GENOMIC APPROACHES TO IDENTIFYING TRANSCRIPTIONAL
TARGETS OF AP-1, CREB AND JNK SIGNALING IN THE NERVOUS
SYSTEM OF DROSOPHILA MELANOGASTER

by

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ABSTRACT

Although a few regulators of memory and addiction have been identified, the biochemical pathways that mediate the development of addiction and memory remain poorly understood. In addition, important questions remain as to how these two phenomena can persist for so long, sometimes for the entire life of an individual.

Signaling molecules and transcription factors are activated in response to stimuli that induce long-term neuronal plastic changes. The transcription factor CREB (cAMP-responsive element binding protein) is clearly involved in triggering processes of addiction and memory, but its sustained activation following a course of chronic drug exposure (or learning) returns to baseline within days [1]. Even the enduring increased levels of ΔFosB (a Fos family transcription factor that couples with other proteins in the AP-1 family to form transcriptional activator/repressor complexes) observed in regions of the mammalian brain following chronic drug exposure, persists for only weeks or months. Thus, although CREB and ΔFosB probably initiate the very stable behavioral changes seen with addiction and memory, their alterations cannot mediate those behavioral changes per se [1]. Long-term up- or down-regulation of molecules downstream of these transcription factors, or others, must be responsible for the enduring modifications in synaptic connectivity and structure believed to be required for the maintenance of these durable behavioral states [2].

Many believe that more rapid progress will be made toward understanding the molecular basis of addiction if research efforts proceed hand-in-hand with, rather than in
isolation from, the overlapping neurobiological study of learning and memory [1, 2]. The importance and utility of using simple model systems such as *Drosophila* and *Aplysia* to identify and characterize genes involved in long-term synaptic plasticity, and hence memory formation, is well documented [3-5]. Identification and functional analyses of neuronal genes transcriptionally regulated by AP-1 and CREB in *Drosophila* would elaborate on molecular mechanisms of long-term plasticity and hence help us understand, and perhaps manipulate, processes that underlie addiction and memory.
Overview and Significance

Behavioral addiction and memory derive from synaptic plasticity

Stable changes occur in the brain following chronic use of drugs of abuse and experiences that lead to memory formation. Both sets of changes appear to involve similar molecular and cellular adaptations. Learning and memory and drug addiction are modulated by the same neurotrophic factors (e.g. BDNF [6, 7]) and involve the regulation of the same excitatory neurotransmitter receptor molecules and transcription factors (e.g. the GluR2 AMPA [α-amino-3-hydroxy-5-methyl-4-isoxazole] glutamate receptor subunit and CREB, respectively) [8, 9]. Consistent with similar underlying mechanisms, addiction and memory are also associated with similar morphological changes in synaptic structure such as differences in the number of dendritic spines in the regions of the brain they affect [10, 11]. Synaptic plasticity, the ability of neurons to modify the strength and number of their synapses, is thought to mitigate both of these long-lasting processes.

Two of the most extensively studied cellular models of activity-dependent synaptic plasticity (and long-term memory) are long-term potentiation (LTP) and long-term depression (LTD) in the mammalian hippocampus. Both phenomena involve AMPA or NMDA (N-methyl-D-Aspartate) glutamate receptor-mediated alterations in certain intracellular signaling pathways and subsequent differences in the post-
translational modification, activity, and trafficking of glutamate receptor subtypes. These modifications cause changes in synaptic efficacy in affected neurons. Cocaine and other drugs of abuse cause LTP- and LTD-like changes at glutamatergic synapses in the ventral tegmental area (VTA) and the nucleus accumbens (NAc), likely involving regulation of glutamate receptors [12, 13]. Memory and addiction are also modulated by activation of the transcription factor, CREB, and one of its downstream targets BDNF (brain-derived neurotrophic factor), the most prevalent neurotrophin in the brain [6, 7, 14]. Thus, with many commonalities between the mechanisms underlying both processes, it is likely that determining basic mechanisms of synaptic plasticity will contribute to our understanding of both memory and addiction, as well as other forms of behavioral change.

Current knowledge and outstanding questions in synaptic plasticity

The establishment of long-term plasticity requires *de novo* gene expression and protein synthesis. Short-term plastic changes, on the other hand, do not require the production of new proteins and are thought to involve only post-translational modifications (e.g. phosphorylation) of existing proteins such as ion channels within neurons. Conserved processes that underlie long-term plasticity can be blocked in a variety of model systems by the application of protein synthesis inhibitors or the manipulation of critical transcription factors [4].

Various intracellular signaling cascades have been identified that are critical for mechanisms of plasticity. In outline, synaptic activity results in membrane depolarization as well as local signaling via second messengers including \( \text{Ca}^{2+} \) and cAMP. When synaptic signaling achieves specific qualitative and quantitative features, kinases
activated by synaptic signals begin to influence gene expression. Nuclear gene expression influences growth and strength of originally active synapses [14]. Kinases that link synaptic activity to transcription factors include the cAMP dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinases (CaMKs) and the mitogen-activated protein kinases (MAPKs) – extracellular signal-regulated kinase (ERK) and Jun N-terminal Kinase (JNK) [15, 16]. These activated kinases translocate to the nucleus where they control the phosphorylation of several transcription factors, including, most importantly, the transcription factor CREB, which is believed to drive the expression of downstream genes responsible for synaptic change [14].

The activity of CREB is regulated by phosphorylation and is induced in neurons by a variety of extracellular signals including neurotrophins, neuromodulators and neurotransmitters leading to the transcription of CREB targets [5, 17, 18]. Thus, various external stimuli that activate diverse signaling pathways appear to converge on CREB to produce plasticity at the level of altered gene expression [1]. CREB’s control over plasticity is reflected in its critical role in behavioral memory in diverse organisms, creating another important link between synaptic plasticity and learning and memory. CREB’s function in the establishment of long-term memory was first demonstrated in an Aplysia sensory: motor-neuron co-culture system. The injection of oligonucleotides that contained consensus CREB binding sites, but not mutant sites or binding sites for other transcription factors, into the nucleus of presynaptic cells blocked long-term facilitation (LTF), a simple form of non-associative memory, without affecting short-term facilitation [19]. Also, CREB reporter genes showed induction following treatments that induce
LTF, and activation of CREB (by inhibition of an repressor isoform) reduced the threshold of synaptic activity required for LTF formation [20, 21]. Similar effects of manipulating CREB activity on synaptic and behavioral plasticity were also simultaneously or subsequently demonstrated in flies [22-24] and in mammals [25, 26].

The mechanism of CREB action has been extensively studied. Activated CREB binds as a dimer to the *cis*-regulatory element, CRE (cAMP response element), in the promoters of target genes where it recruits transcriptional co-activators to drive their expression. Differential CREB-dependent gene expression from CRE-containing promoters is achieved by varying the sequence composition and position of CREs within the promoters of target genes [27]. Approximately 100 genes that have functional CREs have been identified including many that control growth and a majority that function in cellular metabolism. Quite a few function in the nervous system and include neurotransmitters, signaling molecules and even the transcription factors *junD*, *c-fos*, and CREB itself. In addition, recent reports have identified many potential CREB targets in rodents and humans using *in vitro*, *in silico*, and in one case *in vivo*, genomic approaches similar to the ones described in this thesis [28-32].

One such CREB target is particularly intriguing because of its role in addiction. That protein, dynorphin, is a naturally occurring opiate in the brain that binds κ opiate receptors and is associated with an aversive dysphoric syndrome in humans and rats [33]. Dynorphin protein levels are increased in a CREB-dependent manner in response to psychostimulants, and *prodynorphin* (the gene from which the dynorphin protein is translated) mRNA levels are increased in response to repeated exposure to cocaine and in
post-mortem brain tissues from cocaine abusers. *prodynorphin’s* expression is regulated by CREB and requires three non-canonical CREs in the gene’s promoter sequence.

Many “immediate early genes” (IEGs) are induced in response to diverse stimuli in the brain, including electrical stimulation, physiological perturbations, stress, psychotropic drugs and learning [34, 35]. It is likely that these IEGs are involved in synapse-specific plasticity and memory formation, although their precise roles in these processes haven’t been resolved [1]. c-Fos and JunD and other AP-1 members are in this class of IEGs and require CREB activation for their expression following stimulation [36]. c-Fos and JunD are induced rapidly, yet transiently, in response to different forms of activity-inducing stimuli in certain brain regions and are thought to mediate short-lived alterations in downstream gene expression in those areas [34]. However, a splice variant of the *fosB* gene, ΔFosB, has recently been identified that shows increased levels following chronic perturbations of the brain, including repeated drug use and electroconvulsive seizures (ECS), that persist for a much longer time period than other IEGs [37]. ΔFosB activity is an exciting prospect in the field of addiction research because it was shown to control sensitivity to cocaine and natural rewarding behaviors in a mouse model and because its increased levels last long enough to possibly cause more persistent alterations in gene expression than other IEGs [8, 38].

Even still, the protein’s activity doesn’t persist nearly as long as the behavioral adaptations it seems to regulate. It is hypothesized that ΔFosB must initiate changes in gene regulation that last far beyond its initial activity. Several of ΔFosB’s downstream targets have been identified by Eric Nestler’s group using microarrays and other methods,
which include cyclin-dependent kinase 5 (cdk5) and the GluR2 AMPA glutamate receptor subunit [8, 29, 37]. GluR2 was further shown to partially mediate ΔFosB’s increase in responsiveness of an animal to the rewarding and locomotor-activating effects of cocaine.

While investigators in the fields of addiction and memory have largely succeeded in identifying the initial molecular and cellular adaptations that eventually lead to these processes, the specific adaptations that directly underlie or sustain addiction and memory have yet to be identified [1]. There is a lot of interest in identifying the genes downstream of AP-1 and CREB, and more specifically those which are repressed or activated by ΔFosB, because they may be responsible for the long-term adaptations that occur at synapses in the brain following chronic drug exposure or experiences that lead to long-lasting memories. AP-1 is obviously involved in these phenomena, yet its complete role and the mechanisms behind its action have not been completely defined in mammalian systems. Identification of its downstream targets in simple and easily manipulated model organisms such as *Drosophila* could prove very useful in the fight to combat disorders of behavioral addiction and memory.

* *Drosophila melanogaster* is an important model for analyzing plasticity mechanisms

The potential to apply classical (rather than population) genetic analyses to the study of behavioral plasticity was first demonstrated in *Drosophila* by Seymour Benzer’s lab in 1974 [39]. The role of the cAMP cascade in memory formation established in this seminal study, and PKA’s function also first described in flies [40], have been verified in other systems. Many of the signaling cascades, second messenger systems, and
transcription factors described in other models of plasticity and memory have been subsequently shown to effect behavioral memory in *Drosophila*.

An unexpected discovery is that the highly accessible *Drosophila* larval motor synapse shows activity-dependent changes in synapse number and synaptic strength that occur through biochemical pathways similar to those involved in better established models for long-term plasticity [41]. Like the mammalian CNS, the excitatory synapses of the larval neuromuscular junction (NMJ) are glutamatergic and show activity-dependent alterations in their structural and functional properties. Neural activity, cAMP, and CREB activation lead to increased evoked transmitter release, synaptic arborization and postsynaptic sensitivity much like that observed in other models of long-term synaptic plasticity such as LTP in the mammalian hippocampus and LTF in cultured *Aplysia* neurons [41].

The utility of using *Drosophila* for the study of human diseases, such as cancer and pathologies of the brain like Alzheimer’s disease, has become increasingly apparent in the past few years [42-44]. Homologous molecules and pathways mediate conserved biological processes. However, functional analyses of genes and pathways in *Drosophila* are not only more rapid, but also often more incisive. The unique combination of genome resources, sophisticated genetic perturbation methods and the ability to directly analyze the cellular underpinnings of plasticity make *Drosophila* ideal for the identification and analysis of novel plasticity genes.
Fos and Jun play important regulatory roles in the establishment of long-term plasticity

Although the importance of AP-1 (a Fos/Jun dimer) regulation in the control of the process that underlies cocaine addiction is widely appreciated, until recently there was little insight into processes of synaptic and neural plasticity controlled by AP-1. Recent work in the Ramaswami lab, published in Nature, has shown that AP-1, under regulation by JNK, positively regulates both synaptic growth and synaptic strength at the Drosophila neuromuscular junction [45]. Importantly, AP-1 shows a wider range of influence than CREB whose effects at the same synapse are limited to control of synaptic strength [24]. This study demonstrated that neural induction of Fos and Jun together, but not individually, is sufficient to cause increased synaptic size and efficacy at the NMJ. In contrast, inhibition of either Fos or Jun (via dominant-negative transgenes) has the opposite effect, namely reduced number of synaptic boutons and decreased synaptic strength. A variety of genetic perturbation and double-mutant (epistasis) tests reveal the following additional points: a) AP-1 function is under direct modulation of endogenous JNK singaling; b) AP1 recruits (and requires) CREB for its effect on synaptic strength; c) AP-1 regulates synaptic growth though a CREB-independent pathway (see Figure 1.1) [45].

The identification of neuronally expressed genes that are transcriptionally regulated by AP-1 and CREB in Drosophila will increase our understanding of the molecular mechanisms responsible for the phenotypes we observe at the NMJ and
perhaps shed light on the processes of long-term plasticity that underlie addiction and memory.

Rationale and health relevance for the project

Genetic factors are estimated to account for 40-60% of the risk for developing an addiction. For instance, a missense mutation in the human fatty acid amide hydrolase gene, a gene possibly involved in the catabolism of endogenous cannabinoids, was shown to be associated with problem drug use and may confer susceptibility to addiction in humans [46]. Other loci are bound to be uncovered soon. I believe the existence of common molecular and morphological substrates for addiction and long-term synaptic plasticity suggests that genes implicated in one process are likely involved in the other. Elucidating the precise molecular basis of the maintenance of memory and addiction could provide important avenues for the development of medications that combat addiction and improve memory [1].

As mentioned earlier, genes shown to be responsive to AP-1 in the Drosophila nervous system, and required for regulating synapse plasticity in vivo, might represent genetic loci for further study in animal models. They may provide useful diagnostic predictors of individuals at risk for drug-related disorders. These genes may also be good targets for drug therapy design targeting the prevention of the enduring consequences of drug use in susceptible and non-susceptible humans alike. In the near future a repository of cell lines and/or genomic DNA samples from admitted addicts, similar to the ones available for other neurological disorders, may exist. These samples could be used to
analyze the genotypes of those individuals to check genetic loci discovered by methods like the ones described herein and through research by others taking similar approaches. Relevant SNPs at these loci may confer susceptibility to drugs of abuse and, potentially, other behavioral disorders.
Figure 1.1. A model for plasticity regulation at the *Drosophila* larval neuromuscular synapse. Originally published in Sanyal, et al. 2002 [45]. Used by permission of the authors.
CHAPTER 2
PILOT SCREENS, CUSTOM MICROARRAY CONSTRUCTION AND
PRELIMINARY AP-1 INDUCTION EXPERIMENTS

Abstract

The concept of a *cis*-regulatory code, which underlies differential gene expression, has emerged over the years. Computational methods that identify putative transcription factor binding site sequence motifs provide powerful tools for discovery of the *cis*-regulatory elements in the promoters of eukaryotic genes, which control their regulation. Such bioinformatic methods can be used to identify novel enhancers in sequenced genomes [47]. Identification of the sequences controlling activity-dependent transcription in neurons is a major goal of modern neuroscience. Here, we use computational methods to identify putative targets of AP-1- and CREB-dependent signaling in the fly genome. We tested the validity of these candidates using quantitative real-time RT-PCR (Q-PCR) and with custom DNA micorarrays containing the best positives from the computational searches.

Introduction

Signal-dependent transcription factors coordinate cellular responses to external stimuli, such as neuronal activation, by promoting the expression of specific genetic programs [28]. Differential gene activity is controlled primarily by complex enhancer
Bioinformatic methods have been successfully used to identify many putative, and in some cases functional, enhancers (transcriptional targets) in diverse biological contexts [31, 49, 50]. DNA microarray analyses performed with mRNA from whole fly heads has been successfully used to profile gene expression in the Drosophila brain [51-56].

In an attempt to identify direct targets of the transcription factors, AP-1 and CREB, we used a PERL-based search program to detect potential transcription factor binding sites in the upstream sequence of all predicted Drosophila genes (see Figure 2.2)[57]. The 'best candidates' chosen for further analysis were spotted on a custom microarray (see Table 2.2, Figure 2.3). The array, containing probe targets for approximately 100 candidate genes (including most of the 82 'best candidates' from the computational searches) as well as almost 200 other genes spotted for signal normalization purposes, was used to profile gene expression changes following AP-1 induction in the nervous system of flies. Q-PCR analysis was used to validate changes detected on the arrays.

Results

Computational screens for AP-1 and CREB binding sites in the Drosophila genome

In order to identify direct targets of AP-1 and CREB transcription we developed a computational search tool (Pilot) capable of searching any sequenced genome for sequence elements within 3kb upstream of the translational start site of all predicted genes (or transcriptional starts if that information is available). The AP-1 and CREB...
binding-site sequences used in our searches were chosen based on a careful analysis of
the literature looking for studies demonstrating functional interactions between AP-1 or
CREB and target promoters by gel mobility shift assay, deletion experiments with
reporter constructs or oligonucleotide competition methods (see Figure 2.1). As was
expected, the pseudo-palindromic consensus AP-1 binding sequence (TGACTCA) and
the palindromic consensus CRE sequence (TGACGTCA) were the functional sites
identified most often in these studies. For this reason we restricted our searches to those
sequences most represented in the literature, including the consensus and two degenerate
AP-1 sites (TGACTG, TGCGTCA), the consensus CRE and a CRE half-site
(TGACGT) found in many CREB-responsive promoters. Figure 2.2 depicts the search
scheme for all of the Pilot screens. Pilot is able to identify any combination of sequence
elements within a given window, up to 3kb in size, upstream of all predicted translation
start sites in a given genome.

Table 2.1 lists the total number of genes identified in the various searches in
Drosophila. Genes with at least one conserved CRE or AP-1 site in their predicted
promoter region are indicated by the number of candidates with sites in a 3kb window.
The occurrence of both conserved sequences is approximately the number expected by
chance within 3kb of ~14000 genes; however, the count drops ~10-fold for both when
you query for only two conserved sites within the same window. Relatively few genes
have multiple conserved CREB or AP-1 binding sites in their presumptive promoters.

Recent reports have demonstrated that many functional CREB-responsive
promoters are bidirectional in nature (Table 2.2)[31]. A significant proportion of the
candidate CREB and AP-1 responsive promoters identified in our screens (21/62, ~34%) appear they could regulate the expression of two genes as they are organized in a head-to-head arrangement. In this same study, using an ingeniously modified SAGE protocol which incorporates chromatin immunoprecipitation, called SACO, CREB was shown in PC12 cells to bind the homolog of one of the candidate genes (Wnt2) identified in our screens as a putative CREB and AP-1 target [personal communication][31].

Microarray construction, hybridization and analysis

Table 2.2 lists the genes that were spotted on the array along with housekeeping genes and a random set of EST clones from the Berkeley Drosophila Genome Project (BDGP - targets courtesy of the GATC facility at the University of Arizona), which were included for signal normalization purposes, as well as other expected targets of AP-1 and CREB such as homologs of mammalian IEGs. This list of 'best candidates' was chosen as a first pass attempt to confirm the validity of the computational screens, assuming that genes with multiple elements in close proximity were more likely to respond to AP-1 induction, since a typical eukaryotic enhancer is approximately 500bp in length and contains roughly ten binding sites for two or more sequence-specific transcription factors [48]. Probe targets for the ~100 candidate genes and ~150 controls (including multiple probes for some housekeeping genes spanning different regions of the transcript) were synthesized and spotted on glass slides (see Materials and Methods).

An outline of the microarray screen design is illustrated in Figure 2.4. The analysis compared mRNA levels in heads of 1-3 day old adult flies, with or without acute AP-1 induction in the nervous system using the conditional ElavGS-GAL4 strategy
(described in Chapter 4). Briefly, each array “experiment” included sibling flies split into
two groups: the experimental steroid treated (AP-1 induced) population and an untreated
control group. Sibling, age-matched controls used in each experiment ensured that
 genetic background, which can have a large effect on transcriptional variance [58], was
not a confounding factor in our analyses. Thus, each experiment allowed transcript levels
to be compared between experimental and control samples. Heads were isolated from
flies with or without six hours of RU486 treatment to induce AP-1 in the nervous system.
RNA was extracted and labeled cDNA was created with either of the fluorescent dyes,
Cy3 and Cy5. Fluorescently labeled cDNA was mixed and hybridized to the custom
array overnight. Fluorescence signals were calculated and ratios were compared using
Excel. Candidate AP-1 responsive transcripts would be identified as those with average
induced/control fluorescence ratios significantly different from 1.

Reproducible hybridizations and an empirical estimate of experimental noise are
essential to evaluate confidence in array experiments. One measure of microarray
hybridization reproducibility often employed is a “self-self” experiment, which consists of
two cDNA samples derived from the same RNA sample labeled with different Cy-dyes
(Cy3 and Cy5) and hybridized together on an array. In principle, there should be no
detectable differences in the normalized fluorescence values in such an experiment;
however, reverse transcription reactions will differ slightly and the two dyes have
different coupling efficiencies. As a result, small, false expression changes will be
detected the extent of which is one measure of unavoidable experimental noise. The
correlation coefficient \( R^2 \) value, or how well the log of the two fluorescence values for
each spot on the array regresses to a straight line, with a common normalization value (the slope of the line), indicates how well all spots fit this regression line. This $R^2$ value is an inverse measure of the experimental noise (false-positives), or in other words, how reproducible your results are. The closer this value to unity, the better and thus we optimized conditions to the point that $R^2$ values were consistently at or above .95 (see Figure 2.4).

Figure 2.5 shows the results from four repetitions of this basic experiment. The data is represented as the log(2) of the average ratio between induced/control fluorescence signals for each of the approximately 250 genes (error bars - SEM). A ratio of 1.0 thus represents a two-fold difference between fluorescence signals detected on the array. *fos* and *jun* levels were consistently increased as detected by two separate targets for each gene, although the alterations were not as large as expected. A few other transcripts showed moderate changes compared to controls following AP-1 induction. One of the control genes from the EST collection (CG11191) on the array showed consistent increases in mRNA levels following AP-1 induction in fly heads by array analysis (Figure 2.5)

**Microarray screen validation**

To confirm positives, a handful of the most robustly changing AP-1-responsive genes, exhibiting consistent up- or down-regulation in the microarray analysis, were selected as candidates for Q-PCR validation using gene specific primers. To confirm AP-1 induction we assessed *fos* and *jun* mRNA levels in *ElavGS-GAL4-AP1* fly heads following 6 hours of RU486 treatment compared to untreated controls (see Materials and
Methods for a detailed description of the procedure and [59]). We consistently achieved ~2.5- and 9-fold induction of *fos* and *jun* transcripts in fly heads, respectively (Figure 2.6 and Figure 4.7A). Further experiments to validate the increases in the mRNA levels of *CG11191* observed on the array demonstrated that this gene with unknown function responds to RU486 treatment alone (see Chapter 4 and Figure 4.7B)[59]. No other changes detected on the array were confirmed in follow-up Q-PCR analysis (data not shown).

One pitfall to the use of cDNA microarrays is that other labeled cDNAs, which possess sequence homology to a gene target represented on your array, can bind to the target DNA on the glass slide during hybridization. This can potentially adversely affect the signals detected for many gene targets and poses a large problem for researchers using cDNA microarrays. An example of this problem is discussed below.

Figure 2.6 demonstrates that CG1871, which was a strong candidate from the computational screens having been identified in both the 2CRE and 1CRE 1AP-1 site searches, transcripts showed consistent increases by array analysis following AP-1 induction in the nervous system. However, when we attempted to confirm this difference on the same mRNA samples using Q-PCR, no change was apparent. This led to the hypothesis that another gene with sequence homology to CG1871 may be binding to the cDNA probe during hybridization.

When the CG1871 sequence spotted on the array (~800 base pairs long) was blasted against the genomic sequence of *Drosophila*, a few genes with significant sequence similarity were discovered. One gene, *stripe* (a transcription factor homologous
to the early growth response (egr-1) gene - a mammalian NGF-responsive immediate-early gene[60]), has ~60% sequence identity over two different 250 base pair (bp) stretches of its mRNA sequence. Q-PCR, using primers for stripe, performed on the same RNA samples used for the array experiments demonstrated its transcript levels were increased on average in the 4 AP-1 induced samples used on the cDNA arrays. Although stripe levels were increased in 3/4 of the AP-1 induced samples, it was lower in the 4th (depicted by the large variance for sr in Figure 2.6) and in later treatments comparing transcript levels in 2 untreated samples for each (data not shown). The changes observed by Q-PCR were consistent with the signals detected for the CG1871 target on the array in these samples; however, further analysis demonstrated stripe levels appear to simply be dynamic and vary in populations of fly heads from day to day and in untreated samples from the same day. There is no probe for stripe on the cDNA array.

Discussion

Observations made during our analysis of gene expression following AP-1 induction in the fly nervous system, though not particularly informative for helping to explain the mechanisms behind AP-1 and CREB dependent transcription and its control over synaptic plasticity in Drosophila. They do, however, demonstrate the need for independent verification of microarray results and point out that researchers should be wary of non-specific hybridization when using cDNA arrays. Investigators must also employ some external measure of random variability to ensure their results are being interpreted correctly. These preliminary studies were useful, as they paved the way for a
more careful analysis of AP-1 dependent gene expression in the fly head. The problems with cDNA microarrays and dynamic transcript variability raised in this chapter were strongly considered before the next set of experiments, described in Chapter 4, were embarked upon [59]. Non-specific binding is theoretically less of a concern with oligonucleotide arrays; although, recent evidence suggests it can lead to consistently inaccurate signals on Affymetrix GeneChips [61].

Since it is not known what an AP-1 responsive promoter looks like in *Drosophila melanogaster*; it was our hope that an analysis of the promoters of true AP-1 targets discovered by the means described above would have lead to the identification of additional sequence elements conserved between target promoters that could be used to predict AP-1 and CREB responsiveness more precisely. It may be that the location as well as the order of binding sites is important, and it is likely that coordinated interactions with other transcription factors (such as CREB) are required for AP-1 dependent transcription in the nervous system. Confirmed AP-1 responsive genes are likely to share commonalities in promoter sequences, such as cofactor binding sites, which can be deduced by cluster analysis. If a bonified list of real AP-1 target genes had been identified we could have then compared the promoters from the true targets to a random set of non-responsive promoter sequences. Furthermore, we sought to discover sequences which could be used to build an AP-1 responsive reporter construct in flies. None of these were possible however, due to the lack of positive candidates.

Additionally, we may have actually missed many AP-1 and CREB responsive genes in our searches since the Pilot program only allowed identification of binding sites
in the promoter sequence within 3kb upstream of each gene. A significant percentage of known CREB-regulated genes, and presumably AP-1, possess internal or intronic promoters [31]. In fact, the SACO method described above identified a significant proportion of the putative CREB interaction sites were located in introns or at the 3’ end of genes in the rat.

Refer to Chapter 4 for a detailed description of the more comprehensive analysis of AP-1 responsive gene induction in the Drosophila head using whole-genome oligonucleotide microarrays and for a discussion of why these methods may not have yielded the results one might hope (also see discussion in Chapter 5)[59].
Figure 2.1. Promoters of known CREB and AP-1 target genes. Results from literature search for functional AP-1 and CREB binding sites. Binding sites were deemed functional if characterized by gel mobility shift assay, deletion experiments with reporter constructs or oligonucleotide competition methods. Tick marks in promoters = 50bp.

Figure 2.1. references:
c-fos [62-65]  
somatostatin [66, 67]  
tyrosine hydroxylase [68, 69]  
synaptophysin [69]  
PEPC-kinase [72, 73]  
vgf [17, 70, 71]  
zif268 [74]  
BDNF [78]  
NOR-1 [81]  
calspermin [83, 84]  
glycoprotein-H [85]  
HDH [324, 85]  
tPA [87]  
OTR-1 [89]  
CMV [92]
<table>
<thead>
<tr>
<th>Protein/Enzyme</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV-1 [94-96]</td>
<td></td>
</tr>
<tr>
<td>LDH [99]</td>
<td></td>
</tr>
<tr>
<td>neurofilament-L [101]</td>
<td></td>
</tr>
<tr>
<td>cyclin-D1 [103]</td>
<td></td>
</tr>
<tr>
<td>chromogranin-B [105]</td>
<td></td>
</tr>
<tr>
<td>Djun [107]</td>
<td></td>
</tr>
<tr>
<td>collagenase [109, 110]</td>
<td></td>
</tr>
<tr>
<td>PFK [112]</td>
<td></td>
</tr>
<tr>
<td>dopamine β-hydroxylase [114]</td>
<td></td>
</tr>
<tr>
<td>VIP [116, 117]</td>
<td></td>
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<tr>
<td>MCP-1 [119]</td>
<td></td>
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<tr>
<td>α 2u-globulin [120]</td>
<td></td>
</tr>
<tr>
<td>prodynorphin [122, 123]</td>
<td></td>
</tr>
<tr>
<td>secretogranin II [97, 98]</td>
<td></td>
</tr>
<tr>
<td>aromatase [100]</td>
<td></td>
</tr>
<tr>
<td>11-β-hydroxylase [102]</td>
<td></td>
</tr>
<tr>
<td>PAI-2 [104]</td>
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<tr>
<td>insulin-1 [106]</td>
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</tr>
<tr>
<td>clusterin [108]</td>
<td></td>
</tr>
<tr>
<td>SP-A-1 [111]</td>
<td></td>
</tr>
<tr>
<td>5-HT1A [113]</td>
<td></td>
</tr>
<tr>
<td>hMTIIA [109, 115]</td>
<td></td>
</tr>
<tr>
<td>Dca [118]</td>
<td></td>
</tr>
<tr>
<td>SV40 [109, 115]</td>
<td></td>
</tr>
<tr>
<td>MMP-19 [121]</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.2. Pilot: a computational tool for promoter-based gene discovery.

Searching for clusters of specific AP-1 and CREB binding sites (CRE) in the promoters of all predicted genes in a genome.
Table 2.1. Search results from Pilot screens looking for occurrence of AP-1 and CREB binding sites in the fly genome. Search results highlighted in green were chosen for further analysis with cDNA microarrays.

<table>
<thead>
<tr>
<th>Search criterion</th>
<th>window size (bp)</th>
<th># of candidates</th>
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<td>1 cCRE</td>
<td>3kb</td>
<td>536</td>
</tr>
<tr>
<td>2 cCRE</td>
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<td>34</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>18</td>
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<tr>
<td>3 cCRE</td>
<td>3kb</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>11</td>
</tr>
<tr>
<td>1 cAP-1 site</td>
<td>3kb</td>
<td>3089</td>
</tr>
<tr>
<td>2 cAP-1 sites</td>
<td>3kb</td>
<td>389</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>91</td>
</tr>
<tr>
<td>3 cAP-1 sites</td>
<td>3kb</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td>4 cAP-1 sites</td>
<td>3kb</td>
<td>6</td>
</tr>
<tr>
<td>1 cCRE, 1 cAP-1 site</td>
<td>500</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>31</td>
</tr>
</tbody>
</table>
Table 2.2. 'Best candidate' list of genes from Pilot searches spotted on the custom cDNA microarray. Genes identified in both searches are indicated with bold type.

Red/bold type identifies putative bidirectional AP-1 and CREB responsive promoters. Blue type indicates the promoter identified may actually be the functional promoter for an upstream gene that lies between the gene in blue and the elements. NI = not identified in the computational screen described.

### 2 conserved CRE within 500bp (18)
- CG11128
- CG11350
- CG11584
- **CG11761**
- CG12239
- CG13871
- **CG14132**
- **CG14185**
- CG14845
- CG17210
- **CG1871**
- CG2056
- CG4427
- CG5214
- **CPTI** *(same promoter as CG11761)*

### 1 conserved CRE, 1 conserved AP-1 site within 500bp (64)
- **14-3-3ζ** *(same promoter as sites in Jra 1st intron - perhaps co-regulated)*
- **alpha-Adaptin**
- BcDNA:GH02636
- **betaTub56D**
- CG7744 *(NI - sites in betaTub56D intron)*
- **CG10337**
- CG10338
- CG1091
- CG11237
- CG11370
- **CG11709**
- **CG11761**
- **CG11791**
- **CG13871** *(same promoter as CG11761)*
- **CG14132** *(NI - 6.7kb from CG14132)*
- **CG14185** *(4 cCRE between these two genes)*
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CG12433</td>
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</tr>
<tr>
<td>CG12517</td>
<td></td>
</tr>
<tr>
<td>CG1287</td>
<td></td>
</tr>
<tr>
<td>CG12987</td>
<td></td>
</tr>
<tr>
<td><strong>CG14400</strong></td>
<td>CG9336</td>
</tr>
<tr>
<td>CG14629</td>
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</tr>
<tr>
<td><strong>CG14989</strong></td>
<td>Gad1 (NI - 4.3kb from CG14989)</td>
</tr>
<tr>
<td>CG15237</td>
<td>(same promoter as CG3450)</td>
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<tr>
<td>CG15702</td>
<td></td>
</tr>
<tr>
<td>CG15761</td>
<td></td>
</tr>
<tr>
<td><strong>CG17085</strong></td>
<td>porin</td>
</tr>
<tr>
<td>CG17086</td>
<td>(same promoter as CG17085)</td>
</tr>
<tr>
<td>CG17137</td>
<td>(Porin2; same promoter as porin)</td>
</tr>
<tr>
<td>CG18173</td>
<td></td>
</tr>
<tr>
<td><strong>CG18584</strong></td>
<td>CG3450</td>
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<td>CG1871</td>
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<td>CG2179</td>
<td></td>
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<tr>
<td><strong>CG3203</strong></td>
<td>CG14439 (NI - 7.7kb from CG3203)</td>
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</tr>
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<td><strong>CG6613</strong></td>
<td>CG11073 (NI - 3.5kb from CG6613)</td>
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<td><strong>CG6818</strong></td>
<td>CG14081 (NI - 4.3kb from CG6818)</td>
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<td>CG8980</td>
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<td>CG9188</td>
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<td>CG9339</td>
<td></td>
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<tr>
<td>CG9447</td>
<td></td>
</tr>
<tr>
<td><strong>CPTI</strong></td>
<td>(same promoter as CG11761)</td>
</tr>
<tr>
<td>CrebB-17A</td>
<td></td>
</tr>
<tr>
<td>Ddc</td>
<td></td>
</tr>
<tr>
<td>Gapdh2</td>
<td></td>
</tr>
<tr>
<td><strong>Gliolectin</strong></td>
<td>CG6637 (NI - 6.3kb from Gliolectin)</td>
</tr>
<tr>
<td>huntingtin</td>
<td></td>
</tr>
<tr>
<td>lbm</td>
<td></td>
</tr>
<tr>
<td>Pka-R1</td>
<td></td>
</tr>
<tr>
<td><strong>Wnt2</strong></td>
<td>CG8809 (NI - 6.7kb from Wnt2)</td>
</tr>
<tr>
<td>zfh1</td>
<td>CG11315 (NI - 17.3kb from zfh1)</td>
</tr>
</tbody>
</table>
Figure 2.3. The custom cDNA microarray. Example of a hybridization performed with the custom microarray containing the best candidate AP-1 and CREB target genes from the Pilot screens (Table 2.2). Included are all candidates with either 2 consensus CRE or 1 conserved CRE and one consensus AP-1 binding site within 500 base pairs of each other and within 3kb upstream from the predicted translational start site of all predicted Drosophila genes (determined by Adams et al. [57]), as well as many housekeeping and other control genes.
Figure 2.4. Observing AP-1 dependent gene expression in fly heads using custom microarrays. Experimental design of AP-1 induction experiments. RNA isolated from heads of flies with or without 6 hours of RU486 treatment to induce AP-1 in the nervous system was used to make fluorescently labeled cDNA. Fluorescently labeled cDNA was mixed and hybridized to the custom array; fluorescence signals were calculated and ratios were compared. Q-PCR was used to confirm alterations detected on the array.
Figure 2.5. Average expression change from 4 AP-1 induction experiments.

Average ratio between fluorescence values from four cDNA microarray hybridizations for ~250 gene targets is depicted on the y-axis. Positive values indicate increased transcript levels in RU486 treated (AP-1 induced) RNA samples compared to untreated control samples, negative values a decrease. *fos* and *jun* are represented by two different target DNA sequences on the array. Both show consistent increases in the head following their induction in the nervous system in *ElavGS-GAL4-AP1* flies.
Figure 2.6. Microarray result validation using quantitative real-time RT-PCR. Quantitative comparisons of transcript levels in adult head RNA from RU486 treated (AP-1 induced) versus control samples as assessed by microarray and Q-PCR analysis on the same RNA samples. Micorarray results are shown as the log(2) of the average ratio between induced/control samples. Q-PCR values represent average cycle difference in PCR product between induced/control samples (N=4). Positive values indicate an increase in transcript compared to unchanging reference gene, negative values a decrease. *jun* and *stripe* RNA levels are increased in the fly head after AP-1 induction in the nervous system.
CHAPTER 3

THE UPS AND DOWNS OF DAILY LIFE: PROFILING CIRCADIAN GENE EXPRESSION IN DROSOPHILA

Summary

Circadian rhythms are responsible for 24-hour oscillations in diverse biological processes. While most central genes governing circadian pacemaker rhythmicity have been identified, clock-controlled output molecules responsible for regulating rhythmic behaviors remain largely unknown. Two recent reports from McDonald and Rosbash\textsuperscript{[52]} and Claridge-Chang et al.\textsuperscript{[53]} address this issue. By identifying a large number of genes whose mRNA levels show circadian oscillations, the reports provide important new information on the biology of circadian rhythm. In addition, the reports illustrate both the power and limitations of microarray-based methods for profiling mRNA expression on a genomic scale.
Regulation of circadian rhythm

Several biological functions of bacteria, plants and animals show circadian oscillations [124-126], rhythmic fluctuations whose periodicity coincides with the rotation of the earth on its axis. Circadian outputs are regulated by a central pacemaker, or molecular clock, that is ultimately responsible for keeping circadian time. Like any good clock, circadian pacemakers are insensitive to temperature fluctuations and can be reset by various external stimuli [127]. Individual cells show circadian oscillations that arise from an intracellular clock [124]; oscillations in groups of cells must be synchronized or maintained at specific phase differences through intercellular signaling. This is particularly evident for behavioral rhythms. For instance, circadian locomotor activity rhythm in the hamster is regulated by rhythmic release of TGFβ from neurons in the suprachiasmatic nucleus [128, 129].

The first genetic circadian clock component was discovered by Ron Konopka in a mutagenesis screen that successfully identified a circadian rhythm mutant in the fruit fly Drosophila melanogaster. Flies with a mutation in the period (per) gene exhibited perturbed eclosion and activity rhythm phenotypes [130]. This groundbreaking study paved the way for the identification of other clock components not only in Drosophila, but also in Neurospora, cyanobacteria, plants and mouse [131]. Although molecular activities of individual clock components identified in metazoa may differ across species,
the proteins have characteristic regulatory properties. Central clock elements assemble into transcription factors whose activities show rhythmic oscillations [132].

In flies, two transcription factors, Clock (Clk) and Cycle (Cyc) form a heterodimer that controls the expression of two other clock components Period (Per) and Timeless (Tim). The Clk:Cyc heterodimer binds to the promoters of *per* and *timeless* (*tim*) and activates their transcription [133, 134]. Interestingly, Clk, but not cyc, oscillates at both the mRNA and protein level peaking near subjective dawn [133, 135]. Both *per* and *tim* cycle at the mRNA level, as do their protein products, with a delayed peak phase with respect to their transcripts [136, 137]. PER and TIM physically associate into a different complex that acts to repress Clk-mediated transcription [133, 134, 138, 139]. Thus, when Per and Tim protein levels peak, they shut down their own expression. This transcriptional feedback loop is responsible for rhythmic gene cycling and ultimately circadian behaviors in flies. Clk and Cyc are positive elements of the loop, while Per and Tim are negative feedback elements. Post-transcriptional and post-translational regulation of mRNA and protein turnover contributes importantly to the time period of oscillation [140]. Other transcription factors may also participate in the central clock but their functions are poorly defined [129]. For example, dCREB2 exhibits a *per*-dependent 24-hour oscillation in activity; a mutant CREB2 allele dampens the oscillation of *per* and shortens normal fly locomotor rhythms [141].
Identifying novel circadian transcripts in the fly

The identification of genes controlled by the central clock is important because it would provide an avenue for understanding circadian oscillations in diverse biological processes including locomotor activity, feeding behavior, hormone secretion, digestion and the onset of sleep. For instance, sleep is a biological process mechanistically and physiologically independent of, yet integrally influenced by circadian rhythms. The discovery of new clock target genes could lead to the identification of critical circadian inputs into the neural circuitry that regulates sleep [142]. Identification of clock output elements that impart circadian regulation to sleep physiology would not only be of considerable scientific interest, but may also have direct medical relevance leading to the development of new treatments for sleep disorders, for example [131, 142]. Despite the tremendous interest, to date only a handful of clock output genes have been identified using conventional genetic and biochemical approaches.

Two exciting reports published late last year in Cell and Neuron utilized genomic approaches to identify novel candidate circadian genes in Drosophila [52, 53]. Completion of the fly genome [57] and the recent availability of the Affymetrix whole genome fly microarray has made it possible for researchers to investigate the transcriptional state of the fly over multiple time points or following different biological manipulations. Two studies, that we discuss here, describe such experiments. Although global patterns of circadian gene expression using oligonucleotide microarrays have previously been described in Arabidopsis thaliana using similar analysis to the present
reports [143, 144], these studies represent the first time microarray technology has been used to assess circadian gene expression on a genome-wide scale in the animal kingdom. They are also among the first published accounts using the Affymetrix Drosophila genome chip (GeneChip) and reveal both the power and limitations of such an approach.

Both groups designed analogous procedures for determining rhythmically cycling transcript levels in the fly brain (Figure 3.1). Flies were first entrained to a standard 12:12 hr light dark (LD) cycle for three days. RNA was then isolated from whole fly heads at 4 hr intervals in the circadian cycle and used to generate a set of labeled probes for all expressed transcripts. The probes were hybridized to GeneChips that contain immobilized DNA elements representing each of the roughly 13,600 Drosophila genes. The strength of labeling observed at each spot provided an estimate for the relative mRNA expression level at each time point in the circadian cycle.

In the study by McDonald and Rosbash, RNA was isolated every four hours on the fourth day (the first following entrainment) while the flies were in constant darkness to avoid light regulated genes not under circadian control; 3-5 replicates were performed for each time point. They identified 134 genes as showing circadian cycling based on the following criteria. First, the transcript should show a minimum 1.5 fold change in expression level between the highest and lowest expression level measured at the different time points. Second, the wave form of a graph plotting the deduced mRNA level against time should fit a cosine wave of 24 hr periodicity with a cross-correlation coefficient of more than 0.90. The cosine waves could be randomly phased; this allowed detection of circadian mRNAs whose levels peaked at different time points in the
day/night cycle. Because circadian mRNAs are expected to be under control of Clk, the group examined expression levels in clk mutants at two different time points. All cycling transcripts exhibiting differences at these two time points in wild-type flies failed to cycle in the mutant. McDonald and Rosbash interpret this as indicative of Clk regulation of all identified genes, though an alternative explanation remains tenable (see next section).

Many of the cycling transcripts identified in this study share functions that can be rationalized from a circadian standpoint. Several of these genes cluster in specific genomic regions and appeared to be transcriptionally co-regulated.

In the second study, by Claridge-Chang et al., RNA was isolated every four hours over the course of a fourth day of LD entrainment and on a fifth day in constant darkness. Three replicate samples were taken for each time point. For each gene, the three separately observed 48 hr mRNA level profiles were normalized and combined into a single 144 hr plot. These plots were then fit to alternatively phased oscillatory waves with 24 hr periodicity. For each gene a “Fourier score (F24)” was assigned that represents how well the data correlated with a perfect 24 hr oscillator. The F24 values from the real data were compared with values obtained from a set of 10,000 random permutations of the data points. For 158 genes, the F24 value for the original data was higher than the randomly permuted data with a probability p-F24 < 0.02. These were selected as likely circadian genes. Northern blot analysis verified the cycling nature for 16 of 16 genes sampled from this set and a few from the less stringent p-24 <0.05 set. Further, 72 of the 158 oscillating transcripts exhibited a significant change when expression levels were assessed in per, tim and Clk mutants. As in the study by McDonald and Rosbash, several
apparently co-regulated genes with similar functions were found to physically cluster in the genome. The molecular function of many of the genes could be grouped into classes potentially relevant to the circadian cycle.

The power and limitations of microarray analysis

The power: Of the 134 genes identified by McDonald and Rosbash, and 158 by Claridge-Chang et al., 30 were common to both lists. Both microarray analyses identified most of the major clock genes previously shown to oscillate in flies, such as Clk, per, tim, vri and takeout (cry did not appear in the Neuron report); their patterns of expression were in agreement with previously published accounts. Beyond these 5 known central circadian genes 25 other new candidate genes were identified by both analyses (see Table 3.1). The discovery of 30 common genes in these two studies is highly significant, more than 24 standard deviations from the mean of about 1.5 common genes expected to occur by random chance. The expression of most if not all of these 30 genes is likely to truly be under circadian control. Their identification, by enabling new analyses into circadian control of behavioral phenomena, represents major progress in circadian biology.

Limitations: But what of the other genes? There remain 104 from McDonald and Rosbash, and 128 from Claridge-Chang et al. that are not listed in the two independent studies. Are these true circadian genes? The non-overlapping genes could represent subtle differences in experimental approach used in the two studies rather than “false positives” that passed the criteria laid out by the investigators. For example, Claridge-Chang et al.
sampled from a day of free running clock activity in their experiments in addition to a
day in constant darkness, and this might cause real differences in gene expression
profiling. Different fly strains were used in the two studies and this may have lead to
important differences in transcriptional state and/or response [145]. Details of the
statistical analyses applied to the two data sets also differed; in this context, it would be
interesting to see the same analysis applied to the opposite data sets to see if there is more
concurrence. This consideration is supported by an independent experiment in which
McDonald and Rosbash determined a small number of the genes identified in the
microarray assay to be direct targets of Clk regulation.

However, it appears unlikely that these experimental differences alone contribute
to the extensive divergence between the two data sets. It seems more probable to us that
the majority of genes not identified by both studies represent experimental noise
associated with analyses of a large number (~ 13600) of genes. For instance, in the
Claridge-Chang study, 158 transcripts were found to show circadian regulatory
properties, passing a probability filter of p-F24 < 0.02 for rhythmicity. While true
circadian genes should be enriched by this analysis, the laws of probability suggest that
by chance alone 200 of 10,000 genes would be expected to pass through this filter.
McDonald and Rosbash show that mutations in the central clock abolish oscillatory
expression properties for genes identified in their screen. While potentially instructive,
this observation should not be construed as an independent test of the validity of the
candidate circadian genes. In a clock mutant background, false positives also would not
be expected to show circadian oscillations in gene expression. Thus, although it is
conceivable that a large fraction of the genes identified are indeed regulated by the circadian clock, there is currently insufficient supportive evidence. Such ambiguity is likely to be an essential part of microarray analyses and without more detailed confirmation of each candidate gene, analyses of the entire panel of potential circadian genes must be carried out with due caution. For example, although Claridge-Change et al. find a slight enrichment of binding sites for “circadian transcription elements” such as Clk and CREB in promoters of the 158 candidate genes when compared with a randomly selected set of genes, they carefully do not conclude from these data that other transcription factors play a major role in circadian gene expression. The value of analyzing promoters from groups of co-regulated genes is significantly reduced if a large number of false positives are included in the group of analyzed genes.

Conclusions

In conclusion, these papers show the power of microarray analysis for discovering candidate genes with relative ease and rapidity. By identifying several novel circadian genes, these expression profile studies have made a dramatic contribution to the circadian community. It is likely that many of the genes identified in these studies will prove integral to the fly circadian clock and that their homologs will be important components of the mammalian circuit as well. In the near future, characterization of these genes may lead to greater understanding of how circadian clocks feed into diverse physiological and behavioral processes. For instance, many of these candidate genes may also be involved in learning and memory consolidation since CREB, which has a well established role in
synaptic plasticity, shows circadian regulation and several studies indicate memory consolidation occurs during sleep [141]. But, as the reports acknowledge, more detailed investigation must be undertaken to validate their involvement in the *Drosophila* circadian clock as well as to assess their importance to physiology and behavior.

Note added in proof

After completion of this essay, a third microarray based analysis of circadian gene expression in *Drosophila* was published by Ueda et al. in the Journal of Biological Chemistry [54]. Using an analogous approach and further RT-PCR based confirmation of the best positives, the authors identify another collection of candidate circadian genes that include 26 genes found in both studies discussed in this article. The interested reader is referred to this publication for procedural details and some unique findings.
Figure 3.1. Profiling circadian gene expression in *Drosophila*: experimental design.

RNA was isolated at 4 hr intervals in the circadian cycle from heads of flies entrained to a 12:12 hr light dark cycle for 3 days (see text for details). The RNA was labeled and hybridized to GeneChip arrays and fluorescence intensity was measured for each gene. Expression levels were plotted for each time point. Gene 1 and gene 2 show oscillatory expression properties, gene 3 does not.
Table 3.1. **List of genes identified by both studies.** The list of genes showing significant 24 hour rhythmicity identified in both studies [52, 53].

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahcy89E</td>
<td>adenosylhomocysteinase</td>
</tr>
<tr>
<td>ATPCL</td>
<td>ATP-citrate (pro-S)-lyase</td>
</tr>
<tr>
<td>B4</td>
<td>unknown</td>
</tr>
<tr>
<td>Clk</td>
<td>RNA polymerase II transcription factor</td>
</tr>
<tr>
<td>Cyp305a1</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Cyp4d21</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Cyp6a21</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>per</td>
<td>PAS domain clock protein - transcription factor</td>
</tr>
<tr>
<td>Slob</td>
<td>signal transduction</td>
</tr>
<tr>
<td>tim</td>
<td>clock protein - transcription factor</td>
</tr>
<tr>
<td>takeout</td>
<td>ligand binding or carrier</td>
</tr>
<tr>
<td>Ugt35b</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>vri</td>
<td>RNA polymerase II transcription factor</td>
</tr>
<tr>
<td>Zw</td>
<td>glucose-6-phosphate 1-dehydrogenase</td>
</tr>
<tr>
<td>CG10513</td>
<td>unknown</td>
</tr>
<tr>
<td>CG10553</td>
<td>unknown</td>
</tr>
<tr>
<td>CG11891</td>
<td>unknown</td>
</tr>
<tr>
<td>CG12116</td>
<td>sepiapterin reductase</td>
</tr>
<tr>
<td>CG14275</td>
<td>unknown</td>
</tr>
<tr>
<td>CG1441</td>
<td>unknown</td>
</tr>
<tr>
<td>CG15093</td>
<td>similar to short chain alcohol dehydrogenases</td>
</tr>
<tr>
<td>CG17386</td>
<td>crystallin beta gamma domain (RNA binding)</td>
</tr>
<tr>
<td>CG4784</td>
<td>structural protein</td>
</tr>
<tr>
<td>CG4919</td>
<td>glutamate cysteine ligase regulatory protein</td>
</tr>
<tr>
<td>CG5156</td>
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</tr>
<tr>
<td>CG5798</td>
<td>ubiquitin-specific protease</td>
</tr>
<tr>
<td>CG5945</td>
<td>unknown</td>
</tr>
<tr>
<td>CG9363</td>
<td>tyrosine and phenylalanine catabolism</td>
</tr>
<tr>
<td>CG9645</td>
<td>endopeptidase</td>
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<td>CG9649</td>
<td>endopeptidase</td>
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CHAPTER 4
SYNAPTIC AND GENOMIC RESPONSES TO JNK AND AP-1
SIGNALING IN DROSOPHILA NEURONS

Note from Author: This manuscript appeared in BMC Neuroscience [59]. It is presented here with minor organizational modifications to conform to the overall layout of the thesis. It was written entirely by myself and Radhakrishnan Narayanan with editorial suggestions from Mani Ramaswami. It appears with permission from the editors.

Abstract

Background
The transcription factor AP-1 positively controls synaptic plasticity at the Drosophila neuromuscular junction. Although in motor neurons, JNK has been shown to activate AP-1, a positive regulator of growth and strength at the larval NMJ, the consequences of JNK activation are poorly studied. In addition, the downstream transcriptional targets of JNK and AP-1 signaling in the Drosophila nervous system have yet to be identified. Here, we further investigated the role of JNK signaling at this model synapse employing an activated form of JNK-kinase; and using Serial Analysis of Gene Expression and oligonucleotide microarrays, searched for candidate early targets of JNK or AP-1 dependent transcription in neurons.
Results

Temporally-controlled JNK induction in postembryonic motor neurons triggers synaptic growth at the NMJ indicating a role in developmental plasticity rather than synaptogenesis. An unexpected observation that JNK activation also causes a reduction in transmitter release is inconsistent with JNK functioning solely through AP-1 and suggests an additional, yet-unidentified pathway for JNK signaling in motor neurons. SAGE profiling of mRNA expression helps define the neural transcriptome in *Drosophila*. Though many putative AP-1 and JNK target genes arose from the genomic screens, few were confirmed in subsequent validation experiments. One potentially important neuronal AP-1 target discovered, *CG6044*, was previously implicated in olfactory associative memory. In addition, 5 mRNAs regulated by RU486, a steroid used to trigger conditional gene expression were identified.

Conclusions

This study demonstrates a novel role for JNK signaling at the larval neuromuscular junction and provides a quantitative profile of gene transcription in *Drosophila* neurons. While identifying potential JNK/AP-1 targets it reveals the limitations of genome-wide analyses using complex tissues like the whole brain.

Background

Conserved neuronal signaling pathways regulate synaptic plasticity, the ability of neurons to modify synaptic connections. “Long-term” forms of neuronal plasticity require new gene expression that results in persistent synaptic change (altered synaptic strength and
morphology). Thus, long-term forms of plasticity may be perturbed, in a variety of model systems, by protein synthesis inhibitors, or manipulation of either specific signaling kinases or critical downstream transcription factors [4, 5, 15, 146].

A major requirement in long-term plasticity for the ERK/MAPK (extracellular signal-regulated kinase subfamily of mitogen-activated protein kinases) signaling cascade acting through CREB (the cAMP response element binding protein, a basic leucine zipper - bZIP - transcription factor) has been supported in diverse experimental paradigms [5, 15, 147]. Activation of CREB has been reported to enhance long-term memory in Drosophila and rodents, and long-term facilitation in the sea slug Aplysia [21, 23, 26, 148-150]. Normal ERK signaling is required for hippocampal LTP formation, for BDNF-induced forms of structural plasticity, as well as for several forms of learning and long-term memory [151-154]. For example, ERK activation is necessary for the formation of conditioned taste aversion and spatial learning in rodents, and blockade of ERK signaling affects long-term, but not short-term, fear conditioning [155-157]. In addition, ERK regulates synapse plasticity in flies and LTF and memory in Aplysia [158-160].

Many “immediate-early genes” (IEGs), including members of the AP-1 family of transcription factors (heterodimeric transcription factor complexes consisting of the bZIP proteins Fos and Jun), are induced in response to diverse stimuli in the brain, such as electrical stimulation, stress, psychotropic drugs, novel experience and spatial learning [34, 161]. Induction of AP-1 mRNA in neurons requires CREB activation [36]. Although roles have been established for AP-1 components ΔFosB and c-fos in synaptic and
behavioral plasticity [8, 35, 162, 163], the specific mechanisms and signal transduction pathways that initiate and sustain AP-1 dependent neuronal processes have yet to be elaborated [1]. For instance, the requirement for kinase-mediated modification of immediate-early transcription factors remains poorly studied in the context of neural plasticity, and early-response genes downstream of these critical IEGs, have not yet been identified.

While the majority of plasticity studies have focused on CREB and the ERK signaling cascade [16, 147, 164], recent studies, especially of other MAP-kinase family members [165], lead to a broader view of the molecules involved in neuronal plasticity and memory formation. The JNK/MAPK (Jun N-Terminal kinase) signaling cascade and AP-1 proteins have recently been shown to play critical roles in long-term plasticity and memory formation in mammals [34, 35, 162, 163, 166]. Similarly, p38/MAPK mediates memory formation in the rat hippocampus [167] and both short- and long-term synaptic depression in Aplysia [165].

As suggested by its importance in the control of processes underlying cocaine addiction [1], new data indicate that AP-1 may widely influence transcriptional events that underlie long-term synaptic plasticity. AP-1, under regulation by JNK, positively regulates both synaptic growth and synaptic strength at the Drosophila neuromuscular junction (NMJ) [45]. At this synapse AP-1 shows a wider range of influence than CREB whose effects at the same synapse are limited to controlling synaptic strength [24]. Thus, while neural induction of Fos and Jun together is sufficient to cause increases in synaptic size and efficacy at the NMJ, CREB activation, apparently dispensable for synaptic growth, is
only essential for AP-1 induced changes in synaptic strength [24, 45, 168]. In other neural contexts, the exact roles and mechanisms of AP-1 and JNK signaling in long-lasting forms of plasticity are largely unknown [35, 45, 162, 163, 166, 169]. While many genes regulated during CREB and ΔFosB (a splice variant of the FosB gene) mediated cocaine reward [29] have been recently identified, this study identified exclusively late-response genes whose expression levels were altered following 5-days to 8-weeks of either ΔFosB and CREB overexpression in the nucleus accumbens, or cocaine injection. Here, we address two outstanding questions regarding JNK and AP-1 function in synaptic change. First, using temporally controlled induction of a JNK-activating kinase in the fly nervous system, we address synaptic consequences of JNK activation at the Drosophila neuromuscular junction. Second, using DNA microarray or SAGE (serial analysis of gene expression) to profile neuronal transcripts in control and experimental animals, we identify groups of neuronal genes potentially regulated by either: a) JNK, or b) AP-1 in the fly CNS within 6 hours of pathway activation. Some of these changes were confirmed by quantitative real-time RT-PCR (Q-PCR), including CG6044, that was previously identified in a screen as a potential gene required for normal memory formation in Drosophila [170]. We found five genes are responsive to the progesterone-related steroid RU486 commonly used for temporal control of GAL4-restricted transgene expression in Drosophila [45, 171]. In addition, the mini-white gene, a common marker used in most Drosophila transgenes, is induced strongly by AP-1 and JNK signaling. These observations and their wider implications are discussed below.
Results

Neuronal JNK activation triggers synaptic growth

To assess the effect of neuronal JNK activation on synaptic change we expressed an activated JNK-kinase - *hemipterous (hep\textsuperscript{act})* [172] – in the nervous system and analyzed associated phenotypic consequences. Chronic overexpression of *hep\textsuperscript{act}* with neuronal GAL4 drivers (C155, C380, D42 and OK6) caused lethality ranging from late embryonic to early 2\textsuperscript{nd} instar larval stages.

In order to overcome this deleterious effect, we drove expression using the inducible GeneSwitch GAL4 (GS-GAL4) system to express *hep\textsuperscript{act}* acutely in postembryonic neurons [45, 171]. Age-selected larvae were exposed to the inducing ligand RU486 between late 1\textsuperscript{st} instar and early 2\textsuperscript{nd} instar larval stages and allowed to develop to climbing 3\textsuperscript{rd} instar larval stage. Neural overexpression of *hep\textsuperscript{act}* resulted in a 30% increase in bouton number at the larval NMJ compared to the non-RU486 fed sibling controls (Figure 4.1). Changes in synapse size may not be attributed to the effect of RU486 since wild-type animals fed the steroid did not show a significant change in bouton number (Figure 4.1C). Thus, postembryonic activation of JNK signaling in the CNS leads to synaptic growth at the larval motor synapse.

JNK activation disrupts transmitter release and alters presynaptic composition

To evaluate the effect of JNK activation on transmitter release and postsynaptic response, we measured both spontaneous and evoked junctional potentials with and without JNK
activation in postembryonic CNS. Increased hep\textsuperscript{act} resulted in an unexpected 60% decrease in the amplitude of excitatory junctional potentials (EJP) (Figure 4.2A, B). Increased neuronal JNK signaling also decreased the amplitude of spontaneous responses by ~50% (Figure 4.2C). The quantal content of presynaptic transmitter release shows a 30% decrease when hep\textsuperscript{act} is overexpressed in postembryonic neurons (Figure 4.2D). Therefore, while sustained postembryonic JNK activation in the CNS triggers synaptic growth, the strength of the synapse is reduced. A potential cellular mechanism that underlies this reduction in quantal content was suggested by immunohistochemical analyses of NMJs in JNK-activated animals.

Presynaptic proteins including synaptic vesicle proteins, Synaptotagmin, Csp and antigen(s) recognized by anti-HRP were substantially decreased when JNK was activated in motor neurons (Figure 4.3 A-F). Levels of Syt staining were reduced by 45%, Csp by 30% and anti-HRP by 50% (Figure 4.3G). In contrast, postsynaptically enriched proteins, Fasciclin II and Dlg, do not show any change in intensity.

Because JNK has been implicated in axonal transport, we asked whether transport defects could possibly explain how JNK alters presynaptic composition [173, 174]. Defects in axonal cytoskeletal assembly or anterograde axonal transport cause accumulation of Syt positive puncta on axonal tracts [174]. Such organelle jams were not present in axonal tracts of larvae overexpressing hep\textsuperscript{act}. The nerves were indistinguishable from control animals, indicating that visible axonal transport defects are not present after overexpression of hep\textsuperscript{act} (data not shown). For the purposes of this study, these results simply point to potential effects of JNK activation in the CNS that go beyond its
previously defined role as a positive regulator of AP-1 and, thereby, of synaptic growth and synaptic strength [45].

Genome-wide screen to identify JNK targets in neurons

To identify transcriptional targets of JNK signaling in the nervous system we performed a genome-wide analysis of JNK-responsive genes in the Drosophila larval CNS using Serial Analysis of Gene Expression (SAGE). SAGE is an approach that has been extensively used in analyzing expression changes in cancer cells and other disease states as well as to analyze gene expression in the Drosophila embryo and developing eye [175-177]. SAGE is based on generating unique 14 bp tags at a defined position in almost every transcript and, following random sequencing of some 20,000 cDNAs, analyzing the frequency at which each tag (and hence each transcript) occurs in a sample RNA. We used SAGE to a) profile gene expression in the fly central nervous system; and b) identify transcriptional targets of neuronal JNK signaling. To identify early mediators of synaptic change, we analyzed RNA expression 6 hours after JNK activation. We induced a 6-hour burst of neural hep<sup>act</sup> expression in third-instar larval nervous systems using RU486 feeding to induce transcription mediated by neural GS-GAL4. In experimental ElavGS-GAL4-hep<sup>act</sup> animals, we confirmed that JNK signaling was significantly activated by performing the following tests. Quantitative PCR demonstrated a 16-fold induction of hep mRNA in larval CNS after hep<sup>act</sup> overexpression (P<0.001) (Figure 4.5). RNA in situ hybridization of RU486 exposed larval CNS showed a marked increase in hep mRNA localization in the entire larval CNS (Figure 4.4A). Immunostaining with an antibody specific for phsophorylated JNK showed induction of hep<sup>act</sup> mRNA leads to
activation of JNK (Figure 4.4B). Finally, we observed that downstream gene expression of a JNK target gene puc occurs after hep act induction in the CNS. puc mRNA is induced nearly 3-fold (P<0.01) by Q-PCR analysis (Figure 4.5) and in an “enhancer trap” lacZ line we were able to visualize puc promoter activity in the larval CNS (Figure 4.4C). Thus, our protocol to stimulate neural JNK is sufficient to induce an established downstream target of JNK signaling. Exposure of identically cultured, wild-type animals to RU486 did not lead to induction of hep or puc mRNA or lead to activation of JNK (data not shown). Hence, changes observed between RU486 treated and untreated animals should be largely attributable to JNK signaling in the larval CNS.

We sequenced approximately 20,000 tags from individual libraries prepared from dissected larval nervous systems of either control or hep act expressing animals. About 9900 unique SAGE tags represented in these libraries were associated with specific genes/genomic sequences using either a database containing predicted tags of all genes annotated by the BDGP [57] or BLAST searches to identify other transcription units [175, 177]. Approximately 10% of tags with less than 3 matches to the genome mapped to regions with no predicted gene. About 12.5% of all tags did not match the genome probably due to polymorphisms, errors in sequencing or possible gaps in the published Drosophila genome sequence. Comparison of the top 60 expressed genes in the CNS SAGE library to embryonic and photoreceptor SAGE libraries revealed that while 32% of these genes are highly expressed in all 3 tissues such as the cytoskeletal protein betaTub56D, 37% are enriched in the nervous system like the translation elongation factor Ef1alpha100E (see Table 4.1). Such comparisons could prove useful for
understanding transcriptional regulation and other processes in different tissues (see Discussion).

Potential JNK-target genes were identified by comparing the relative representation of specific tags in control and hep\textsuperscript{act} expressing nervous systems (Figure 4.4D). A tag was considered up or downregulated when present 3 or more times in a given library and changed at least 3-fold between the two libraries. By these criteria, 346 tags were increased while 271 were decreased following JNK induction. Of these, 25 were “upregulated” and 32 “downregulated” more than 8-fold. Approximately 50\% of the induced or repressed tags in the hep\textsuperscript{act} library mapped to genes that fell into different functional classes, ~35\% of these tags mapped to genes that have no predicted function and ~10\% mapped to parts of the genome without any predicted genes (see Figure 4.4E, F).

To determine whether predictions of SAGE could be confirmed by more careful single gene analyses, we performed Q-PCR to measure relative levels of expression of selected candidate JNK-target genes in control and JNK-induced nervous systems. We selected 61 candidate genes for such Q-PCR verification based on: 1) an abundance of tags for that gene in the hep\textsuperscript{act} induced library (9 genes); 2) an interesting known function for the gene (12 genes); 3) presence of AP-1 binding sites in the promoter region of the gene (11 genes); and 4) random selection of genes that did not fall into the above criteria (29 genes). From the 61 genes so examined, 15 that showed induction in at least two independent RT-PCR analyses were analyzed further, namely more extensive Q-PCR analyses using RNAs from 5 independent JNK-induction experiments. In the end, only
three genes showed consistent JNK-responsiveness ($P<0.05$) (Figure 4.5). *white* showed consistent and robust increases in mRNA levels, whereas *appl* and *cher* showed smaller magnitude inductions. To test if some of the candidate JNK target genes were robustly regulated in subsets of neurons, but diluted out in the Q-PCR analysis, we examined the expression of RNA in the larval CNS using *in situ* hybridizations with probes for several candidate mRNAs. We did not see a clear increase in expression in any of the putative target genes in *hep* act expressing larval CNSs other than *white* (Figure 4.8; also see microarray screen validation).

The small number of SAGE-predicted JNK target genes confirmed by RNA *in situ* and Q-PCR analyses was difficult to explain without multiple repetitions for which SAGE, being expensive and time-consuming, is not ideally suited. Also, we speculated that genes expressed at lower levels than those identified by SAGE may be true JNK/AP-1 target genes. To test and further these considerations, we used a different genomic approach - oligonucleotide microarrays - to search for neuronal AP-1 target genes.

**Whole-genome microarray screen to identify direct AP-1 target genes in the nervous system**

We performed comprehensive analyses of transcript levels in fly heads using Affymetrix *Drosophila* Genome1 GeneChip arrays representing the entire annotated genome at the time of its release (~13600 unique genes). An outline of the microarray screen design is illustrated in Figure 4.6. The analysis compared mRNA levels, with or without AP-1 induction using the same conditional GS-GAL4 strategy described for the previous SAGE analysis. After treatment with RU486 for 6 hours to induce *fos* and *jun* we
consistently achieved, respectively, ~2.5- and 9-fold induction of *fos* and *jun* transcripts in fly heads (quantified by Q-PCR in 1-3 day old adult flies, Figure 4.7A). Untreated control animals showed no significant difference of either gene when levels were compared between age-matched siblings from the same experiment (average untreated change).

Each array “experiment” included sibling flies split into three groups: group A was the experimental (AP-1 induced) population (and groups B and C were independent controls). Thus, each experiment typically allowed transcript levels (normalized hybridization signals) to be compared between experimental and control samples ("A/B" or "A/C" comparisons), and between two identically treated controls (a "B/C" comparison). Candidate AP-1 responsive transcripts would be identified as those with "A/B" and "A/C" ratios significantly different from control "B/C" ratios. This experimental design was useful because hybridization signals for some mRNAs varied significantly more than others and could potentially confound a more straightforward analysis. Through 5-7 repetitions of this basic experiment, we obtained 12 independent experimental versus control ratios, and 5 control-control ratios from which means, variances and SEMs could be determined. Sibling, age-matched controls used in each experiment ensured that genetic background, which can have a large effect on transcriptional variance [58], was not a confounding factor in our analyses.

Based on analyses of 19 hybridizations we established that basic elements of the array technology, probe labeling, hybridization and scanning, were working efficiently and reproducibly (See Methods for a complete description). Microarray hybridization data
were passed through three statistical filters to select the most promising AP-1 responsive genes (Methods). Filter 1: We asked that the average ratio of hybridization signal from AP-1 induced versus control mRNA hybridization was significantly ($P<0.01$) different from 1.0 by Student’s $t$-test. Filter 2: We asked that the signal ratio be greater than 1.2. Filter 3: Through analysis of variation observed in identical control-control comparisons, we ensured that genes passing filters 1 and 2 did not show wide variability, for instance based on physiological states of the flies.

Using filter 1: of the ~5200 genes considered for analysis (those with relatively strong and specific hybridization signals), 269 showed altered expression after AP-1 induction, with a significance of $P<0.01$ (Student’s $t$-test). Strikingly, 167 genes showed significant upregulation while only 102 were downregulated, a skew consistent with AP-1’s expected role as a transcriptional activator. 269 candidates, at $P<0.01$, is substantially larger than predicted by random chance (52 genes - 0.01 X 5200 genes). However, when a second filter – a requirement that the signal ratio modulus be greater than 1.2 – was applied, the number of candidates dropped to 115. Though small, such signal ratios could correspond to higher mRNA ratios and have been reported as meaningful in previous microarray experiments. Filter 3, to eliminate “variable” genes, trimmed the list of candidate genes that respond consistently to AP-1 overexpression in the fly head to either 4 ($P<0.01$) or 16 ($P<0.05$, listed in Table 4.2) for which “A/B” and “A/C” ratios were significantly greater than control “B/C” values by Student’s $t$-test.

An internal control for the array screen and analysis was provided by Drosophila Jun ($Jra$), whose mRNA was experimentally induced. We found that $jun$ ranked highest once
all three filters were applied and showed robust induction with an average log<sub>2</sub> treated expression ratio of 1.26 (P=5.9e-11) and an average untreated expression change of only -0.07 (Table 4.2). In contrast, *fos* did not pass these stringent filters although we consistently observed an average 2.3-fold increase in *fos* transcript levels by quantitative RT-PCR (Figure 4.7A). This discrepancy may arise from either of two limitations: a) that *fos* is a low-abundance transcript in the fly head, below the threshold for quantifiable gene expression change detection using Affymetrix GeneChip arrays; or b) the *fos* probe on this particular array may not perform reliably [178], perhaps hybridizing to other non-specific RNA probes. Many gene probes could have similar problems; indeed, other transcripts may exhibit altered expression levels beyond the scope and sensitivity of this assay.

We searched promoters (sequences 3kb upstream of the translation start sites) of the top 15 candidate AP-1 responsive genes for conserved AP-1 or CREB binding sites and compared their frequencies of occurrence in this group with frequencies observed in a control group of 15 genes that appeared insensitive to AP-1 induction. This analysis revealed no significant enrichment of CREB or AP-1 binding elements in promoters selected based on the microarray experiments (data not shown).

**Microarray screen validation**

A major task after initial microarray screening has been completed is confirmation of candidate gene transcript level changes using secondary, independent tests for gene expression. Although the frequency of false-positives is substantially reduced through
repetition, a subset of observed expression differences should be validated by other methods.

To confirm positives, a subset of the most robustly changing AP-1-responsive genes, exhibiting significant up- or down-regulation by microarray analysis, were selected as candidates for real-time quantitative RT-PCR validation using gene specific primers (see Table 4.2). 12 genes chosen from the group of 15 top candidates mentioned above, in addition to more than 30 genes from outside this stringent set - those with very low “$P$” values or specific predicted biological functions - were selected for these more careful confirmatory experiments. Increases in transcript levels following AP-1 overexpression, detected by Q-PCR, for $fos$, $jun$ and 2 confirmed candidate genes ($white$ and $CG6044$) are shown in Figure 4.7A. All mRNA levels are normalized to the control gene $rp49$.

Transcript levels for a second control gene, $gapdh1$, are shown to demonstrate its levels do not change significantly by either AP-1 induced versus control (“A/B”, “A/C”) or control-control (“B/C”) comparisons.

Five uncharacterized genes ($CG2016$, $CG11191$, $CG15438$, $CG5853$ and $CG3348$) were confirmed by Q-PCR to be consistently altered in RU486-treated, AP-1 induced samples (Table 4.2, Figure 4.7B - data for $CG5853$ and $CG3348$ not shown). In addition, overexpression of $fbz$ with RU486 treatment also caused a similar change in transcript levels of these 5 genes. When treated with the steroid, wild type flies and all other transgenic lines tested showed consistent alterations of these 4 mRNA transcripts in the head, suggesting they are hormone-responsive genes in the fly.
As in the larval CNS, Q-PCR experiments confirmed *white* gene induction in the adult fly head. *white* transcript levels are significantly increased in the head following AP-1 overexpression in the brain (Figure 4.7A, Table 4.2, Figure 4.8). Further Q-PCR experiments demonstrated *white* transcripts are increased to an even greater extent when *hep*\textsuperscript{act} is induced in combination with AP-1 or by itself in the adult nervous system (Figure 4.8A), although its levels are not increased to the degree seen in the larval CNS (Figure 4.5). *white* is not induced when *fbz* is overexpressed, nor in wild type flies treated with RU486. Only primers designed to the 3’ portion of the *white* transcript showed altered levels (Figure 4.8A), which is consistent with the background strain used in all the experiments (\textit{w}\textsuperscript{1118}). This strain lacks the 5’ portion of the *white* gene locus [179], yet still contains sequence for and expresses the second through fifth exons (data not shown) that are induced in response to JNK signaling in the fly head. The same transcriptional induction profile is observed in *ElavGS-GAL4-hep*\textsuperscript{act} flies with the wild-type (\textit{w}+) copy of the *white* gene on the X chromosome as well as in a *white* null (\textit{w}\textsuperscript{11E4} [180]) background (data not shown). This suggests *white* induction occurs via the *mini-white* cassette present in pUAST transgenes. RNA \textit{in situ} hybridization experiments confirmed the increase in *mini-white* transcript levels in the CNS of larvae in which *hep*\textsuperscript{act} has been induced (Figure 4.8B). Increases were also observed in larvae overexpressing AP-1 (data not shown), albeit with smaller magnitude changes consistent with our findings from Q-PCR analyses in the adult head (see Figure 4.8A).

\textit{CG6044} induction following AP-1 overexpression was also confirmed in independent Q-PCR experiments (Figure 4.7A). Consistent increases in transcript levels were observed
in all AP-1 overexpressing heads but not in treated fbz or wild type heads (data not shown). The induction observed by quantitative RT-PCR and microarray experiments was not reflected in follow-up in situ experiments; however, this is likely because the small magnitude increases in transcript levels observed by other means (1.2-fold - microarray; 1.4-fold - Q-PCR) are below the detection range for this method.

Discussion

This extensive study makes three contributions: (A) it demonstrates unexpected and novel interactions between JNK and cellular processes that underlie synapse plasticity; (B) by SAGE analyses, it provides a genomic profile of mRNAs expressed in the fly larval nervous system; (C) it presents two large-scale genomic approaches to identify JNK and AP-1 targets in the fly CNS providing useful data pertinent to JNK/AP-1 signaling in neurons as well as to genomic analyses in the Drosophila nervous system.

Effects of JNK activation in postembryonic motorneurons

The immediate-early transcription factor AP-1 positively regulates both synapse size and synapse strength at the Drosophila larval NMJ [45]. While JNK signaling is necessary for the effect of AP-1 on synapse structure and function, it is not clear whether JNK signaling is sufficient for synaptic change. We show, first, that activation of JNK in post-embryonic neurons leads to significant synaptic alterations; second, that these alterations are inconsistent with JNK functioning solely through AP-1. Our finding that activation of JNK signaling leads to an increase in synapse number but decreases synapse strength
indicates that JNK activates not only AP-1, a positive regulator of growth and strength, but also a pathway that negatively influences synaptic strength.

The neural transcriptome, and its regulation by JNK and AP-1

The ability of SAGE to evaluate absolute expression levels of gene transcripts enables relatively facile, quantitative, profiling of gene expression in any given tissue (or RNA source). Given the intense interest in *Drosophila* neurobiology, a previous painstaking sequence analysis of some 1000 cDNAs from a fly brain cDNA library provided useful new information on the neural transcriptome [181]. The analysis presented here, following sequencing of about 20,000 ESTs from two independent brain libraries, substantially extends the previous study. The use of this resource is demonstrated by our simple survey of highly expressed neuronal RNA-binding proteins, potentially involved in important neural-specific, post-transcriptional functions such as translational repression, mRNA transport or RNA editing. 10% of the 60 most highly expressed (non-ribosomal) mRNAs in nervous system encode RNA-binding proteins, 2 of which are enriched in neurons versus embryonic tissue. A significant fraction of these (3/6) have conserved homologs recently found on RNA granules, organelles containing translationally repressed mRNAs which are actively transported to synaptic sites [182]. We have recently begun functional analyses of some of these RNA-binding proteins. Similarly, we anticipate that identification of tissue-specific genes could provide unanticipated launch points for investigation into their cellular functions.

Given the evidence to indicate wide effects of AP-1 and JNK on synaptic properties, we searched for AP-1 and JNK-target genes using both SAGE and microarray approaches to
determine effects of JNK and AP-1 signaling on neuronal gene expression. Of the two approaches, microarray analysis, being dependent on parameters such as hybridization and labeling efficiencies that vary among individual transcripts, is not ideal for quantitative analyses as outlined in the previous section. However, it provides information on transcripts with low to moderate levels of expression, is fast, and allows multiple iterations of each experiment at a small cost relative to SAGE.

In order to identify early transcriptional targets, most likely to link JNK and AP-1 activation to synaptic change, we used the steroid-inducible GAL4 system, an increasingly popular strategy to achieve conditional, tissue-specific transgene expression in *Drosophila* [45, 171, 183, 184]. SAGE-derived transcript profiles of RNA extracted from whole larval CNSs showed several potentially significant targets. However, very few were confirmed by secondary low-throughput, gene-specific analyses. Microarray-derived transcript profiles of adult head mRNA showed similar results. Several statistically significant targets of AP-1 signaling were initially identified; however, few were confirmed by carefully controlled application of the most commonly used transcript-specific analyses (quantitative RT-PCR and *in situ* RNA hybridizations).

While the implications of these results for neurogenomics are briefly discussed in the next section, we first consider the “positive” genes identified by SAGE and microarray screens.

Quantitative RT-PCR validation of the generated SAGE data resulted in the identification of 3 genes, *cher*, *appl* and *white*, which were consistently upregulated following JNK activation in the larval CNS. Though we were unable to evaluate induction of *cher* and
appl by RNA in situ hybridization, white showed robust increases by this method as it did by Q-PCR. A total of seven expression changes identified in the microarray screen were verified by Q-PCR analysis; remarkably, five turned out to be genes responding to RU486 treatment itself rather than to consequent AP-1 induction. These steroid-responsive genes may be of significant biological interest. However, from our point of view they serve primarily to: a) further establish the bonafides of our experimental and analytical protocols; and b) as a useful caution for Drosophilists and others using the steroid-inducible conditional expression system. The remaining two confirmed AP-1 target genes were white, also identified in the SAGE screen but shown eventually to be expressed from the P-element associated mini-white locus, and CG6044. Of potential significance, CG6044 has been implicated in olfactory associative memory [170]. AP-1 responsiveness of CG6044 was verified in Q-PCR validation experiments (Figure 4.8). The gene was previously found in a mutational screen for putative memory genes required for normal olfactory conditioning in Drosophila [170]. In addition, it is one of the few genes from the list of likely AP-1 targets (listed in Figure 4.9) that has a conserved AP-1 binding site within 500 base pairs of its translation start site. It is therefore a promising candidate warranting further investigation into the role it plays in synaptic plasticity and memory formation.

Lessons and limitations

It appears unlikely, if not inconceivable, that the 4 probable downstream genes enumerated above could mediate the demonstrated effects of AP-1 or JNK induction on motor-synapse properties. Thus, the genomic approaches we have followed, while
informative, have likely not led to the identification of JNK/AP-1 targets that link these signals to synaptic change. One possible interpretation, that the experiments were technically flawed, appears to be ruled out, not only because internal controls (Jun, Hep, Puckered and steroid-responsive genes) were identified in the screens, but also because various standards for microarray hybridization data and SAGE library complexity were evaluated and shown to be well within the technically optimal range. Thus, we are left with the second interpretation, that analysis of whole-brain mRNA may not allow targets of signaling pathways to be unambiguously identified. A major issue is likely to be cell-type heterogeneity within the brain. If different subsets of neurons show substantially different genomic responses to JNK/AP-1 (including the absence of a response), then altered expression of the meaningful JNK/AP-1 targets in a subset of cells may be diluted by the large background of mRNA deriving from other neuronal types.

At a conceptual level, Barolo and Posakony have nicely articulated the concept of “activator insufficiency” and the need for cooperative activation of multiple transcription factors for turning on transcriptional pathways governing developmental processes [185]. Considerable evidence argues that neurons are a diverse class of cells with a range of distinct transcriptional ground states. For example, cell-type-specific binding of CREB to known target gene promoters has been shown in various cell types under basal and stimulated conditions [186]. Similarly, the response of different neuronal populations to TGFβ has been shown to be highly context dependent and to derive from variations in expression of specific TGFβ insensitive transcription factors [187]. Thus, genomic
analyses when applied to whole nervous systems may have significant intrinsic limitations.

Nevertheless, some conserved downstream genes may still be revealed [51-54]. For instance, the steroid hormone, RU486, used to induce transgene expression in our experiments presumably activates a set of hormone-responsive genes in a large subset of neural cells. However, for incisive mechanistic analyses for which *Drosophila* is so convenient, we suggest that genome-wide screens described to study signaling responses in the nervous system be applied with specific refinements, such as emerging methodologies to prepare sufficient mRNA from a homogeneous population of cells in which biological function of these signaling pathways have been evaluated [188]. Various GFP transgene lines should make it possible to sort specific cell populations prior to genomic screens to identify transcriptional targets.

The availability of new genetic and molecular tools and refined functional genomic approaches should result in continued understanding of how kinases and transcription factors regulate molecular changes that occur in the *Drosophila* nervous system, as well as intrinsic flexibility and constraints of these signaling pathways.

Conclusions

This study revealed unexpected relationships between JNK signaling and synaptic plasticity in *Drosophila* that are inconsistent with a role for JNK acting solely through AP-1 to affect strength of the synapse. It also presents a profile of the transcriptome of the larval nervous system and, while providing potential transcriptional targets of JNK
and AP-1 signaling in neurons, points out the pitfalls of genome-wide analyses in complex tissues such as the whole fly nervous system.

Authors’ Contributions

PDE and MR conceived of and designed the AP-1 overexpression experiments. PDE performed the microarray experiments and statistical analysis; PDE and CP performed the microarray confirmation experiments. RN, HJ, MR and DB conceived of and designed the SAGE experiments. RN and HJ performed the SAGE experiments and analysis; RN and ZN performed the hep act overexpression and SAGE validation experiments. PDE, RN and MR drafted the manuscript with input from the other authors.

Acknowledgements

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doctoral training grants (T32) 532GM08659 and (T32) AG07434-04 to the University of Arizona.
Figure 4.1. Postembryonic expression of hep\textsuperscript{act} in larval neurons increases synaptic growth. Confocal projections of synaptic arbors show that synapse size is increased after hep\textsuperscript{act} induction in postembryonic neurons (B) compared to control (A). C) A histogram representation of bouton number shows that hep\textsuperscript{act} overexpression leads to a 31% increase in synapse size ($P<0.001$), while exposing wild-type larvae to the inducible ligand does not cause a significant change in synapse size.
Figure 4.2. *hep*<sup>act</sup> expression leads to decreased transmitter release. A) EJP traces from larvae in which *hep*<sup>act</sup> expression is induced (lower trace) or control (upper trace). Expression of *hep*<sup>act</sup> in postembryonic neurons leads to decreases in EJP and miniature (m)EJP amplitude by nearly 50% (*P*<0.01 for both) compared to control (B, C). D) Quantal content of presynaptic transmitter release is reduced by 35% after *hep*<sup>act</sup> induction in postembryonic neurons (*P*<0.04).
Figure 4.3. Presynaptic protein levels decrease with hep\textsuperscript{act} overexpression. Confocal projections of synapses show that levels of presynaptic protein synaptotagmin (Syt) (B) and an antigen recognized by anti-HRP (D) are reduced after hep\textsuperscript{act} induction compared to controls (A, C), whereas levels of the postsynaptically enriched protein dlg is similar to control (E) after hep\textsuperscript{act} induction (F). G) Quantification of fluorescent intensities show that levels of presynaptic proteins Syt, Csp and anti-HRP, are reduced by 45%, 34% and 50% respectively ($P<0.001$ for all) compared to control. Levels of postsynaptically enriched proteins FasII and Dlg go not significantly change after hep\textsuperscript{act} induction in postembryonic neurons.
**Figure 4.4. Acute induction of hep\textsuperscript{act} in larval neurons.** A) RNA *in situ* hybridization using a probe specific for hep shows inducible expression of hep mRNA in third instar larval CNS. B) Western blot analysis of larval CNS protein extracts shows increased levels of activated JNK (P-JNK) after hep\textsuperscript{act} induction. C) A lacZ enhancer trap line of puc shows increased lacZ expression after hep\textsuperscript{act} induction in larval CNS. D) A distribution of up- and down-regulated SAGE tags comparing hep\textsuperscript{act} induced and control libraries, indicates most tags are present in similar numbers in both the induced and control libraries. E) Functional classification of SAGE tags upregulated after hep\textsuperscript{act} induction. Approximately 50% of tags map to genes with no known function and to regions of the genome without an identified gene.
Figure 4.5. Q-PCR validation of SAGE results. Quantitative comparisons of transcript levels in larval CNS RNA from RU486 treated (\(hep^{\text{act}}\) induced) versus control samples. Values represent average cycle difference in PCR product between induced and control samples (N\(\geq\)5). Each cycle change corresponds to a 2-fold difference in mRNA levels (see Methods). After induction of \(hep^{\text{act}}\), \(hep\) RNA levels increase 16-fold relative to control (\(P<0.001\)); \(puc\) RNA, not identified by SAGE, is induced three-fold relative to control (\(P<0.01\)). While SAGE targets \(appl\) and \(cher\) show induction above control RNA levels, \(white\) is induced more than 32-fold (\(P<0.001\)).
Table 4.1. Comparison of the top 60 expressed genes in larval CNS with expression profiles from embryo and photoreceptor cells identified by SAGE. The top 60 highly expressed tags from the hep\textsuperscript{act} CNS control library were compared with control libraries from embryonic [175] and photoreceptor tissues (Jasper and Bohmann, unpublished data). The number of tags for each gene is indicated on the left for all three tissues and all libraries examined, normalized, to the same number - 20,000 - of total tags sequenced. Tag rankings are sorted in descending order for the control library for each tissue using hep\textsuperscript{act} CNS control library as reference, after eliminating tags with more than 3 matches to the genome and excluding any ribosomal RNA binding proteins (highly enriched) and selecting only tags that mapped to an identified gene. 19/60 (green) highly expressed genes in the CNS libraries were also in the top 60 of highly expressed genes in the embryo and photoreceptor libraries while 22/60 (white) are found in the top 60 only in the CNS libraries. There were 13/60 (pink) genes found only in the top 60 of CNS and photoreceptor libraries and 6/60 (gray) genes found only in the top 60 of CNS and embryo libraries.
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Figure 4.6. **Microarray experimental design, analysis and validation.** To induce AP-1 in the nervous system, 1-3 day old adult *ElavGS-GAL4-AP1* flies were treated with the synthetic steroid hormone RU486 in 2% sucrose or sucrose alone for six hrs. Biotinylated RNA from heads was created and hybridized to Affymetrix *Drosophila* Genome1 GeneChip arrays. Gene expression changes between AP-1 induced and control samples were considered significant if they passed a statistical (*P*<0.01, Student’s *t*-test) and secondary filters looking at variance in untreated control samples from the same experiment. Validation of candidate gene expression changes was carried out using quantitative real-time RT-PCR and *in situ* hybridization experiments.

**Observe gene expression changes in *ElavGS-GAL4-AP1* flies**

**Treatment:**
- A: AP-1 induced
- B: Control 1
- C: Control 2

**Hybridization:**
- Labeled RNA from heads hybridized to Affymetrix *Drosophila* GeneChip™ oligo arrays

**Analysis:** Average ratio of A/B and A/C compared to expected ratio of 1.0
- Statistical filters based on replicate untreated control samples (Figure 9)

**Validation:** Quantitative real-time RT-PCR and *in situ* hybridization
Figure 4.7. **Q-PCR validation of AP-1 induction and microarray results.** A) Quantitative comparisons of transcript levels in adult head RNA from RU486 treated (AP-1 induced) versus control (black) and control-control (gray) samples. Values represent average cycle difference in PCR product between samples being compared (N=5). Positive values indicate an increase in transcript compared to unchanging reference gene, negative values a decrease. *fos, jun, white* and *CG6044* RNA levels are increased in the fly head after AP-1 induction in the nervous system while untreated control levels show no significant difference. B) Comparisons of transcript levels in adult head RNA from RU486 treated versus control samples. *CG2016, CG11191* and *CG15438* levels are induced in all samples from flies fed the steroid hormone RU486 (N=3). X-axis in panel B indicates the UAS-transgene(s) induced by *ElavGS-GAL4* (AP1: *UAS-fos;UAS-jun*). *Average difference between samples significant at P<0.05 (Student’s *t*-test).
Table 4.2. Top 15 candidate AP-1 responsive genes identified by microarray analysis. Genes altered following neuronal AP-1 overexpression, passing statistical (Student's t-test, $P<0.01$) and secondary filters based on ratio thresholds and variance in untreated control samples from the same experiment. Arrows on left indicate directionality of expression change listed in order of magnitude from largest positive ratio (induction) on top to largest negative ratio (repression) on bottom. jun induction shows the largest expression change by these criteria. Predicted functions from Flybase [189]. Bold type highlights expression changes confirmed by Q-PCR (n.t. = not tested). Blue type highlights RU486-responsive genes. *VGA – volatile general anesthetic.

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<tr>
<td>Cys4a3</td>
<td></td>
<td>cytochrome P450</td>
<td>n.t.</td>
<td>.001</td>
</tr>
</tbody>
</table>
Figure 4.8. *white* transcript levels are induced when positive JNK pathway components are overexpressed in the fly nervous system. A) Quantitative comparisons of *white* transcript levels in adult head RNA from RU486 treated versus control samples. PCR primers designed to the 3’ end of *white* (black), but not 5’ primers (gray), show increased levels in response to AP-1 and *hep*\(^{act}\) induction (N=3). Wild-type flies exposed to hormone or flies overexpressing *fbz* do not show induction of *white*. X-axis indicates the UAS-transgene(s) induced. *Average treated difference significant at P<0.05 (Student’s t-test). B) *in situ* confirmation of increase in *white* transcript levels following *hep*\(^{act}\) induction in the larval CNS.
Why didn't it work?

There are many reasons why the whole-genome approaches described in this thesis may not have provided as many real AP-1 targets as hoped. These include: 1) dilution of real, biologically meaningful changes by RNA in other tissues present in the fly head, such as glia, muscle and cuticle or by differing responses in diverse neuron sub-types; 2) altered expression may only occur in a small number of critical neurons where the transcript is normally expressed; 3) our protocol may not have induced the activation of other necessary co-activators or the cooperative activation of multiple signaling pathways that are required for gene induction in neurons.

The computational searches may have identified true targets of AP-1 and CREB signaling that simply could not be verified by our assays. Some genes may only respond to relevant signaling pathways in specific tissues other than the brain or in a subset of cells within the nervous system. Alternatively, AP-1 may cause different transcriptional responses in diverse neuron sub-types, which could significantly muddy gene expression analyses in the whole brain. Diverse responses to TGFβ signaling in Drosophila neurons have been documented, delineating a set of transcription factors (or a transcription factor code) that must be expressed in the same neuron in order for a downstream gene, *FrmFamide*, to be activated [190]. This suggests other signaling pathways may also
require a transcription factor code that is only active in a small number of neurons within
the head. For instance, 14-3-3ζ (*leonardo*) was identified as a strong candidate for AP-1
and CREB dependent transcription in our computational screens searching for 1CRE and
1AP-1 site or multiple AP-1 binding elements, but was not confirmed in any of our gene
expression experiments. This may be explained by the fact that the promoter identified in
the screen actually lies in the first intron of the upstream *Djun* gene. AP-1 and CREB
proteins have been shown to bind and modulate *Djun* expression through these sites
[9, 10], and thus may not regulate 14-3-3ζ expression at all. However, 14-3-3ζ
does have a defined role in learning and short-term memory in *Drosophila*, originally
identified as a learning and memory mutant because of its preferential expression in the
mushroom body (the presumptive 'learning center' in flies)[189, 191]. Thus, 14-3-3ζ may
indeed display altered expression following AP-1 induction in the small subset of
competent neurons where it is normally expressed; however, such changes would not be
detected with the sensitivity of our methods.

Recently, cell-type-specific binding of CREB to CRE sites and recruitment of
CREB binding protein (CBP), which may require additional CREB regulatory partners,
at target promoters has been demonstrated in other systems [32, 186]. Hence, it could be
that chromatin structure and/or a lack of other critical activators may inhibit the binding
of AP-1 and CREB complexes to promoters in some cells within the brain where they
might otherwise activate transcription. Presumably in motor neurons, where we know
AP-1 induction causes phenotypic changes we can measure at the synapse, AP-1 and
CREB are able to bind accessible AP-1 binding sites and CREs in order to exert their
effects; whereas, in other neuronal sub-types within the fly brain, AP-1 and CREB may not be able to exert the same transcriptional responses.

As mentioned in Chapter 2 the rat homolog of Wnt2, identified in the Pilot screens as a likely AP-1 and CREB target (see Table 2.2), was shown to be a putative CREB target by Richard Goodman's group who assayed global CREB binding in PC12 cells [personal communication][31]. Thus, it is likely that many of the genes from our searches are real CREB targets, but that our protocol for AP-1 induction, though requiring CREB for its action, does not induce CREB or increase its activity to a level sufficient to activate the transcription of these genes - at least not to a point that we can detect by the methods employed.

Additionally, there is evidence that glucocorticoid receptor signaling actually acts to inhibit AP-1 signaling in other systems, perhaps through direct interaction with AP-1 proteins and blockade of its necessary interaction with CBP and the basal transcriptional machinery [192]. Though there is no glucocorticoid receptor (GR) present in flies, which RU486 antagonizes, at least one gene (the estrogen-related receptor - ERR) shows sufficient homology to the rat GR to perhaps act to repress AP-1 signaling in the fly. The fly ERR shows 57% identity over a 68 amino acid stretch and 28% id over another 198 amino acids, with 70% and 48% positive amino acid similarity over these same stretches, respectively. In all, approximately 1/3 of the fly protein shows considerable similarity to the rat protein. If not this protein, some other protein must bind RU486 to cause transcriptional changes in the fly head in response to RU486 feeding to
the whole animal, as demonstrated by the altered expression of at least five genes after steroid treatment (see Chapter 4, Figure 4.7 and Table 4.2).

Computational methods have been successful before

Recently, others have successfully used computational methods to identify real transcriptional targets in other systems, such as targets of the Dorsal gradient and other transcription factors involved in patterning of the *Drosophila* embryo as well as targets of CREB in the human genome [32, 47, 50, 193]. In all cases the binding sites were very well-defined and had been extensively characterized in the model system being investigated. In our case, we still are not certain if conserved CRE and AP-1 binding sites are the best targets for CREB and AP-1 dependent transcription in *Drosophila*. To date the only functionally characterized CREB and AP-1 binding sites are the three AP-1 sites (two degenerate and one conserved) and the one consensus CRE in *Djun*’s own promoter [107], a degenerate yet functional CRE in the *Ultrabithorax* gene [194] and two non-canonical CREs which control cardiac expression of *tinman* [195]. As a result, we may have been looking in the right place for the wrong suspects.

Emerging methods for isolation of homogenous cell populations and tissue-specific pools of mRNA

Recent methods have been developed that would have streamlined our analysis if they could have been utilized, including more restricted, motorneuron-specific Gal4-driver lines available in *Drosophila*. Fluorescence activated cell sorting (FACS) methods could now be used to sort dissociated neurons expressing GFP to isolate a relatively homogeneous population that express GFP along with AP-1 only in motorneurons using
the newer, more specific, Gal4-drivers that could be compared to motorneurons only expressing GFP. Such methods have already been used to identify sensory neuron type-specific genes in *C. elegans* [188].

In addition, protocols for isolating mRNAs from specific tissues in *Drosophila* and methods for cell-type-specific 'mRNA tagging' in other organisms have recently been described [196-198]. The first technique, published only weeks ago, uses a FLAG-tagged poly-A binding protein (PABP) to tag mRNA in a given tissue where the construct is expressed and immunoprecipitation to isolate mRNA bound to the recombinant PABP construct from RNA derived from other tissues. AP-1 certainly could be expressed in conjunction with the FLAG-PABP to isolate AP-1 dependent changes in transcription in a given neuron sub-type or in other tissues in the fly. The second approach, which employs biosynthetic labeling of RNA with uracil phosphoribosyltransferase to isolate cell-specific mRNA, has not yet been published to work in flies, but it certainly will be tried.

These newly emerging procedures should be able to overcome many of the pitfalls and hurdles associated with profiling transcription in complex tissues such as the brain, and offer exciting avenues for future research in the field.
CHAPTER 6
MATERIALS AND METHODS

Fly strains and genetics

We used the following strains: wild type (Oregon R; D. Brower); GAL4-responsive UAS-
hep\textsuperscript{act} (M. Mlodzik), UAS-fbz, UAS-fos, UAS-jun (M. Bienz), puc-lacZ line - puc\textsuperscript{e69} (A.
Martinez Arias); neural GAL4 lines - C155, C380, D42 and OK6 were from C.
Goodman, V. Budnik, G. Boulianne and B McCabe, respectively; ElavGS-GAL4 line was
from T. Osterwalder and H. Keshishian.

Postembryonic and acute induction in neurons

**Induction in larvae:** All animals were generated by crossing males homozygous for
UAS-transgenes (or wild-type males) with virgin females homozygous for the ElavGS-
Gal4 driver. All animals were raised at 25°C and parents transferred to a new vial each
day for age-selection of larval instar stages. For postembryonic induction, larvae in vials
that should contain a majority of late 1\textsuperscript{st} instar-early 2\textsuperscript{nd} instar were transferred into a
standard vial containing 0.015 mg/ml RU486 (Sigma) for 48 hrs before climbing third
instar larvae were selected for further analysis. Control animals were exposed to food
containing only 4% ethanol (same as treated) and analyzed accordingly. For acute
induction in 3\textsuperscript{rd} instar larvae, age-selected larvae were transferred to a 1.5 ml sample tube
containing 0.5 ml of 3 mg/ml RU486 for 2 min, before they were washed and transferred
into a standard vial containing 0.015 mg/ml RU486 (Sigma) for 6 hrs before the CNS was dissected for further analysis.

**Induction in adults:** As in larvae, all animals were generated by crossing males homozygous for UAS-transgene constructs (or wild-type males) with virgin females homozygous for the *ElavGS-GAL4* driver. Progeny reared at 25˚C were aged to be 1-3 days old at time of treatment. 16-64 hour old adults were starved for 8 hours in a Tupperware container filled with desiccant to keep the humidity level at ~16% and ensure ingestion of the treatment medium. Flies were then split into separate bottles and fed for 6 hours. Each treatment consisted of *ElavGS-GAL4-UAS* flies handled identically (aged and starved in the same bottle) except RU486 was added to the sucrose fed to the experimental group. Experimental animals were fed on a kimwipe soaked with RU486 in 2% sucrose at a final concentration of 0.04 mg/ml, taped to the bottom of a large, dry, empty bottle. Sibling control flies of the same genotype were fed sucrose alone.

**Computational searches using Pilot**

The Pilot program was designed by Michael Robinson to identify sequence elements in the presumptive promoters of all genes in a sequenced and annotated genome. The program allows user-friendly identification of putative transcription factor binding sites in the 5' upstream regulatory sequence of genes within a given window up to 3kb in length.

**cDNA microarray construction, hybridization and analysis**

Probes specific for the genes of interest were amplified from genomic DNA using standard PCR protocols and gene specific primers designed to recognize the 3’ end of
desired transcripts. BDGP EST clones were amplified in a 96-well plate using standard PCR protocols and vector specific primers. Purified DNA, at a concentration of ~200ng/µl in a 384-well plate, was spotted on amino-silane slides (Sigma) using the BioRad ChipWriter-PRo.

Total RNA was extracted from 200-300 heads of RU486-treated (AP-1 induced) and untreated control ElavGS-GAL4-AP1 flies (w¹¹¹8;UAS-fos/+;UAS-jun/ElavGS-GAL4) using the RNeasy kit (Qiagen). RNA was amplified using MessageAmp II aRNA Amplification Kit (Ambion). cDNA was created prepared using 2µg aRNA using random hexamers and amino-allyl dUTP (Ambion). aa-cDNA was labeled with fluorescent Cy3 or Cy5 dyes (Molecular Probes). Hybridizations were performed overnight for 16 hrs at 62˚ in GeneMachines HybStation (Genomic Solutions).

Fluorescently-labeled arrays were scanned with an arrayWoRx Biochip Reader, and fluorescence intensities were calculated using softWoRx Tracker software (Applied Precision). Ratios and averages were computed in Excel.

Immunostaining

Larvae were raised at 25°C after postembryonic induction of UAS transgenes, dissected, stained with anti-Syt antibody and mounted. Bouton number was counted from projections of confocal sections at 60X magnification. Boutons at segment A2 in muscle 6 and 7 were manually counted without knowledge of the genotype (blind counting), using Metamorph imaging software. No significant difference in muscle surface area, measured using a drawing tool in Metamorph was observed in the different genotypes.

To quantify levels of synaptic proteins synapses labeled with specific antibodies; anti-syt,
anti-csp, anti-HRP, anti-fas II, anti-dlg, were identically imaged for control and induced animals and the average pixel intensity of terminal boutons (3-4) was measured and analyzed. To quantify organelle accumulation on axons after \( \text{hep}^{\text{act}} \) induction, larval segmental nerves were imaged at high resolution using a cooled charge-coupled device camera (Princeton Instruments) and Metamorph imaging software (Universal Imaging). After background subtraction, images were analyzed for organelle jams and compared with control animals.

Electrophysiology

All electrophysiological recordings were made from muscle 6 within A2, with the larval preparation immersed in a low volume of the HL3 saline with 1mM Ca\(^{2+}\).

Electrophysiology was performed as described previously. In all experiments, the CNS was gently removed to prevent endogenous motor firing. Motor nerves were stimulated with glass-tipped suction electrodes. For intracellular recordings, electrodes pulled from borosilicate capillary tubes were backfilled with 3M KCl, yielding resistances of 6-10 M\( \Omega \). To ensure good recordings, preparations with resting potentials more positive than \(-60\)mV were discarded. For recording excitatory junctional potentials (EJPs), an isolated pulse stimulator (A-M systems, Everett, WA) was used to deliver 1 msec pulses at a frequency of 1 Hz to elicit an evoked response. All recordings were acquired with an axoclamp 2B amplifier in conjunction with pClamp 6 software (Axon Instruments, Foster City, CA). The EJP amplitude for each preparation was determined from an average of 15 consecutive evoked responses. For quantifying mini frequencies, the number of mEJPs occurring consecutively within 30 sec was counted for each preparation. The
mEJP amplitude for each preparation was determined from an average of 30 consecutive mEJPs. At least 5 animals were analyzed for each genotype. For each animal examined that was exposed to RU486 treated food, we examined control animals and expressed the quantal content of transmitter release as a percentage of control.

Serial Analysis of Gene Expression (SAGE)

SAGE was performed as previously described [175, 177]. Briefly, polyA mRNA from 50 CNSs dissected out of drug treated 3\textsuperscript{rd} instar larvae was purified with dynabeads mRNA direct kit (Dynal). Double-stranded cDNA was synthesized on the beads and digested with the anchoring enzyme (NlaIII; NEB). After linker ligation, digestion with the tagging enzyme (BsmFI, NEB), and ligation of the ditags, PCR amplification (29 cycles) was carried out with 20\% of the ligation product as template. The 100 bp PCR products were purified and submitted to a secondary PCR (10–12 cycles) with biotinylated primers to generate enough material for the concatemerization. After NlaIII digestion, the released ditags were purified by polyacrylamide gel electrophoresis and subsequently incubated with 100 \textmu l of Dynabeads Streptavidin to eliminate any remaining biotinylated linkers. Concatemerization was carried out for four hours. Concatemers were cloned into the SphI site of pZero1 (Invitrogen), and resulting colonies were screened for inserts by PCR and submitted for sequencing. All sequencing reactions and SAGE tag generation was performed at Agencourt Inc. (Boston).

Analysis of SAGE Data and Annotation of SAGE Tags

Sequenced SAGE concatemers were analyzed using the SAGE2000 program obtained from The Johns Hopkins University (see also [199]). The database linking SAGE tags to
data of the Berkeley Drosophila Genome Project was built using datasets downloaded from the BDGP site [200] and extracting the 10 bp sequence downstream of the 3′-most CATG site. These putative tags were linked to the GadFly site of the corresponding gene. Annotation of experimental data was performed using Microsoft Access to link the experimental dataset and the Tag annotation database [175, 177].

Affymetrix oligonucleotide microarray analysis

Total RNA was extracted from 200-300 heads of RU486-treated (AP-1 induced) and untreated control ElavGS-GAL4-AP1 flies (w1118;UAS-fos/+;UAS-jun/ElavGS-GAL4) using the RNeasy kit (Qiagen). 5 μg of total RNA was used as a starting template for 19 microarray hybridizations (7 treated and 12 untreated RNA samples). Two sets of control flies were included for analysis in five of the seven experimental AP-1 overexpression treatments used for the microarray hybridizations. Transcript quantification was performed with Affymetrix Drosophila Genome1 GeneChip [201] arrays using biotinylated cRNA targets prepared according to standard Affymetrix protocols by the GATC Affymetrix Core Facility at the University of Arizona [202]. Hybridized arrays were scanned using Affymetrix MicroArraySuite software as described in the manufacturer’s protocol. All hybridizations were normalized with a global scaling factor of 500 so that transcript levels could be compared directly. Text files containing raw, normalized values were exported into Excel for further analysis.

Internal control, 3′-5′ probe signal ratios (a measure of how well the biochemical reactions went prior to hybridization of the biotinylated probe to the oligonucleotide array) were within the range recommended by the manufacturer for all hybridizations. $R^2$
values for all comparisons of control versus control samples from the same experimental group were high (≥0.97).

Between 41% and 49% of all genes were scored present or marginal on each of the arrays by MicroArraySuite. In order to avoid spurious data, only the 5188 genes present or marginal in all 7 AP-1 induced samples were considered for further analysis (~38% of all probes on the array). Ratios between AP-1 induced and the 1 or 2 control samples from a given treatment were calculated in Excel. Fold-differences were converted to log₂ values so that increasing and decreasing levels of mRNA could be compared directly. Log₂ values (n=12) were tested against the value of 0, expected if there were no change in expression, using the Student’s t-test (unpaired t-test, two-sided P, samples with unequal variance estimates). The P-values accepted for our analysis (P<0.01) therefore reflect a 99% probability that the null hypothesis (there is no difference in the expression of a given transcript in AP-1 induced samples) should be rejected.

Secondary filters to eliminate false positives and randomly fluctuating transcripts included: 1) the average AP-1 induced versus control ratio (n=12) for a given gene had to be 1.2 or higher; 2) Log₂ values for the expression ratio, comparing AP-1 induced to control signals for a given gene (n=12), were tested against the values of control versus control ratios (n=5), again using the Student’s t-test - genes passing this statistical filter (P<0.05) were considered to be changed beyond the dynamic nature of the transcript.

Quantitative real time RT-PCR and in situ hybridization

Larval CNS: To quantify RNA expression, approximately, 25 larval brains were dissected for each sample. PolyA mRNA was isolated using the Dynabeads mRNA
direct kit (Dynal) and oligo dT-primed cDNA was synthesized with the Omniscript cDNA synthesis kit (Qiagen). The cDNA was diluted 1:5 for Q-PCR reactions performed on a Cepheid SMARTCycler using QuantiTect SYBR Green PCR kit (Qiagen). Transcript levels were determined using gene-specific primer sets (details available on request). Expression differences are shown as the average change in cycle number at which PCR product (determined by fluorescent signal) is detected as statistically significant above background. This is referred to as the crossing threshold and the more cDNA template present at the start of the reaction, the fewer number of cycles it takes to reach this point. A one-cycle difference represents a two-fold difference in starting template concentration. All transcript levels are normalized to the control gene, ribosomal protein 49 (rp49), as previously described [45].

**Adult heads:** Independent RNA samples were extracted as for microarray experiments for all Q-PCR comparisons. Equal amounts of total RNA (4 μg) for RU486-treated (induced) and untreated control samples were purified from genomic DNA with the DNA-free DNase kit (Ambion) prior to oligodT-primed cDNA synthesis using the Omniscript cDNA synthesis kit (Qiagen). The cDNA was diluted 1:20 with nuclease-free H2O (Invitrogen) for Q-PCR reactions performed as described above. Each PCR reaction was repeated in triplicate for 3-5 independent RNA preparations from separate RU486 treatments. Sample sets were compared using the Student’s t-test as for the array analysis and only results showing a P-value <0.05 were considered statistically significant.
*In situ* hybridizations were performed using probes prepared with PCR DNA (400-600 bp) from primers specific for gene of interest containing T7 RNA polymerase binding site in the sense orientation and SP6 RNA polymerase-binding site in the antisense orientation. RNA probes were labeled with DIG and visualized using either NBT/BCIP (blue reaction product). Larval CNSs were dissected after drug treatment and the tissue was processed using standard protocols [203].
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