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Unwrapping Glial Biology: Gcm Target Genes Regulating Glial Development, Diversification, and Function

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Summary

Glia are the most abundant cell type in the mammalian brain. They regulate neuronal development and function, CNS immune surveillance, and stem cell biology, yet we know surprisingly little about glia in any organism. Here we identify over 40 new *Drosophila* glial genes. We use *glial cells missing* (*gcm*) mutants and misexpression to verify they are Gcm regulated in vivo. Many genes show unique spatiotemporal responsiveness to Gcm in the CNS, and thus glial subtype diversification requires spatially or temporally restricted Gcm cofactors. These genes provide insights into glial biology: we show *unc-5* (a repulsive netrin receptor) orients glial migrations and the *draper* gene mediates glial engulfment of apoptotic neurons and larval locomotion. Many identified *Drosophila* glial genes have homologs expressed in mammalian glia, revealing conserved molecular features of glial cells. 80% of these *Drosophila* glial genes have mammalian homologs; these are now excellent candidates for regulating human glial development, function, or disease.

Introduction

Recent studies demonstrate far-reaching, instructive roles for glia in brain development and function. During development, glia provide growth factors that regulate neuronal proliferation and survival (Ebens et al., 1993), modulate axonal growth and fasciculation (Booth et al., 2000; Gilmour et al., 2002), establish boundaries that delimit regions of neurite outgrowth (Oland and Tolbert, 2002; Wang et al., 2002), and secrete factors essential for the formation, growth, and maintenance of synapses (Barres and Raff, 1999). In the adult, glia ensheath and insulate axons and synapses, buffering ions, pH, and neurotransmitters, and secrete multiple factors that regulate synaptic efficacy and synaptogenesis (Haydon, 2001; Pfrieger and Barres, 1997). Glia are the primary immune cells of the nervous system that engulf dying cells or invading pathogens and help form the blood-brain barrier that isolates and protects neural tissue. Glia mediate many brain responses to injury and neurodegenerative diseases (Wyss-Coray and Mucke, 2002), and abnormal glial growth results in gliomas—the most deadly forms of cancer—and other neurological diseases (Baumann and Pham-Dinh, 2001; Wingerchuk et al., 2001). Nevertheless, despite the importance of glia in the nervous system, we know surprisingly little about this remarkable cell type.

Drosophila offers an excellent opportunity to study glial biology in a genetically tractable organism; *Drosophila* glia are strikingly similar to their vertebrate counterparts based on developmental, functional, and morphological criteria. For example, developing *Drosophila* glia migrate long distances and undergo extensive interactions with axons and neuronal cell bodies, similar to vertebrate glia. A number of these interactions are known to be essential for aspects of neuronal development including axon pathfinding (Bastiani and Goodman, 1986), regulation of neural stem cell proliferation (Ebens et al., 1993), axon fasciculation (Hosoya et al., 1995; Jones et al., 1995), or neuronal trophic support (Oland and Tolbert, 2002). In the mature *Drosophila* nervous system, cell body glia perform modulatory functions, similar to vertebrate astrocytes; longitudinal glia ensheath CNS axons, similar to vertebrate oligodendrocytes; peripheral glia ensheath the nerves that project from the CNS, similar to vertebrate Schwann cells; and multiple glial subtypes remove apoptotic cell corpses from the CNS (Auld et al., 1995; Ito et al., 1995; Sonnenfeld and Jacobs, 1995), similar to vertebrate microglia. Finally, the *Drosophila* embryonic CNS is protected by a bilayered sheath consisting of several subtypes of glia, similar to the blood-brain barrier of vertebrates (Auld et al., 1995; Ito et al., 1995; Leiserson et al., 2000).

Nearly all *Drosophila* glial development requires the transcription factor encoded by the *glial cells missing* (*gcm*) gene: in *gcm* mutants, glia are transformed into neurons by both morphological and molecular criteria, whereas misexpression of *gcm* turns many neurons into glia (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Currently, there are four known Gcm target genes—*repo*, *loco*, and *pnt*, which promote glial fate (Campbell et al., 1994; Granderath et al., 1999; Klaes et al., 1994; Klambt, 1993; Xiong et al., 1994), and *tramtrack*, which represses neuronal fate (Giesen et al., 1997)—each of which appear to be expressed in all Gcm^{+} glia. The only other *Drosophila* genes known to be required for glial development or function are *ana*, which encodes a glycoprotein secreted by glia to negatively regulate neuroblast proliferation (Ebens et al., 1993), *not*, which encodes a ubiquitin-specific protease required for glial migration (Poeck et al., 2001), and *axo* (Yuan and Ganetzky, 1999), *gli* (Auld et al., 1995), *fray* (Leiserson et al., 2000), *nrx* (Baumgartner et al., 1996), *htl* (Shishido et al., 1997), and *wrapper* (Noordermeer et al., 1998), which are all required for neuronal ensheathment.

Here we use multiple genomic approaches to identify 45 *Drosophila* glial genes, and we verify all of them as

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glial-expressed and Gcm-regulated in vivo. The genes can be clustered into groups with common expression in specific glial subtypes or at specific times during gliogenesis; each group has a unique response to ectopic Gcm, revealing the existence of spatially and temporally restricted Gcm cofactors. Genes expressed early or in subsets of glia are excellent candidates to regulate glial development (e.g., specification, migration, glia-neuron interactions) while genes expressed later may regulate glial function (e.g., synaptic buffering, axon physiology, and immune roles). We perform loss-of-function or gain-of-function analysis on one gene from each class and show that *unc-5* (encoding a repulsive netrin receptor) can direct glial migration out of the CNS, and *draper* (encoding an EGF-repeat transmembrane protein) is essential for apoptotic neuron engulfment and larval locomotion.

Results

Computational Identification of *gcm* Target Genes in Glia and Macrophages

Computational algorithms have been designed that identify predicted genes within the entire genomic sequence (Jones et al., 2002) and potential cis-regulatory modules within genomic DNA (Berman et al., 2002; Markstein et al., 2002). Here we use a novel Gcm binding site search algorithm as a tool to rapidly identify potential direct targets of Gcm, and therefore potential new glial genes. We wrote Perl scripts designed to search the *Drosophila* genome for clusters of Gcm binding sites meeting our cluster criteria (see below) and identify genes in the flanking genomic DNA. We trained our algorithm to identify three known Gcm direct target genes (*repo*, *pnt*, and *loco*; Figures 1A and 1B); it narrowly misses the fourth (*ttk*). This training led us to define a Gcm binding site cluster as ≥8 Gcm DNA binding sites (A/GCCCGCAT), each spaced less than 1 kb apart, no site within a predicted exon, and all sites must fall within 10 kb of a predicted exon (Figure 1C). When run on the EMBL March 2000 *Drosophila* gene database, the algorithm identified 384 candidate target genes (Figure 1C; see Supplemental Data Set S1 at http://www.neuron.org/cgi/content/full/38/5/567/DC1). The search criteria used here represent a balance between sensitivity and selectivity: relaxing any criterion resulted in the identification of many thousand genes, while increasing the stringency failed to identify the known Gcm targets. More recently we have developed a simple interactive web site for performing DNA binding site pattern searches on the most recently annotated *Drosophila* genome using any user-defined sequence and cluster criteria (http://flycompute.uoregon.edu/cgi-bin/seqseek.pl; see Supplemental Figure S1 for instructions).

To determine which of these 384 candidate Gcm target genes are regulated by Gcm in vivo, we analyzed the mRNA expression pattern of each gene that had an available cDNA (n = 204) in wild-type embryos and embryos misexpressing *gcm* throughout the CNS. We scored for genes expressed in two Gcm-dependent tissues: glia (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) and macrophages (Bernardoni et al., 1997). We found 20 genes (10%) expressed in glia and/or macrophages in wild-type embryos (Figure 2; Table 1), clearly enriched compared to 0.5% glial/macrophage genes annotated in Flybase (http://flybase.bio.indiana.edu). Seventeen of these genes are expressed in glia, and all showed a loss of glial expression in *gcm* mutant embryos and/or upregulation in embryos where *gcm* is misexpressed throughout the CNS (Figure 2; Table 1).

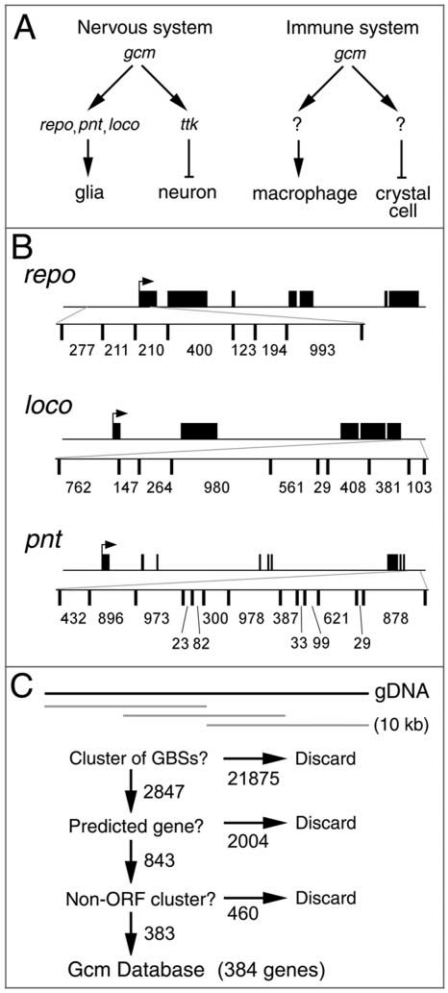

Figure 1. Computational Identification of Gcm Target Genes

(A) Known Gcm target genes in the nervous system and immune system.

(B) Spacing *gcm* binding sites (A/GCCCGCAT) adjacent to known Gcm direct target genes. Numbers indicate spacing in base pairs between sites.

(C) The Gcm binding site algorithm. We segmented the *D. melanogaster* genome data set (BDGP, version 2.9) into overlapping 10 kb pieces and searched for clusters of Gcm binding sites (GBSs; see text for clustering criteria). Fragments were retained if they contained a predicted *Drosophila* gene and the GBS cluster was in noncoding DNA. A total of 384 genes met these criteria; all genes and attributed data are provided (see Supplemental Data Set S1 at http://www.neuron.org/cgi/content/full/38/5/567/DC1).

Previously there were no known targets for *gcm* in immune cell lineages. We found nine genes expressed in embryonic macrophages (Figure 2B). The expression of all these genes was normal in *gcm* mutant embryos (e.g., Figure 2A; data not shown). However, *gcm* has recently been shown to act redundantly in macrophage

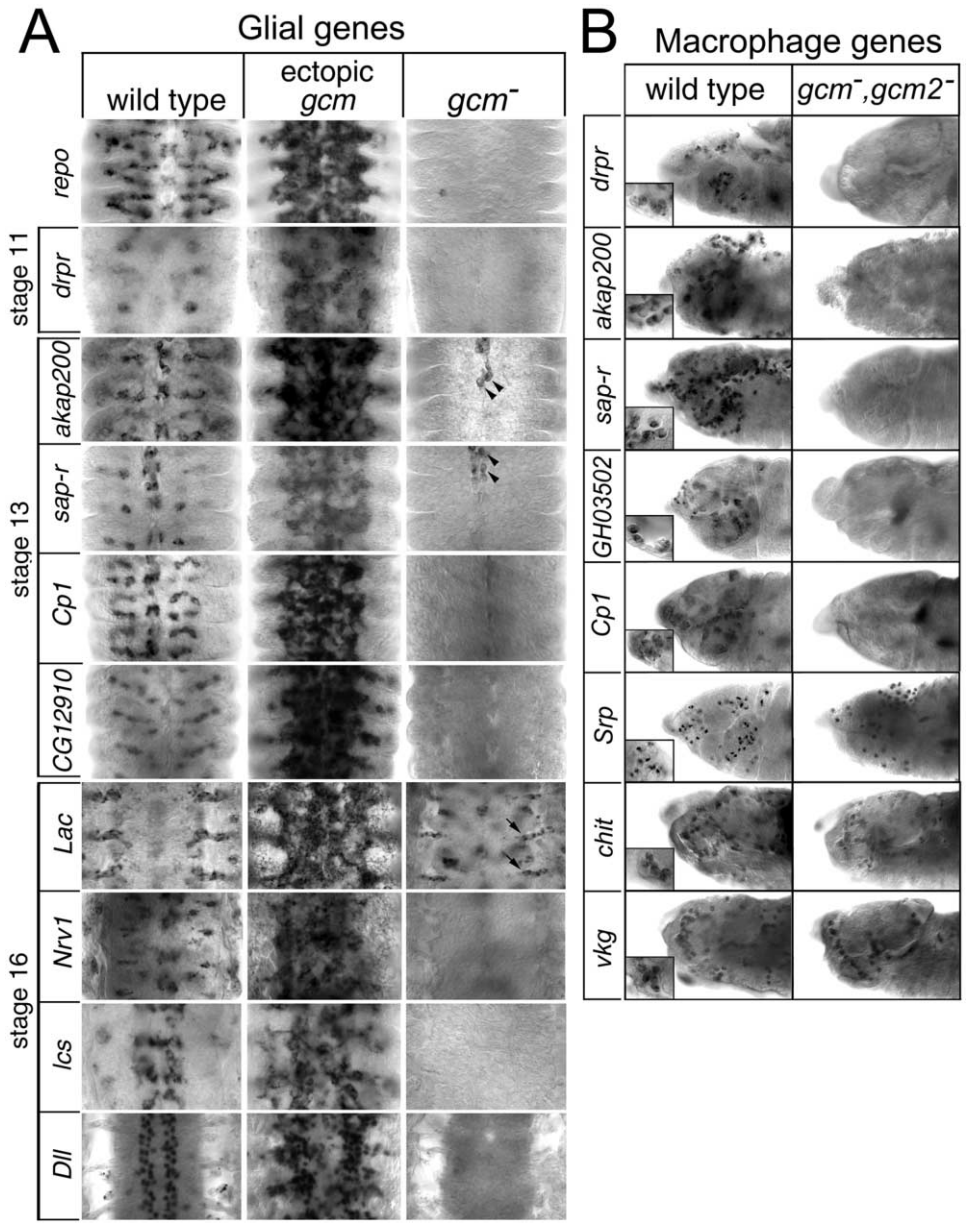

Figure 2. Computationally Identified Glial and Macrophage Genes Are Gcm Regulated In Vivo

Gene expression of candidate Gcm target genes in glia (A) and macrophages (B) assayed by RNA in situ hybridization to whole embryos. Genotypes are wild-type, *y w*; *gcm*$^{-}$, *gcm*$^{N7\text{-}4}$; ectopic *gcm*, *Sca-Gal4/UAS-gcm; UAS-gcm/+*; *gcm*$^{-}$ *gcm2*$^{-}$, *Df(2L)132*. Dll and Srp expression were assayed using antibody probes.

(A) Glial genes. Ventral views of three segments of the *Drosophila* embryonic CNS at the indicated stages of development. *repo* serves as a control for the pattern of embryonic glia (top; stage 13). Macrophage expression persists in *gcm* single mutants (shown for *sap-r* and *akap200*, arrowheads). Anterior, up; ventral midline is at center of each panel.

(B) Macrophage genes. *Serpent* and *viking* are known macrophage-expressed genes (Rehorn et al., 1996; Yasothornsrikul et al., 1997); *drpr*, *akap200*, *sap-r*, *dll*, *BcDNA:GH03502*, *Cp1*, and *chit* are newly identified macrophage genes. Lateral views of the anterior region of stage 13 embryos. Anterior, left; dorsal, up. Insets show a high-magnification view of macrophages.

development with the closely related *gcm2* gene (Alfonso and Jones, 2002), so we assayed their expression in *Df(2L)132* homozygous embryos lacking both *gcm* and *gcm2* function (Kammerer and Giangrande, 2001). We observed five of the nine genes are not expressed in embryos lacking *gcm* and *gcm2* function, the same subset that can be induced by ectopic Gcm expression in the CNS (Figure 2). In summary, our computational approach has identified 17 new Gcm-regulated genes in glia, and the first 5 Gcm/Gcm2-regulated macrophage genes.

Identification of Glial Genes Using cDNA Microarrays

Gene expression profiling using cDNA microarrays provides an independent method for identifying genes regu-

Table 1. Glial and Gcm-Regulated Genes Identified in This Study

	Gene	Wild-Type Expression[a]	Ectopic *gcm*[b]	*gcm*$^-$	Expression Class[c]	Predicted Product	Functional Class	Human Sequence Homolog
computational target	*chit*	macrophages, other	n.c.	n.c.		glycosyl hydrolase	extracellular	chitotriosidase
	vkg	macrophages, other	n.c.	n.c.		collagen type IV	extracellular	collagen α 2 type IV
	srp	macrophages, other	n.d.	n.c.		GATA-domain	transcription factor	GATA-4
	axo	glia	n.d.	-[d]		neurexin superfamily	signal transduction	contactin-associated protein 2
	aly	glia, other	+	-	S, T	transcriptional coactivator	transcription factor	ALY
	loco	glia, other	+	-	G	regulator of G-proteins	signal transduction	RGS12TS-S isoform
	akap200	glia, macrophages, other	+	-	G	kinase A anchor protein	signal transduction	--
	dri	glia, other	+	-	S	ARID transcription factor	transcription factor	dead ringer-like 1
	pnt	glia, other	n.d.	-[d]	G	ETS transcription factor	transcription factor	c-ets-1
	nrv1	glia, other	+	-	T	Na/K-exchanging ATPase	transporter	β1 polypeptide, Na/K ATPase
	lac	glia, other	+	-	S, T	immunoglobulin-MHC	cell adhesion	unnamed protein BAC03858
	dll	glia, macrophages, other	+	-	S	homeodomain	transcription factor	AAH36189, similar to DLX-1
	sap-r	glia, macrophages, other	+	-	G	sphingolipid activator	lipid metabolism	prosaposin
	GH03502	glia, macrophages, other	+	-	G	novel	other	hypothetical protein FLJ11838
	unc5[e]	glia, other	+	-[d]	S, T	netrin receptor	signal transduction	UNC5C
	repo	glia	+	-	G	homeodomain	transcription factor	cartilage homeoprotein 1
	cp1	glia, macrophages, other	+	-	G	cysteine proteinase	enzyme	cathepsin L
	draper	glia, macrophages, other	+	-	G	EGF repeats, transmembrane	cell adhesion	MEGF10
	CG12910	glia	+	-	G	novel, calcium/lipid-binding	other/lipid metabolism	--
microarray target	*rac2*	glia, other	+	n.d.		rho small GTPase	cytoskeleton	GTP-binding protein rac1
	CG8054	glia, macrophages	+	-	G	substrate transporter	transporter	hypothetical protein FLJ14936
	CG3426	glia, other	+	n.d.		novel, chitin binding	other	--
	CG3702	glia, other	n.d.	n.d.		novel	other	cleft lip and palate associated protein 1
	CG6218	glia	+	-	G	n-acetylglucosamine kinase	enzyme	n-acetylglucosamine kinase
	CG11100	glia, other	+	-	G	novel	other	--
	Jhl-21	glia	+	n.d.		amino acid transporter	transporter	L-type amino acid transporter 2
	CG10668	glia, macrophages	+	-	S	novel, RNA-binding	nucleic acid binding	CDC2/CDC13 suppressor
	CG5822	glia	+	n.d.	G	novel, ankyrin repeat	other	hypothetical protein for BC031303
	argk	glia	+	n.d.	S	arginine kinase	enzyme	brain creatine kinase
	CG8776	glia, other	+	n.d.		CO monooxygenase	enzyme	duodenal cytochrome b
	CG11045	glia, macrophages, other	+	n.d.		nucleoside transporter	transporter	Equilibrative nucleoside transporter 1
	htl	glia, other	+	-	G	FGF receptor	signal transduction	fibroblast growth factor receptor 3
	CG4389	glia, other	n.d.	n.d.		enoyl-CoA hydratase	lipid metabolism	hydroxyacyl-coenzyme A dehydrogenase
Other[f]	*CG4322*	glia	+	-	G	G-protein coupled receptor	signal transduction	G protein-coupled receptor 84
	CG7117	glia	+	-	S, T	dehydrogenase/reductase	lipid metabolism	Locus BAC11591
	CG5453	glia	n.d.	-	S, T	novel	other	--
	CG6783	glia	+	-	S, T	fatty acid-binding protein	lipid metabolism	--
	DAT	glia, other	+	-	S	dopamine transporter	transporter	Na-dependent noradrenaline transporter
	CG9977	glia, other	+	-	S, T	adenosylhomocysteinase	other	adenosylhomocysteinase
	Mdr65	glia	+	-	S, T	ABC transporter	transporter	ATP-binding cassette, B4A
	CG2310	glia	+	-	S, T	novel	other	--
	CG11902	glia	+	-	G, T	C_2H_2 zinc finger domain	transcription factor	zinc finger protein 91
	GS2	glia, other	+	-	S, T	glutamine synthetase	other	glutamate-ammonia ligase
	EAAT1	glia	+	-	S, T	amino acid transporter	transporter	Excitatory amino acid transporter 4
	EAAT2	glia	+	-	S, T	amino acid transporter	transporter	glial high-affinity glutamate transporter
	nrg	glia, other	+	n.d.	G	immunoglobulin-fibronectin	cell adhesion	NrCAM protein
	nrv2	glia, other	+	-	G	Na/K-exchanging ATPase	transporter	Na+/K+ transporting ATPase, beta 1
	CG8965	glia, other	+	n.d.		RA domain	other	--

[a] Analysis was focused on CNS and macrophage expression, expression in other tissues is listed as "other". "glia", Gcm$^+$ lateral glia.
[b] "ectopic *gcm*," *yw*; *Sca-Gal4/UAS-gcm*; *UAS-gcm/+* embryos; "*gcm*$^-$," *gcm*$^{N7-4}$ mutant embryos (a *gcm* null allele); n.d., not determined; -, no detectable expression; +, upregulation of expression; n.c., no change.
[c] G, expression in all Gcm$^+$ glia; S, expression in a subset of glia (presumably requiring a spatially-restricted Gcm cofactor); T, temporally restricted expression (presumably requiring a temporally restricted Gcm cofactor).
[d] Yaun and Ganetzsky, 1999; Jones, 2001; Keleman and Dickson, 2001.
[e] Identified in separate computational Gcm binding site screen (M.R.F., E.J., and C.Q.D., unpublished data).
[f] Glial genes identified from Berkeley *Drosophila* Genome project databases, or a candidate gene approach.

lated directly or indirectly by Gcm in the CNS. We made Cy3-labeled RNA from wild-type embryos and Cy5-labeled RNA from embryos overexpressing *gcm* throughout the CNS and probed cDNA chips contained 9710 unique spots representing 4386 unique genes, which includes ~31% of all predicted *Drosophila* genes (Figure 3A). Each experiment was performed ten times from independent pools of embryos, and the observed changes in gene expression were highly reproducible between experiments (see Supplemental Data S2 at http://www.neuron.org/cgi/content/full/38/5/567/DC1 for microarray statistics).

As we would predict, a number of known *gcm* target genes are highly enriched by ectopic *gcm* expression in microarray assays. For example, the known *gcm* target gene *repo* shows a ~3-fold enrichment, and a number of our computationally identified genes including *draper*, *CG12920*, *Cp1*, and *rac2* were also in the top 5% of genes upregulated by ectopic *gcm* (Figure 3A). These data indicate that our microarray experiments enriched for many known glial genes. We note, however, that a number of other known glial genes including *loco*, *ttk*, *pnt*, and many of our computationally identified glial genes were not dramatically enriched on microarrays (Figure 3A; see Discussion). To identify additional glial genes, we analyzed the embryonic expression patterns of 153 genes that were among the most highly upregulated in microarray experiments. This collection included all genes for which there was an available cDNA, regardless of predicted function or known protein motifs. We found 18 genes (12%) expressed in embryonic glial lineages; all genes tested were upregulated in response to ectopic *gcm* in the CNS and not expressed in the CNS of *gcm* mutant embryos (Figure 3B; Table 1).

To investigate whether these Gcm-regulated genes are likely to be direct or indirect Gcm targets, we analyzed the 10 kb of DNA flanking each gene for Gcm DNA binding sites using our interactive web search algorithm (http://flycompute.uoregon.edu/cgi-bin/seqseek.pl). Interestingly, we find that three genes have Gcm binding site clusters meeting our original search criteria; however, these were not annotated in the original EMBL

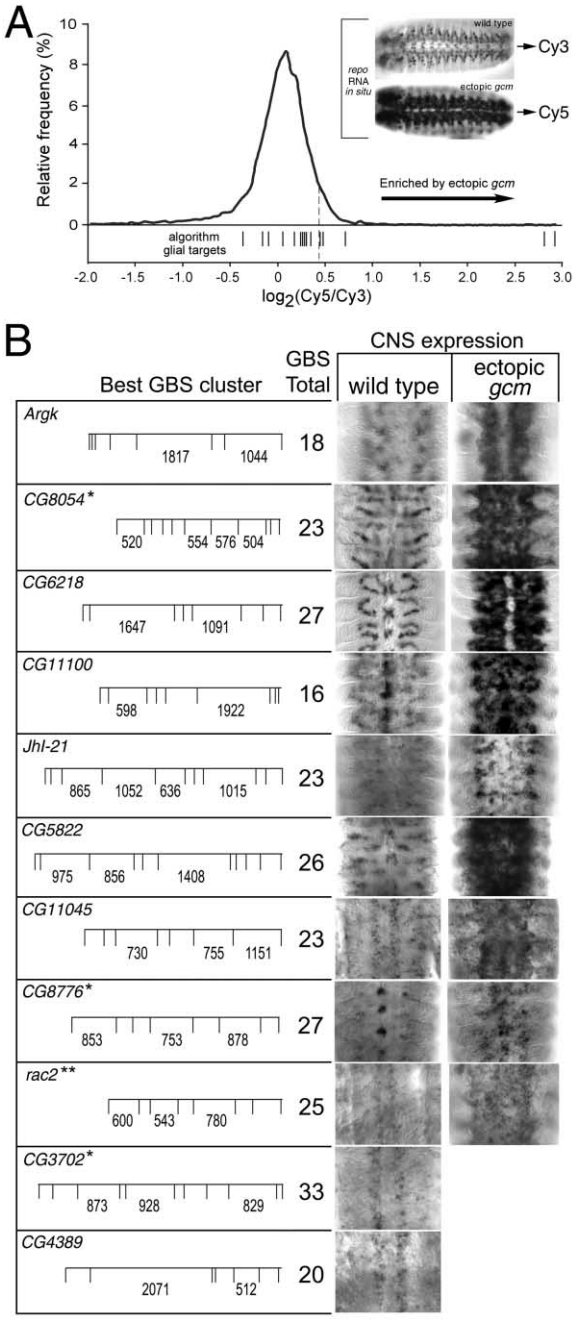

Figure 3. Microarray Identification of Gcm-Regulated Genes in the CNS

(A) mRNA was isolated from staged wild-type embryos and embryos with ectopic *gcm* in the CNS (*Sca-Gal4/UAS-gcm ; UAS-gcm/+*), processed for Cy3 or Cy5 fluorescent labeling, and used to probe DNA microarrays. Distribution of fluorescence intensities in DNA microarray experiments shown on a $\log_2$ scale; 0 indicates equal mRNA levels in wild-type and ectopic *gcm* embryos, whereas genes upregulated by ectopic *gcm* fall to the right (positive numbers). Bin size is 0.01. Genes identified computationally are shown below the *x* axis (from right to left): *CG12910*, *repo*, *drpr*, *Cp1*, *rac2*, *loco*, *dri*, *GH03502*, *sap-r*, *pnt*, *lac*, *akap200*, *Aly*, *nrv1*, *dll*.

(B) Glial expression within three segments of the embryonic CNS using RNA in situ hybridization to wild-type embryos (*y w*) and ectopic *gcm* embryos in the CNS (*Sca-Gal4/UAS-gcm ; UAS-gcm/+*). Ventral view; anterior, up; genes and embryonic stages as indicated. GBS total, the number of Gcm binding sites within 10 kb of each gene. Asterisk, genes with Gcm binding site clusters meeting our original search criteria (these were not annotated in the EMBL2000 data set we searched); double asterisk, *rac2* was identified by both computational and microarray methods.

2000 data set and were therefore missed. A number of other genes also have a high number of Gcm binding sites in their regulatory regions (Figure 3B and data not shown), suggesting that a number of microarray-identified genes may be direct Gcm targets. A bias toward direct targets may be due to the relatively short time interval between Gcm induction and harvesting embryonic mRNA in these experiments (see Experimental Procedures).

Exploiting *Drosophila* Public Databases to Identify Glial Genes

Due to the excellent progress of the Berkeley *Drosophila* Genome Project, there are currently several publicly available expression and sequence annotation databases. To identify additional *Drosophila* glial genes, we searched these databases by two methods. First, we examined two *Drosophila* gene expression databases that provide images of mRNA expression patterns in staged whole-mount embryos: the newly released BDGP Imago database (http://www.fruitfly.org/cgi-bin/ex/insitu.pl) and a previously published database (Kopczynski et al., 1998). Second, we identified *Drosophila* sequence homologs of vertebrate glial genes involved in glutamate neurotransmitter metabolism (a well-described role for vertebrate glia). We obtained cDNAs for each candidate glial gene (n = 24) and assayed expression by RNA in situ hybridization to wild-type embryos and embryos with panneural *gcm*. We found an additional 15 genes expressed glia, many of which show highly specific expression in various subsets of embryonic CNS glia. All genes tested are positively regulated by *gcm* and not expressed in the CNS of *gcm* mutant embryos (Figure 4; Table 1).

Together our computational, microarray, and database searching approaches have yielded 45 CNS glial genes, 39 of which are newly identified. Almost 50% of these fall into one of three classes of predicted protein products: transporter molecules (20%), transcription factors (13%), or proteins involved in signal transduction (13%); this is a significant enrichment over their representation in the *Drosophila* genome (see Discussion). Based on their expression patterns and predicted functions, these genes are excellent candidates to regulate both early aspects of glial development (specification, migration, glia-neuron interactions) and later aspects of glial function (synaptic buffering, axon physiology, and immune function). In the following section, we use this collection of glial genes as a tool to investigate the mechanisms by which *gcm* acts to generate glial diversity in the *Drosophila* embryonic CNS. In subsequent sections we verify the functional significance of this collection of glial genes by performing loss-of-function and gain-of-function analyses on one predicted glial development gene (*unc-5*) and one predicted glial function gene (*draper*).

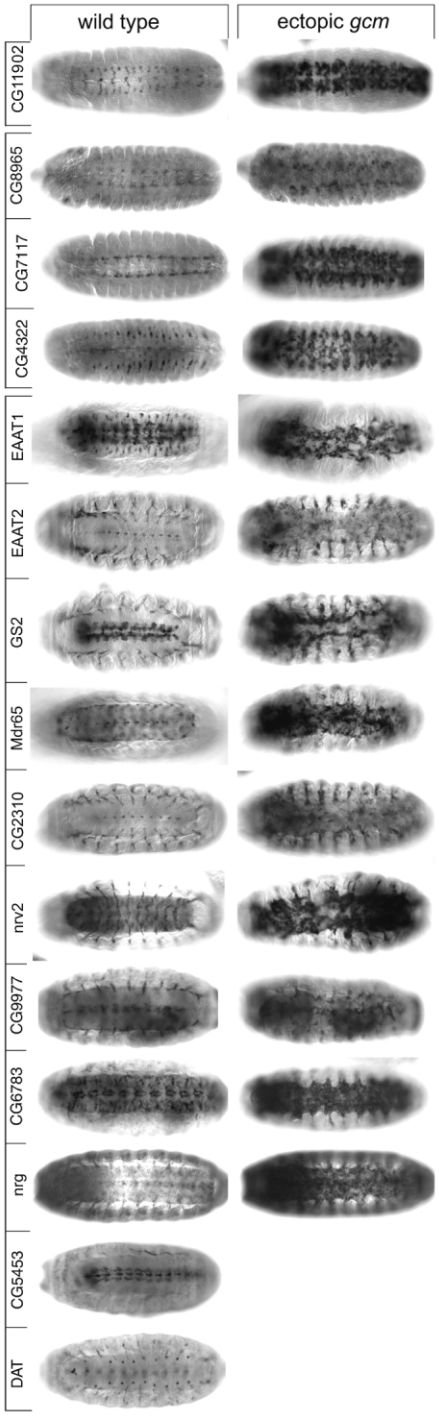

Figure 4. Gcm-Regulated Glial Genes Identified in Public Databases

Glial expression within the embryonic CNS using RNA in situ hybridization to wild-type embryos (*y w*) and embryos with ectopic *gcm* in the CNS (*Sca-Gal4/UAS-gcm ; UAS-gcm/+*). Ventral view; anterior, left; genes as indicated.

Regionalization of *Drosophila* Glia: Gcm Requires Spatially and Temporally Restricted Cofactors to Activate Target Genes

It is well established that *gcm* is required for nearly all embryonic glial development (Hosoya et al., 1995; Jones et al., 1995), but it is not known whether Gcm activates the same target genes in all glial lineages, or if Gcm acts with different cofactors to activate distinct target genes in each glial lineage, thereby generating glial subtype diversity. To address this issue, we analyzed the spatio-temporal expression pattern of our glial genes in detail, first in wild-type embryos (Figure 5) and then in embryos with ectopic Gcm throughout the CNS (Figures 5 and 6). We found that glial genes could be clustered into groups that share distinct spatial or temporal patterns. Temporal profiles included genes expressed throughout gliogenesis and genes that are first expressed early, midway, or late in gliogenesis (Figure 5A); spatial profiles include all glia, longitudinal glia only, peripheral glia only, CNS glia only, and other characteristic subsets of glia (Figure 5B).

Interestingly, each spatial and temporal group of Gcm target genes showed a different response to ectopic Gcm expression. Genes expressed in all CNS glial lineages could be induced throughout the entire CNS by Gcm overexpression (Figure 6A); thus, Gcm is sufficient to activate their expression in the CNS. In contrast, genes expressed in subsets of glia showed Gcm-induced upregulation primarily in the same regions of the CNS (Figures 6B–6D); therefore, Gcm requires a spatially restricted cofactor to induce these target genes. These spatial domains appear to be lineage specific (Figure 6B) or regionally restricted along the dorsoventral axis (Figures 6C and 6D). Genes expressed only late in glial development showed Gcm-dependent upregulation only late in development (Figure 6E); therefore, Gcm requires a temporal cofactor to activate expression of these genes. We found no gene that could be induced by Gcm prior to its normal time of expression. We conclude that most identified Gcm target genes require both Gcm and a spatially or temporally restricted cofactor to be activated. This likely contributes to the generation of glial subtype diversity and adjusts the timing of gene expression for proper glial function.

Glial Development: *unc-5* Orients Glial Migration

There are three types of glia in the *Drosophila* embryonic CNS based on migration patterns: those migrating toward the midline, those migrating away from the midline, and those that remain close to their original birth positions (Bossing et al., 1996; Schmidt et al., 1997). The signaling pathways that regulate these stereotyped glial migrations are poorly understood. We identified the *unc-5* gene as a Gcm-regulated glial gene that it is only expressed in glia migrating away from the midline, and a subset of neurons (Figure 7A; Keleman and Dickson, 2001). *unc-5* encodes a repulsive netrin receptor; netrins are secreted from CNS midline cells and can either attract neurite growth via the Deleted in Colorectal Cancer (DCC) receptor or repel neurite outgrowth via the Unc-5 receptor (Colavita and Culotti, 1998; Hamelin et al., 1993; Hedgecock et al., 1990; Hong et al., 1999; Stein and Tessier-Lavigne, 2001; Stein et al., 2001). To determine if Unc-5 can regulate glial migration, we ectopically expressed Unc-5 in all Gcm^+ glia using a *gcm-GAL4* driver and found that Unc-5 is indeed sufficient to repel glia away from the CNS midline (Figures 7B and 7C). Misexpression of this repulsive receptor was sufficiently

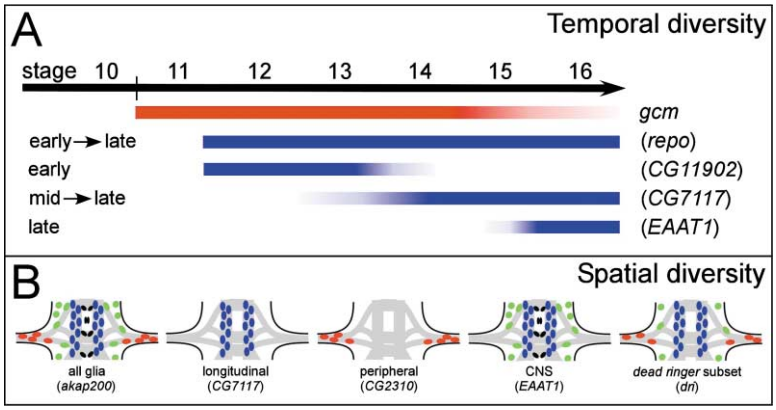

Figure 5. Clustering of Glial Genes Based on Spatio-Temporal Expression Profiles

(A) Summary of glial gene temporal expression patterns. Embryonic stages of development are shown at top. Gcm is activated first in all glial lineages and fades by stage 16 (red). Distinct temporal profiles are observed for subsets of glial genes (blue; one representative gene indicated).

(B) Summary of glial gene spatial expression patterns. Glia in one CNS segment are shown with color-coded subtypes (for simplicity not all glia of each subtype are show). Each glial subtype coexpressed a distinct group of glial genes; one representative from each group is indicated.

strong to block nearly all longitudinal glia from reaching axons tracts to which they normally are strongly attracted and tightly adhere. We conclude that most glia can respond to activation of the Unc-5 signaling pathway and that nonmigratory glia and glia that migrate toward the midline must keep *unc-5* expression off or low. Conversely, the laterally migrating glia are likely to require *unc-5* expression to induce their migration away from the midline.

Glial Function: *draper* Is Required for Glial Immune Function and Larval Behavior

A subset of our panglial genes are likely to play a role in glial function, rather than early developmental events, due to their broad and later expression patterns. We characterized the expression and function of one of these novel genes, *CG2086* (which we have named *draper*; *drpr*), which is expressed in all Gcm^+ glia and macrophages (Figure 2). We obtained and fully sequenced seven cDNAs representing transcripts from the *draper* locus. Each is predicted to encode one of three different splice variants of an EGF-repeat single-pass transmembrane domain receptor molecule (Figure 8B). BLAST homology searches reveal that *draper* appears to be the sequence homolog of the *C. elegans* cell corpse engulfment gene *ced-1* (Zhou et al., 2001). *draper* also shares strong homology with the mouse *jedi-1* gene (Carninci et al., 1996) and the human *MEGF10* and *MEGF11* genes (Nagase et al., 2001). In *C. elegans*, Ced-1 is required for the engulfment of apoptotic cell corpses, though this pathway has not been described in other organisms. Both mammalian and *Drosophila* glia are responsible for removing apoptotic neuronal cell corpses from the CNS; however, the molecular pathways involved have not been identified. Based on the requirement for *ced-1* in cell corpse engulfment in *C. elegans*, we tested whether *draper* performed a similar role in the *Drosophila* CNS glia.

We generated an antibody to the Draper intracellular domain, which is predicted to recognize all known protein isoforms. We detect Draper protein on the plasma membrane of all glia, including glial membranes ensheathing axon tracts, and outside the CNS on the membranes of macrophages (Figures 8C, 8D, 8F, and 8G). All Drpr immunoreactivity is absent in $drpr^{\Delta 5}$ mutant embryos. We next obtained or generated several *draper* mutations (see Experimental Procedures): a predicted null allele ($drpr^{\Delta 5}$) that is embryonic lethal and two hypomorphic alleles ($drpr^{EP(3)522}$ and $drpr^{\Delta 19}$) that are larval lethal (Figure 8B). We found that embryos homozygous for the $drpr^{\Delta 5}$ null allele showed no defects in early CNS development (e.g., glial and neuronal specification, migration, proliferation), but did show clear defects in CNS cell corpse engulfment. Wild-type embryos had 25.9 $\pm$ 0.8 cell corpses per hemisegment, while $drpr^{\Delta 19}$ had 31.0 $\pm$ 0.9, and $drpr^{\Delta 5}$ had 43.6 $\pm$ 1.7 (Figure 8H). Consistent with this phenotype, we found strong Draper immunoreactivity on vesicles within glia that contain neuronal cell corpses (Figure 8G). Together, our data indicate that *draper* is a downstream target of Gcm in glia and macrophages, it encodes a Ced-1-like transmembrane domain receptor expressed on glial and macrophage membranes, and it is required for cell corpse removal in the CNS.

Draper protein is detected at high levels on glial membranes that ensheath motor nerves, so we investigated its role in motor neuron function. Flies homozygous for hypomorphic *draper* alleles died as first instar larvae, which allowed us to assay the role of *draper* in larval locomotion. We found that $drpr^{EP(3)522}$ and $drpr^{\Delta 19}$ larvae were profoundly uncoordinated as judged by quantitative assays (Figure 8E) or by time lapse recordings (see Supplemental Data Set S2 at http://www.neuron.org/cgi/content/full/38/5/567/DC1). This phenotype is characteristic of a failure in glial ensheathment of neurons (Auld et al., 1995; Baumgartner et al., 1996; Granderath et al., 1999) and suggests that Draper may be required for maintaining proper motor axonal physiology.

Discussion

We have used multiple genomic approaches to identify 45 *Drosophila* glial genes, and nearly all have been verified as Gcm regulated in vivo. The genes can be clustered into groups that show a common expression pattern in specific glial subtypes or at specific times during gliogenesis; each group has a unique response to ectopic Gcm, revealing the existence of spatially and temporally restricted Gcm cofactors. Genes expressed early or in subsets of glia are excellent candidates for regulating glial development, whereas genes expressed late in glia are likely to modulate glial function. We show that

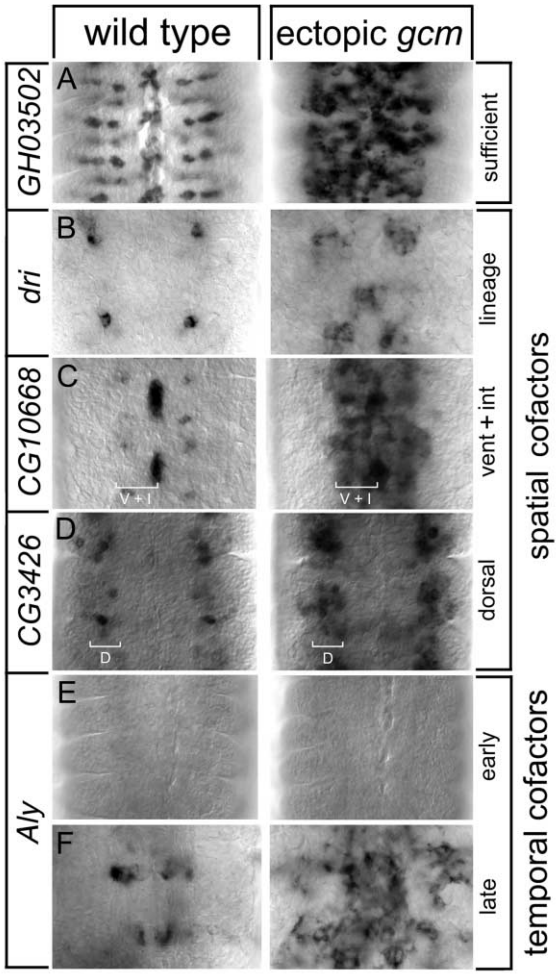

Figure 6. Spatial and Temporal Cofactors Modulate Gcm-Dependent Gene Expression

Glial expression within 2-3 segments of the embryonic CNS using RNA in situ hybridization to wild-type embryos and embryos with ectopic *gcm* in the CNS (*Sca-Gal4/UAS-gcm ; UAS-gcm/+*). V + I and D, ventral and intermediate, and dorsal CNS columns, respectively. Ventral view; anterior, up; all embryos are shown at stage 11–13, except (F), which is at stage 16.

(A) Gcm is sufficient to induce *GH03502* throughout the CNS.

(B) Gcm requires a lineage-specific cofactor restricted to induce *dri* expression.

(C and D) Gcm requires spatially restricted cofactors to induce gene expression of *CG10668* and *CG3426* within the ventral and intermediate, or dorsal column of the CNS (white brackets), respectively.

(E and F) Gcm can only induce *Aly* expression late in embryogenesis, revealing a requirement for a temporally restricted cofactor.

one candidate glial development gene is important for orienting glial migration, and one putative glial function gene is required for glial immune function and larval locomotion. Over 80% of our newly identified *Drosophila* glial genes have clear mammalian orthologs (Table 1), which are now excellent candidates for regulating mammalian glial development and function.

Computational Identification of Transcription Factor Target Genes

Our computational approach proved to be an effective means for identifying Gcm-regulated genes in both glia and macrophages. All but one of the known Gcm targets were identified, and ~10% of the tested candidate genes showed Gcm-regulated expression in glia or macrophages. These genes are excellent candidates for being directly regulated by Gcm in vivo (as are the Gcm targets identified by microarray with a high density of Gcm binding sites in their regulatory regions), due to the high number of Gcm binding sites near each gene and their Gcm-dependent expression, but this can only be confirmed definitively by Gcm chromatin immunoprecipitations. What about the other 90% of the candidate genes that have closely associated clusters of Gcm binding sites—are they Gcm targets or false positives? Many of these genes are expressed in neurons and could be negatively regulated by Gcm in glial lineages (Giesen et al., 1997); such regulation would be difficult to detect in our expression studies. Genes expressed outside CNS and macrophage lineages may be positively regulated by Gcm, such as the Gcm/Gcm2$^+$ body wall ligament cells (M.R.F. and C.Q.D., unpublished data; Kammerer and Giangrande, 2001); we have not yet tested most of these genes for Gcm-dependent expression. In addition, future studies of postembryonic Gcm-dependent development will likely reveal additional Gcm targets. Finally, the number of glial and macrophage genes identified from our full collection of 384 will increase as we assay the remaining ~180 candidate Gcm target genes for which ESTs are not currently available.

This collection of Gcm-regulated genes provides the opportunity to define the optimal binding site for Gcm or its cofactors by analyzing the genomic regions surrounding these genes. For example, do glial-specific Gcm targets share genomic sequence motifs that are distinct from macrophage-expressed Gcm targets? Do all late, dorsal, or panglial expressed genes share distinct genomic sequence motifs that might aid in the identification of relevant DNA binding cofactors? We are currently using our interactive web search algorithm (http://flycompute.uoregon.edu/cgi-bin/seqseek.pl) to perform these experiments (M.R.F., E.J., and C.Q.D., unpublished).

The computational method described here can be used immediately to investigate candidate target genes for any transcription factor, or combination of transcription factors, for which a DNA binding site is known. In the context of glial development, we have begun to search for genes coregulated by both Gcm and Pnt (a positive regulator of glial fate in *Drosophila*) by looking for genes enriched for both Gcm and Pnt DNA binding sites. We have already used this computational approach to identify genes coregulated by Dl, Smad (Dpp effector), and Pnt (EGF effector)—three signaling pathways that coordinately pattern the dorsoventral axis of the early embryo and neuroectoderm (T. von Ohlen, E.J., and C.Q.D.; unpublished results). The combination of multiple binding sites gives a greater degree of specificity than a single site alone: for example, clusters of Pnt and Gcm sites should enrich for glial genes, whereas Pnt and Dl sites should enrich for dorsoventral-restricted genes in the early embryo. This method is already quite powerful and will only become more useful as additional genomes become sequenced, which will help distin-

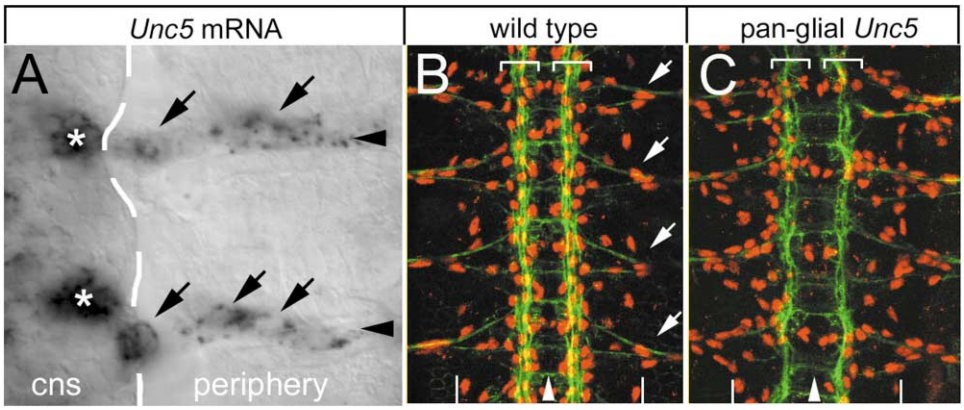

Figure 7. The Unc-5 Repulsive Netrin Receptor Regulates Glial Migration

(A) *unc-5* is expressed in laterally migrating glia. RNA in situ hybridization showing *unc-5*-expressing glia (arrows) that migrate to the lateral edge of the CNS and/or onto the peripheral nerves (arrowheads). Neuronal *unc-5* expression, asterisk. Stage 14 embryo; semilateral view; anterior, top; white dashed line, lateral edge of the CNS.

(B and C) *unc-5* is sufficient to induce lateral migration of glia. Repo nuclear protein marks glia (red); FasII membrane protein marks axons (green). Ventral view; arrowhead, ventral midline; white dash, lateral edge of CNS.

(B) Wild-type embryos have medially migrating longitudinal glia associated with axons of the connectives (brackets) and laterally migrating peripheral glia associated with the nerve roots (arrows).

(C) Embryos with panglial *unc-5* expression (*gcm-GAL4/+*, *UAS-unc-5/+*; *gcm-GAL4* does not drive expression in midline glia) show a block in the medial migration of longitudinal glia and an excess of glia migrating to the lateral edge of the CNS and onto the nerve roots.

guish conserved (functional) and "background" (nonfunctional) DNA binding site sequences.

Identification of *Drosophila* Glial Genes with cDNA Microarrays

We used cDNA microarrays to identify 153 genes that were highly upregulated following Gcm CNS overexpression and found 18 (12%) expressed in glia. In addition, Gcm represses neuronal fate (Giesen et al., 1997), so many direct targets should be highly "downregulated" in our microarray experiments; however, we have yet to investigate this class of genes. Although we clearly enriched for glial genes in these experiments, 88% of the upregulated genes appear to be false positives expressed outside the CNS or ubiquitously. In addition, many known glial genes—*pnt*, *loco*, and many genes identified in our computational analysis (Figure 2)—were false negatives in microarray experiments. This is not due to variability in the mRNA pools used to make probes, as we replicated each experiment ten times with a very high degree of reproducibility (see Supplemental Data S2 at http://www.neuron.org/cgi/content/full/38/5/567/DC1). Rather, we believe this is a problem with the complexity of whole-embryo tissue. False positives may be due to upregulation of target gene expression outside the CNS as an indirect effect of ectopic Gcm within the CNS. In contrast, many false negatives may be due to gene expression outside the CNS, which would mask increased expression within the CNS due to panneural Gcm.

Our results suggest that microarray data alone, without in vivo verification, should be interpreted with great caution. A recent microarray study, using similar genotypes to our microarray experiments, reported over 1500 Gcm-regulated genes (Egger et al., 2002). We observed an 88% false positive rate, and thus we predict a similar rate of false positives among these 1500 putative Gcm-regulated genes. The use of homogeneous target tissue (e.g., purified neurons versus purified glia) should substantially increase true positives and reduce false positives in future microarray experiments.

The Glial Gene Collection: Insights into *Drosophila* Glial Biology

This collection of glial genes provides insight into glial biology, even prior to functional analyses. The most prominent class of gene products identified is predicted molecular transporters. In fact, 20% of the genes in our collection are transporters, whereas transporters account for only 4% of the genes in the fly genome. Glial transporters are likely to regulate the extracellular environment around axons and synapses (e.g., pH, ionic, and neurotransmitter balance), a well-described role for vertebrate glia. For example, one of the best-studied functions of vertebrate glia is the removal of the neurotransmitter glutamate from the synaptic cleft. Vertebrate glia that ensheath synapses abundantly express excitatory amino acid transporters (EAATs) that have a high affinity for glutamate and rapidly remove it from the extracellular environment. Once glutamate has entered the cell, it is converted by *glutamine synthetase* (*GS*) into glutamine for subsequent transport back into neurons and reuse as a neurotransmitter (Figure 9). We identified the *Drosophila* EAATs and a GS sequence orthologs as *Drosophila* glial genes (Figure 9; Table 1); at late embryonic stages, these genes are activated very specifically and at high levels in CNS glia. Thus, the activation of EAATs and GS2 appears to be a late step in *Drosophila* glial differentiation, and *Drosophila* glia are likely responsible for clearing and recycling extracellular glutamate. This suggests there may be an unexpectedly high degree of conservation in the program of glial development between *Drosophila* and mammals, despite the

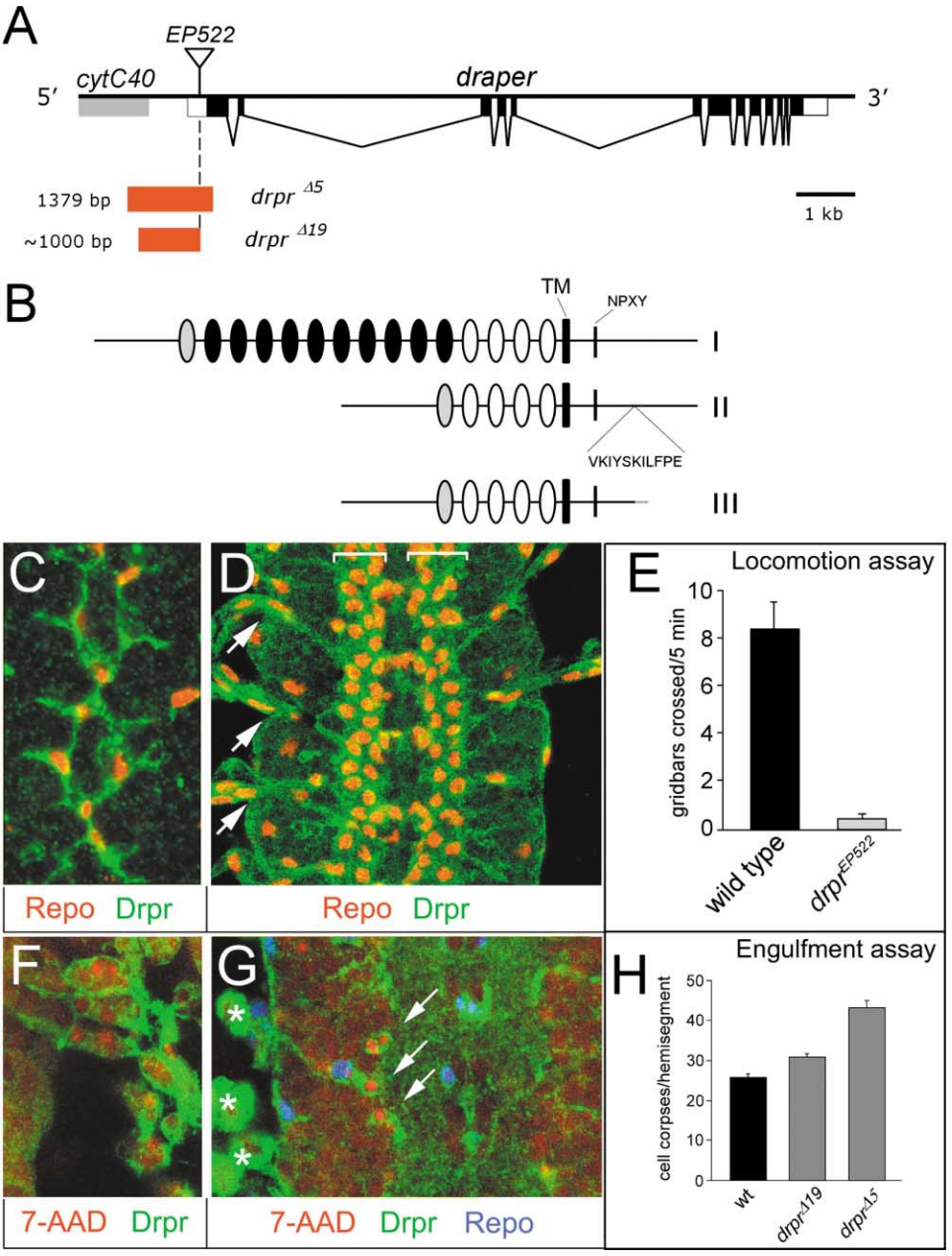

Figure 8. Draper Protein Is Detected on Glia Membranes and Is Required for Engulfing Apoptotic Neurons and Larval Locomotion

(A) The *draper* locus spans approximately 15 kb; transcribed region shown as bars (black, coding; white, noncoding) with the adjacent *cytC40* gene shown in gray. Genomic deletions in $drpr^{\Delta 5}$ and $drpr^{\Delta 19}$ are shown as red bars; the $drpr^{EP(3)522}$ P element insertion is shown as a white triangle.

(B) The three identified *draper* splice variants (I, II, III) are predicted to encode single-pass transmembrane domain receptors with a variable number of extracellular EGF repeats (ovals) and a novel intracellular domain. Splice variant II has an additional 11 amino acid intron in the intracellular domain; splice variant III is identical to II, except that it has an alternative 30 amino acid exon that includes a stop codon (gray).

(C and D) Draper protein is detected on glial membranes. Draper, green; Repo glial nuclear marker, red. Ventral view; anterior, up.

(C) Confocal image showing ventral glial cell bodies in a stage 14 embryo.

(D) Confocal image of a stage 16 embryo showing glial membranes ensheathing the neuropil of the CNS (brackets) and nerve roots (arrowheads). Stage 14 embryos; ventral view; anterior, up.

(E) *draper* mutants have defects in locomotion. $drpr^{EP(3)522}$ larvae show severely reduced motility compared to control ($drpr^{EP(3)522}/+$) larvae. n = 15 first instar larvae per genotype.

(F and G) Draper protein is detected on embryonic macrophages (F) and glia associated with apoptotic cell corpses (arrows, G). Draper, green; apoptotic cells labeled with 7-aminoactinomycin D (7-AAD), red. Macrophages were identified by Serpent staining (not shown) and morphology (F) and glia are identified by $Repo^{+}$ nuclei (blue, G). Asterisks, macrophages adjacent to the CNS.

(H) *draper* mutants have defects in clearing apoptotic neurons from the CNS. Number of $7\text{-}AAD^{+}$ apoptotic neurons per hemisegment in stage 16 embryos is given for the indicated genotypes. $n > 47$ hemisegments scored for each genotype.

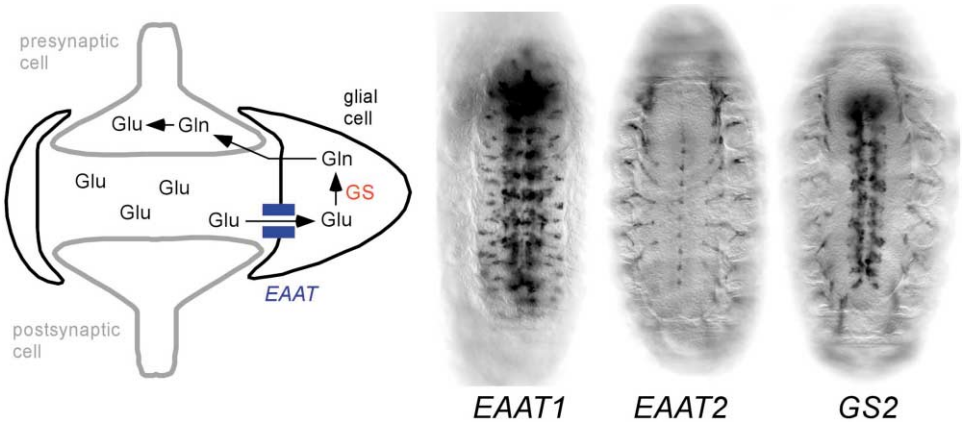

Figure 9. Conservation of Glutamate Neurotransmitter Metabolizing Gene Expression in *Drosophila* and Vertebrate Glia

The recycling of glutamate at synapses by glia (see text for details). Right, expression of *EAAT1*, *EAAT2*, and *GS2* in embryonic CNS glia. Ventral views; anterior, up.

apparent lack of conservation of Gcm function in mammalian glial cell fate induction (Kim et al., 1998).

Consistent with *gcm* acting at the top of a transcriptional hierarchy controlling glial and immune cell development, our collection is enriched for genes encoding transcription factors; ~13% in our collection versus 5% in the *Drosophila* genome. Future studies will be aimed at unraveling how these transcriptional regulators act to generate various subtypes of glial and immune cells. Another abundant class of glial genes are those encoding products predicted to regulate signal transduction; ~13% in our collection versus ~4% in the *Drosophila* genome. Molecules involved in cell-cell communication may play an essential role in glial migration or glial-axon interactions. For example, *CG4322* is predicted to encode a 7 transmembrane domain receptor that may function together with *loco*, which encodes a regulator of heterotrimeric G protein signaling that has been shown to be required for proper glial ensheathment of nerves (Granderath et al., 1999). One of the most exciting classes of *Drosophila* glial genes are those lacking known functional motifs but having strong human orthologs (Table 1). Genetic studies of these glial/macrophage genes is likely to provide novel insights into glial biology in both *Drosophila* and humans.

Generation of Glial Diversity Requires Complex Spatial and Temporal Regulation of Downstream Target Genes by Gcm

Gcm is expressed in all glial lineages throughout most of embryogenesis and is a potent glial inducer, but how does this single transcription factor activate the appropriate target genes for each glial subtype, or at the correct time of glial development? Here we identify at least three temporal phases of glial development distinguished by the expression of specific genes at either early, mid, or late glial developmental stages (Figure 5A); we also identify different glial subtypes that coexpress groups of glial genes (Figure 5B), with each group of genes having a distinctive, shared response to ectopic Gcm (Figure 6). Genes expressed in all glia, for which *gcm* appears to be sufficient to activate their expression, may encode molecules required for biological events associated with all glial/macrophage lineages (e.g., membrane morphogenesis). In contrast, *gcm* targets that are expressed in subsets of glia, for which Gcm requires spatially or temporally restricted cofactors to induce their expression, may have glial subtype-specific functions. For example, *CG7117* is expressed at high levels only in longitudinal glia, and thus *CG7117* may be required for enwrapping the longitudinal axon bundles or regulating cell-specific aspects of their physiology. Similarly, transcription factors such as Dri, Aly, and Dll are expressed in subsets of glia, and may act alone or together with Gcm to regulate glial subtype-specific gene expression. In the future it will be of great interest to identify the spatial and temporal Gcm cofactors and determine if they play an important role in patterning *Drosophila* or vertebrate glial lineages.

unc-5 Regulates Glial Migration

Glia exhibit dynamic migration patterns in the CNS that must be coordinated with neuronal development so that the appropriate neuron-glial and axon-glial interactions take place. Glia follow stereotyped migration routes in the *Drosophila* embryonic CNS: some migrate toward the CNS midline (e.g., the longitudinal glia that enwrap the axon connectives), some migrate away from the midline (e.g., the nerve root glia that ensheath axons entering and exiting the CNS), and others are nonmigratory. We chose one of our early expressed glial genes, *unc-5*, to analyze for a role in glial development, and we found that it regulates glial cell migration. Studies over the past decade have shown that netrins and their DCC/UNC-5 receptors play key roles in directing axon guidance; here we show that the repulsive netrin receptor Unc-5 regulates glial migration as well. *unc-5* is specifically expressed in glia that migrate away from the midline, and misexpression of *unc-5* in medially migrating glia blocks their medial migration. Thus, medially migrating glia must keep *unc-5* expression off to achieve their normal position, whereas laterally migrating glia may require *unc-5* expression for their lateral migration; identification and analysis of the *unc-5* mutant phenotype

will be necessary to confirm this prediction. Interestingly, a similar role for netrins in repelling oligodendrocyte precursors from optic nerve chiasma during vertebrate visual system development has been proposed (Sugimoto et al., 2001); thus, *Drosophila* and vertebrate glia appear to use similar molecular mechanisms to coordinate repulsive migratory events during glial development. It remains to be seen whether glia that migrate toward a netrin source, such as the medially migrating glia in the *Drosophila* embryonic CNS, utilize the Dcc/Frazzled attractive netrin receptor to respond positively to a netrin signal. Interestingly, these results illustrate how a single spatial cue, in this case the netrins, can coordinate the organization of multiple cell types within a single tissue for proper tissue assembly.

draper Regulates Engulfment of Apoptotic Neurons and Larval Locomotion

Both *Drosophila* glia and mammalian microglia are required for removal of apoptotic neurons in the CNS, but the molecular pathways mediating engulfment are poorly understood. Here we identify the Draper/CED-1 pathway in *Drosophila* and show that glia use this molecular pathway to accomplish cell corpse removal. In *C. elegans*, CED-1 is required for engulfment of apoptotic cell corpses derived from several tissues (Zhou et al., 2001). CED-1::GFP fusion proteins driven by the endogenous CED-1 promoter are expressed at high levels on the surface of many engulfing cells; CED-1::GFP appears to accumulate on the surfaces of cells facing apoptotic corpses, and in the absence of CED-1 function, cell corpses remain unengulfed (Zhou et al., 2001). Draper appears to be the CED-1 ortholog based on its structure, localization, and mutant phenotype. Similar to CED-1, Draper contains several extracellular atypical EGF repeats, a single transmembrane domain, and a novel intracellular domain. In the intracellular domain, we find a conserved NPXY motif that appears to be the domain mediating physical interactions between CED-1 and CED-6 (Su et al., 2002), the only known protein acting genetically downstream of CED-1. Draper is detected at high levels on glial membranes and on vesicles within glia that contain apoptotic neuronal cell corpses. In the absence of Draper function, we find an increase in the number of cell corpses present in the CNS, indicating that Draper is essential in glia for removal neuronal cell corpses. We find that *Drosophila* macrophages also strongly express Draper, suggesting that immune cells in higher organisms may also use the Drpr/Ced-1 pathway for more general cell corpse removal. It will be interesting to determine whether CED-6 or *Drosophila* orthologs of the other components of the cell corpse engulfment machinery (e.g., Conradt, 2001) are also involved in glial- or macrophage-dependent cell corpse removal in *Drosophila*.

draper mutant larvae show an uncoordinated phenotype highly similar to known axon ensheathment mutants (Auld et al., 1995; Baumgartner et al., 1996; Granderath et al., 1999), suggesting second potential role for the Draper in neuronal ensheathment. The cellular mechanisms required for cell corpse engulfment and neuronal ensheathment are strikingly similar: recognition of the target cell and extension of membranes to engulf/ensheath the target cell. There are important differences, however, because cell corpse engulfment leads to destruction of the target cell, whereas axon ensheathment leads to survival and nourishment of the target cell. In the future, it will be interesting to test the hypothesis that Draper mediates both cell corpse engulfment and axon ensheathment.

Experimental Procedures

Drosophila Stocks, Generation of *draper* Alleles, and Larval Behavior

Drosophila strains used in this study were $gcm^{N7\text{-}4}$ (Vincent et al., 1996), $gcm^{\Delta P1}$ (Jones et al., 1995), *Df(2L)132* (Lane and Kalderon, 1993), *yw; UAS-gcm; UAS-gcm* (Jones et al., 1995), *UAS-unc-5* (Keleman and Dickson, 2001), *scabrous-gal4*, and *gcm-gal4* (a gift from U. Tepass). Animals from a *y w* genetic background were used as wild-type controls. We obtained *EP(3)522*, an insertion in the 5′ untranslated region of the *draper* gene, from the BDGP collection of P element insertions. The current BDGP annotation lists a second gene, *CG18172*, within *draper*, but our EST sequence data indicates that these are in fact a single gene that we will henceforth refer to as *draper*. Lethal excision lines were generated using the Δ2-3 transposase and screened for loss/reduction of protein product with an anti-Draper antibody (see below); molecular lesions were defined by PCR amplification and sequencing of mutant loci. First instar larval crawling was assayed on 1% agarose plates, using 2.5 mm square grids on a fluorescent light table as previously described (Naimi et al., 2001).

Computational Analyses

Drosophila chromosome assembly Version 2.9 from BDGP was fragmented into 10 kb overlapping pieces (effectively imposing the constraint that Gcm binding site should be within 10 kb of coding sequences). A Perl script was written to filter the 10 kb fragments for those that contained clusters of Gcm binding sites (see Results). 10 kb fragments with Gcm clusters were BLASTed against known *Drosophila* coding sequences from EMBL database (July, 2000). Additional Perl scripts were written to collate the results and generate an output of Gcm binding site positions and positions of coding regions.

cDNA Microarrays

Expression analyses were performed using spotted microarrays constructed from *Drosophila* Gene Collection release 1 (Rubin et al., 2000) and 430 additional cDNA and genomic sequences (see Supplemental Data Set S1 at http://www.neuron.org/cgi/content/full/38/5/567/DC1 for cDNAs). Target label preparation and hybridization protocols are described elsewhere (Fazzio et al., 2001). Briefly, cDNA targets were generated using a standard amino-allyl labeling protocol, where 30 μg each of each "wild-type" and "ectopic *gcm*" total RNAs collected from 5- to 9-hour-old embryos were coupled to either Cy3 or Cy5 fluorophores. Embryos were hand-staged to verify that they were at stage 12-13, the period of maximum gliogenesis. Targets were cohybridized to microarrays for 16 hr at 63°C and sequentially washed at room temperature in 1× SSC and 0.03% SDS for 2 min, 1× SSC for 2 min, 0.2× SSC with agitation for 20 min, and 0.05× SSC with agitation for 10 min. Arrays were immediately centrifuged until dry and scanned using a GenePix 4000 scanner (Axon Instruments, Union City, CA). Image analysis was performed using GenePix Pro 3.0. Normalization and microarray statistical analysis was performed as described elsewhere (Furlong et al., 2001).

Immunohistochemistry and Confocal Microscopy

Standard methods were used for collection, fixation, and immunohistochemistry of *Drosophila* embryos (Spana et al., 1995). cDNAs were purchased from Research Genetics (Huntsville, AL). Digoxigenin-labeled RNA probes were generated according to the manufacturer's instructions (Roche, Indianapolis, IN). RNA in situ hybridization to embryos was carried our as described previously (Broadus et al., 1998). For most genes, we simultaneously assayed control

(*y w*) and ectopic *gcm* (*y w; sca-gal4/UAS-gcm; UAS-gcm/+*) animals. After staining, embryos were mounted in glycerol, viewed on a Zeiss Axioplan microscope, and imaged with a Spot Insight digital camera.

To prepare Draper antisera, a cDNA fragment corresponding to amino acids 849–1016 (from splice form I, derived from the Berkeley *Drosophila* Genome Project cDNA *GH16585*) was subcloned into the *pET28a* vector. Amino acids 849–946 are common to the intracellular domain of all predicted Draper protein variants, and therefore polyclonal antisera raised to this polypeptide is expected to recognize all identified Draper protein isoforms. Fusion protein expression was induced in bacteria by 1 mM IPTG and purified over a nickel column according to the manufacturer's instructions (BioRad, Hercules, CA) under denaturing conditions. Purified proteins were dialyzed into 1× PBS, conjugated to KLH, and used to immunize rabbits.

Primary antibodies were used at the following dilutions: rat anti-Repo, 1:2000; mouse anti-FasII, 1:100; rabbit anti-Drpr, 1:500; and rabbit anti-Dll, 1:100. Secondary antibodies used for immunofluorescence conjugated to Alexa Green, Cy3, or Cy5 (Molecular Probes, Eugene, OR) were used at 1:400. Embryos were mounted in antifade reagent (BioRad, Hercules, CA) and viewed on a BioRad Radiance confocal microscope. Apoptotic cell corpses were identified by staining fixed embryos with 7-aminoactinomycin D as described elsewhere (Franc et al., 1999). The number of cell corpses per hemisegment in stage 16 embryos were scored in double-blind experiments, counting in 3 μm steps from the dorsal to ventral surface of the embryonic CNS.

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