regulation of *dFMRFa* gene expression is indirect. It will be useful to analyze and manipulate ecdysteroid levels and ecdysteroid receptor expression in the candidate target neurons (e.g., Schubiger *et al.*, 1998) to test this possibility.

Although direct negative control of *dFMRFa* gene expression during pre-wandering larval stages appears slight, indirect negative control may still be a significant force to define the acquisition of OL transcriptional competence. For example, inhibition in the larval OL2-A (and first-born OL3 neurons) may keep positive regulators of *FMRFa* repressed (not expressed or inactivated). According to this line of speculation, OL neurons achieve *dFMRFa* transcriptional competence following relief of inhibition through signaling by the molting hormone cascade. Domain C is a candidate *cis*-binding site for such a "repressed" positive regulator, as argued below.

Developmental Control of dFMRFa Peptide Expression

In contrast to OL2-A neurons, OL3 neurons displayed a significant delay between *dFMRFa-lacZ* and dFMRFa peptide expression. The full complement of OL3 neurons produced *dFMRFa-lacZ* by ~18–30 h APF, but OL3 neurons did not produce dFMRFa peptide until late pupal stages (~90 h APF) and then did so in a roughly synchronous manner (Fig. 9). In large part, the later schedule of OL3 differentiation (*dFMRFa-lacZ* and dFMRFa peptide accumulation) could reflect their later birthdates. Yet, the rate of OL3 peptide accumulation lagged significantly behind OL3 reporter accumulation during adult development. Therefore, a second explanation for the greater delay in OL3 peptide accumulation invokes the action of additional inhibitory factors, the sum of which we term "imaginal inhibition" (Fig. 9). We cannot yet specify at which level of gene expression this putative inhibition may act.

Previous studies of transmitter differentiation in *Drosophila* have noted sharp accumulations of choline acetyltransferase activity (Chase and Kankel, 1988), acetylcholinesterase activity (Dewhurst *et al.*, 1970), and GABA immunostaining (cited in Truman *et al.*, 1993) during the final day of adult development. Likewise, small peptidergic and aminergic neurons of the medulla and lobula derived from optic lobe neuroblasts first display transmitter in the final stages of metamorphosis or after adult eclosion (Ohlsson and Nässel, 1987; Ohlsson *et al.*, 1989). We propose that imaginal inhibition is a principal mechanism to ensure temporal order among the majority of imaginal neurons in a functioning nervous system during metamorphosis. It is possible that the majority of imaginal cells serve in adult-specific circuits and so are not made functional until near the end of adult development (cf. Truman, 1976). It will be of interest to expand the scope of these studies to determine, for other groups of *Drosophila* neurons, whether the development of metamorphic changes in embryonically generated neurons (e.g., OL2-A) always precedes that in later born neurons (e.g., OL3).

The Mechanisms of dFMRFa OL Minimal Enhancer

Our observations on the mechanisms of *dFMRFa* transcription in OL neurons suggest that the activity of the OL

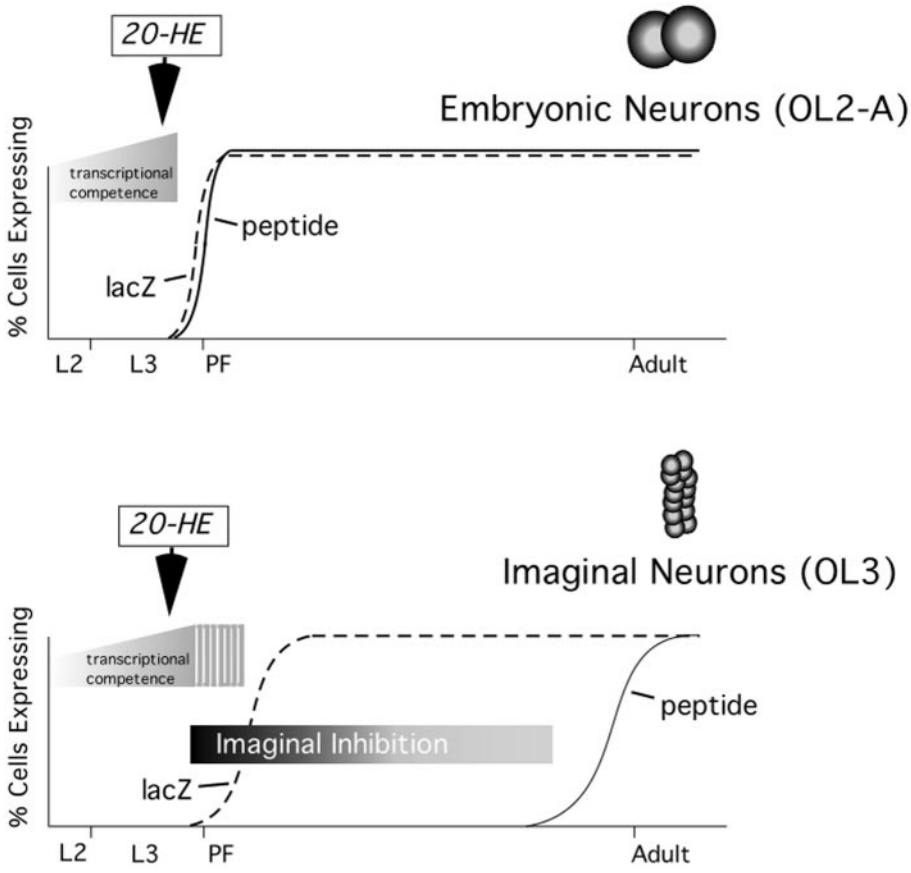

FIG. 9. A model comparing the differentiation of embryonic (OL2-A) and imaginal (OL3) cells during the metamorphosis of *Drosophila.* The time line covers the period from the second larval instar (L2) through the start (puparium formation, PF) and end (Adult) of metamorphosis. Embryonically derived neurons begin *dFMRFa* gene and peptide expression during early periods of metamorphosis; imaginal neurons begin *dFMRFa* gene expression at the same time but do not complete differentiation until the end of metamorphosis. Transcriptional competence: hypothetical steps occurring within OL neurons that permit *dFMRF-lacZ* expression. Imaginal inhibition: a hypothetical process occurring within OL3 neurons that explains their lack of peptide expression prior to late adult development. The model proposes that ecdysone (20-HE) triggers (or completes) the acquisition of transcriptional competence. "lacZ" (dashed lines), the activity of *dFMRFa-lacZ* reporters in OL neurons; "peptide" (solid line), anti-pro-dFMRF immunostaining in OL neurons.

minimal enhancer depends on several sequences distributed throughout its 256-bp length that each make different quantitative contributions. A minority of sites, indicated by their evolutionary conservation and mutagenesis phenotypes (i.e., domains B and C), make large contributions. The sequences of domains B and C suggest specific classes of candidate binding proteins.

Domain B: a TAATNN element. Domain B contains a TAATNN sequence which suggests the involvement of homeodomain-containing proteins (Gehring *et al.*, 1994). A similar conclusion was reached in the case of a separate cell type-specific enhancer within the *dFMRFa* gene (Benveniste *et al.*, 1998). The ~500-bp enhancer controlling transcription in the Tv neuroendocrine neuron contains three conserved TAATNN sequences, regulated in part by the LIM homeodomain protein Apterous. Apterous does not bind the TAATNN site of OL domain B *in vitro* (R. Benveniste, pers. comm.). These findings highlight the importance and specificity of TAATNN-binding proteins in the operation of neuronal cell type-specific enhancers. Their contributions are required in each case from the *dFMRFa* gene (Tv neurons and OL neurons), as the phenotypes resulting from alterations of single TAATNN sites produced measurable losses of activity *in vivo* in both instances. It is noteworthy that a construct containing multiple tandem copies of a region which included domain B (four copies of a 46-bp fragment—pWF73) was not effective in substituting for the entire minimal OL enhancer.

Domain C: TGAC repeats. The similarity of these *cis* sequences to AP1 binding sites is strong, but not absolute (6 of 8 bases). In some contexts, AP1 activity is negatively regulated by retinoid signaling (Caelles *et al.*, 1997). That

observation suggests a simple hypothesis to explain the normal metamorphic delay in OL development whereby juvenile hormone (JH) is a negative regulator. JH displays weak retinoid-like biological activity (Jones, 1995) and could delay OL cell differentiation in premetamorphic stages by negatively regulating the proteins that bind to domain C in the OL2 enhancer.

To what extent do these mechanisms apply to regulation of neuropeptide genes at similar developmental periods in other imaginal neurons? The *Drosophila pdf* gene (Park and Hall, 1998) is expressed by neighboring tangential neurons (Fig. 8G) that also differentiate at metamorphosis (Helfrich-Förster, 1997). Inspection of the upstream region of *pdf* revealed a 13-bp perfect direct repeat that included TGAC sequences: **CCTGCGGATGACA**TGTATTGGT**CCTGCGGATGACA** (direct repeat shown in bold; the first bp is No. 106490 of record AC005813, Berkeley *Drosophila* Genome Project). The *pdf* sequences differ from *dFMRFa* domain C in several details (precise repeat sequence, repeat length, and number of bases separating the repeats). Based on these shared and dissimilar features, we speculate that a set of related transcription factors, interacting with domain C-type repeats, are critical regulators of terminal differentiation in diverse imaginal neurons.

A Model of Tangential Cell Differentiation

We have seen that imaginal neurons differentiate according to schedules that are highly correlated with changes in the steroid signals that instigate metamorphosis. Surprisingly, neurons that otherwise share many cellular properties (OL2-A and OL3) follow remarkably different schedules. The principal difference was the lag of many hours between neuropeptide gene expression and neuropeptide expression by imaginal (OL3) neurons. The highly similar OL2-A neurons, which are born much earlier in development, acquired adult characters in much more rapid fashion. We coined the descriptive terms "transcriptional competence" and "imaginal inhibition" to indicate two events that we hypothesize have substantial developmental significance in this context. We propose that these events define much of the rate and pattern underlying the differentiation of these adult neurons (Fig. 9). A better understanding of transcriptional competence will follow the identification of regulatory factors that act on domains B and C of the OL minimal enhancer. A better understanding of imaginal inhibition will require a molecular definition of the point at which neuropeptide expression is blocked in OL3 neurons. It will then be possible to ask when that block is relieved and what developmental factors regulate its effects.

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