PUTATIVE AXONAL INPUT AND OUTPUT PATHWAYS OF THE CIRCADIAN CLOCK SYSTEM IN THE BRAIN OF THE INSECT *RHODNIUS PROLIXUS* (HEMIPTERA)

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GENERAL ABSTRACT

The lateral clock neurons (LNs) arborize in the accessory medulla (aMe) and the dorsal midbrain. In hemimetabolous insects, this latter region appears highly developed and has been extensively studied in the insect *Rhodnius prolixus* within which it is called the principle protocerebral arborization area (PPA). This study investigated the importance of the PPA in the *Rhodnius* brain clock system. Double label immunohistochemistry and laser scanning confocal microscopy allowed observation of close associations between neurons stained with antibodies against four neurochemicals and the LNs. Neuronal projections more densely innervated the PPA than the aMe. All neurochemicals were produced and released with a daily rhythm, implying clock control, likely via PPA axons. Taken together, these results suggest that the PPA is important in the integration of timing information in the *Rhodnius* brain clock system, perhaps as important as the aMe, containing many potential pathways for input to and output from the system.

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Abbreviations

5HT	5-hydroxytryptamine (serotonin)
5HT-lir	Serotonin-like immunoreactivity
ACTH	Adrenocorticotropic hormone
A-G1	Group 1 AST-7 cells; 7 cells in the dorsal anterior protocerebrum
A-G2	Group 2 AST-7 cells; 2 cells in the lateral protocerebrum
ALP	AST-7-like peptide
aMe	Accessory medulla
AST-7	Allatostatin-7
AST-7-lir	Allatostatin-7-like immunoreactivity
BSA	Bovine serum albumin
BTG	Bovine thyroglobulin
CCAP	Crustacean cardioactive peptide
CCAP-lir	Crustacean cardioactive peptide-like immunoreactivity
C-G1	Group 1 CCAP cells; ~150 cells in the distal optic lobe
C-G2	Group 2 CCAP cells; 4 cells dorsal to the medulla at the base of the optic lobe
C-G3	Group 3 CCAP cells; 1 cell in the lateral protocerebrum
C-G4	Group 4 CCAP cells; 2 cells in the postero-medial protocerebrum
CLP	CCAP-like peptide
CRH	Corticotrophin-releasing hormone
CRY	Cryptochrome; clock protein
CYC	CYCLE; clock protein
DABCO	1,4-diazabicyclo[2.2.2]octane; antioxidant used in mounting medium
dCLK	*Drosophila* CLOCK; clock protein
DNs	Dorsal clock neurons
FITC	Fluorescein isothiocyanate
FLP	FMRFamide-like peptide
FMRFamide-lir	FMRFamide-like immunoreactivity
GABA	γ-aminobutyric acid
JH	Juvenile hormone
La	Lamina; first optic chiasm
LNs	Lateral clock neurons
Me	Medulla; second optic chiasm
PC bridge	Protocerebral bridge
PDF	Pigment-dispersing factor
PDF-lir	Pigment-dispersing factor-like immunoreactivity
PDH	Pigment-dispersing hormone
PER	PERIOD; clock protein
PFA	Paraformaldehyde; fixative
PGs	Prothoracic glands
PPA	Principle protocerebral arborization area

PTTH	Prothoracicotropic hormone
SCN	Suprachiasmatic nucleus
S-G1	Group 1 5HT cells; 4 cells just dorsal to the medulla
S-G2	Group 2 5HT cells; 5 cells just posterior to the base of the medulla near the junction between the optic lobe and lateral protocerebrum
S-G3	Group 3 5HT cells; 3 cells in the anterior protocerebrum
S-G4	Group 4 5HT cells; 2 cells in the lateral protocerebrum
S-G5	Group 5 5HT cells; 5 cells in the postero-medial protocerebrum
SLC	Serotonin-like chemical
TIM	TIMELESS; clock protein
TRITC	Tetramethylrhodamine isothiocyanate
TTO	Transcription/translation feedback oscillator
VIP	Vasoactive intestinal peptide

CHAPTER I:

GENERAL INTRODUCTION

1.1. Life in a Cyclical Environment

We live in a periodic environment – the Earth spins on its axis producing a cycle of day and night, and it revolves around the sun, driving a cycle of seasonal change, while the moon's revolution around the Earth produces rhythmic tides. The adaptive value of a trait which allows organisms to predict repetitive environmental changes and thereby produce anticipatory physiological responses atop a rhythmic planet is undeniable. In fact, circadian rhythms are present in nearly all organisms and are an essential feature of life on earth. The word "circadian" derives from the Latin "circa diem," meaning "about a day." The most basic properties of endogenous circadian rhythms were first extensively investigated in the fruit fly *Drosophila pseudoobscura* by Bruce and Pittendrigh (1957) who proposed that circadian rhythms must: (1) free-run in aperiodic conditions, (2) possess a period length of approximately 24 hours, and (3) possess a period length which remains constant across a range of physiological temperatures (reviewed in detail by Pittendrigh, 1993).

Since endogenous circadian rhythms maintain periods which are distinct from the solar day, each day they re-synchronize to the 24-hour period of the external world primarily via light input – a process known as entrainment (Nishiitsutuji-Uwo and Pittendrigh, 1968). Light is referred to as an external Zeitgeber (German for "time-giver") and the alternation of day and night (i.e. dawn and dusk) is by far the most powerful and

consistent entraining signal, far more reliable than other external Zeitgebers such as changes in temperature and/or humidity (Pittendrigh, 1954).

1.1.1. The necessity of endogenous circadian rhythms for life on earth

Circadian rhythms have been documented at all levels of biological organization with the exception of viruses. In prokaryotes, key physiological processes indispensable for survival are under circadian control including the regulation of nitrogen fixation, cell division and photosynthesis (reviewed by Johnson and Golden, 1999; Dvornyk et al., 2003). In eukaryotes, circadian rhythms have been well studied at the molecular level, as in gene expression (Balsalobre et al., 1998); at the cellular level, as in liver metabolism (Lavery and Schibler, 1993) and renal activity (Rabinowitz, 1996); in autonomic functions, like blood pressure (Portaluppi et al., 1996) and body temperature (Buhr et al., 2010); and in whole organisms, as in the leaf movements of plants (Edwards and Millar, 2007), the phototactic response of the single-celled green alga *Euglena gracilis* (Bruce and Pittendrigh, 1956), the rhythm of luminescence in the single-celled dinoflagellate *Gonyaulax polyedra* (Hastings and Sweeney, 1958), the rhythm of conidiation in the fungus *Neurospora crassa* (Sargent et al., 1966), and the behavioural and activity rhythms of insects and mammals, including the sleep-wake cycle (reviewed by Helfrich-Förster, 2004). Hence, it is clear that circadian rhythms comprise a vital feature of many biological processes.

Temporal organization within organisms is necessary for survival (detailed by Pittendrigh, 1993). A particular example of circadian rhythms lending to the survivability

of an entire species can be found in *Wuchereria bancrofti,* one of a handful of parasitic filarial nematodes that cause lymphatic filariasis in mammals. Hawking (1967) noted that these worms possessed a circadian rhythm of circulation in the bloodstream – the worms spend the day primarily in the lung tissue of their host and only enter the peripheral blood stream at night, during the short window of time when their vector insect is most active, thereby increasing the probability of ingestion by their vector and ultimate transmission to their mammalian host. Only a handful of studies have directly measured the adaptive significance of circadian rhythms, however disruption of the circadian system has been shown to adversely affect all organisms studied (detailed below).

Internal desynchronization caused by disruptions of the circadian clock system (experimentally achieved by subjecting organisms to aperiodic or non-circadian conditions) has led to serious consequences in all organisms studied: impaired growth and even early death in plants (Arthur et al., 1930; Went, 1960); decreased fitness in malarial parasites (*Plasmodium chabaudi*; O'Donnell et al., 2011); decreased lifespan in insects (Pittendrigh and Minis, 1972); and an increased incidence of depression (Turek, 2007), obesity and diabetes (Shi et al., 2013), infertility (Boden and Kennaway, 2006), and various cancers (Savvidis and Koutsilieris, 2012) in mammals, including humans.

Circadian rhythms have been found to be driven by a core molecular oscillator in both prokaryotes and eukaryotes (the insect molecular clockwork is discussed in more detail in *Section 1.3* below). The molecular machinery underlying endogenous circadian rhythms has been elucidated in a wide array of model organisms including: the plant *Arabidopsis thaliana* (Harmer, 2009), the cyanobacterium *Synechococcus elongatus*

(Dong and Golden, 2008), the fungus *N. crassa* (Dunlap et al., 2007), the insect *Drosophila melanogaster* and the mouse *Mus musculus* (Weber, 2009). At the core of this machinery lies a positive-negative transcription-translation feedback loop, a feature that appears to have been conserved through time (Paranjpe and Sharma, 2005). It has been suggested that the molecular machinery that underlies circadian rhythms has evolved independently at least three separate times – once, in cyanobacteria; again, in an ancestor of animals prior to the divergence of insects and mammals (reviewed by Rosbash, 2009); and again in plants. The apparent evolution of a circadian molecular oscillator in three different kingdoms highlights its adaptive significance. Therefore, it seems clear that circadian rhythms are a critically important component of life on Earth and arguably necessary for life as we know it.

1.1.2. Why is it important to study the circadian clock system?

As previously mentioned, disruptions of the endogenous circadian system caused by internal desynchronization can ultimately have severe health-related consequences. Transmeridian travel, shift work and (on a more universal scale) light at night have become major and common causes of internal desynchronization in the human population and have been linked to many adverse health effects (Bernstein, 2002; Knutsson, 2003; Rüdiger, 2004; Lall et al., 2012). With the increasing prevalence of diseases linked to disruptions of circadian rhythms in humans, the importance of studying these rhythms is now widely accepted in the scientific and medical communities. Chronotherapeutics, a relatively new field of medicine, entails the treatment of disease by administration of

drugs at optimal times throughout the 24-hour day (with respect to biological rhythms) in order to reduce the negative effects (e.g. toxicity) and maximize potential benefits of treatment (Lemmer and Labrecque, 1987).

Thus, studying the circadian timing system is important, as it has many implications for human health. However, direct study of the mammalian brain clock, the suprachiasmatic nucleus (SCN), is difficult due to its extremely small size. It also possesses a large number of relatively tiny cells which themselves possess their own projections making the study of intercellular communication particularly trying. Cell cultures have been employed to study the SCN *in vitro* by taking hypothalamic slices, but due to the heterogeneous nature of the cells in these cultures (mixtures of glial and neuronal cells; Welsh et al., 1995) the results from such studies can be relatively unreliable. The evolutionary conservation of the molecular and cellular mechanisms of endogenous circadian rhythms across animal phyla makes possible the use of more tractable experimental systems (e.g. insects) with the ultimate goal of better understanding the complexities of the mammalian system, particularly that of humans.

1.2. The Mammalian Brain Clock

The endogenous circadian clock in the mammalian brain, the SCN, consists of paired nuclei located on either side of the midline in the anterior portion of the hypothalamus just above the optic chiasm, each consisting of ~10 000 cells (Moore, 1991). Early evidence for the SCN as the mammalian brain clock came when it was shown that the SCN, when isolated both *in vivo* and *in vitro*, was capable of sustaining a

circadian rhythm in the rate of spontaneous neuronal firing activity (Inouye and Kawamura, 1979; Green and Gillette, 1982). Upon transplantation of a slice of tissue taken from the SCN region of rhythmic animals into animals previously rendered arrhythmic by targeted ablation of their SCN, rhythmicity was restored with a period matching that of the donor animal (Ralph et al., 1990). This essentially confirmed the SCN as the central circadian clock in the brain of mammals.

It was later determined that individual SCN neurons are capable on their own of producing spontaneous rhythms of firing activity, and that the periods of these rhythms are highly variable between cells and independently phased (Welsh et al., 1995; Liu et al., 1997; Herzog et al., 1998, 2004). Thus, intercellular coupling of cells within and between the SCN is essential to ensure synchronization of neurons and the production of a uniform circadian period (reviewed by Aton and Herzog, 2005; Liu et al., 2007). It has been suggested that individual cells of the SCN are coupled by either neurochemical communication or gap junctions (Colwell, 2000; Albus et al., 2005; Long et al., 2005); and that the bilateral SCN themselves are coupled by physical axonal connectives (Leak et al., 1999; Michel et al., 2013).

1.3. The Insect Brain Clock

The first endogenous circadian clock in the brain of any animal was demonstrated in an insect, the cockroach *Leucophaea maderae* (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Roberts, 1974; Sokolove, 1975; Page, 1982). The insect brain clock was first described as a master pacemaker assumed to control all other slave oscillators (capable of

sustaining rhythmicity only whilst receiving input from autonomous clocks) throughout the organism. It is now understood that nearly all cells possess clocks with varying degrees of autonomy and that these interacting components of the circadian timing system are organized hierarchically with the brain clock having been accepted as the master coordinator and synchronizer (for reviews, see: Herzog, 2007; Allada and Chung, 2010; Tomioka et al., 2012). In general, the organization of the clock in the insect brain is well conserved among insects of disparate orders (reviewed by Helfrich-Förster et al., 1998). *Rhodnius prolixus* (Hemiptera) has been used with great success to study circadian organization in insects and the brain clock has been described in detail for both larvae (Vafopoulou et al., 2010) and adults (Vafopoulou and Steel, 2012a).

1.3.1. The molecular machinery underlying endogenous circadian rhythmicity in insects

The identification of the molecular components of the circadian clock permitted detailed analysis of the insect brain clock. The molecular machinery of the circadian clock is largely conserved throughout the animal kingdom, particularly between insects and mammals (Herzog, 2007). Only the insect clockwork will be elaborated upon here. In *Drosophila,* it was found that overt rhythmicity is driven by a transcription/translation feedback oscillator (TTO) located within clock cells in the brain. The TTO is essentially a feedback loop between clock genes and their RNA and protein products (Scully and Kay, 2000; reviewed by Nitabach and Taghert, 2008). Briefly, the canonical clock proteins PERIOD (PER) and TIMELESS (TIM) are cyclically produced and degraded at 24-hour intervals. Within the nucleus, a third clock protein known as CLOCK (dCLK)

binds a fourth clock protein, CYCLE (CYC). This complex activates transcription of *per* and *tim* mRNA thus up-regulating the expression of the PER and TIM proteins. As cytoplasmic levels of PER and TIM rise, they form a complex that translocates into the nucleus, wherein it binds dCLK, preventing its binding to CYC and thereby inhibiting transcription of their own mRNA. Degradation of PER and TIM occurs in the cytoplasm and begins with the binding of the blue light-sensitive cryptochrome protein, or CRY, to TIM (PER is degraded in the absence of TIM) and allows the dCLK/CYC heterodimer to once again bind and activate transcription of *per* and *tim.* The molecular machinery of the mammalian brain clock is functionally analogous to that of the insect brain clock, with three homologues of PER, two CRY proteins in place of TIM, and BMAL1 in place of CYC (reviewed by Reppert and Weaver, 2001).

This is, of course, a dramatic oversimplification of the molecular mechanism underlying endogenous circadian rhythmicity. Additional interconnected feedback loops, other transcriptional elements as well as many post-translational elements have been implicated as important components of the complex circadian machinery, and though the canonical TTO is widely accepted as being central to the production of rhythmicity, it appears, at least in *Drosophila,* that it is neither necessary nor sufficient to maintain cellular rhythmicity (Yang and Sehgal, 2001; Sathyanarayanan et al., 2004; Harrisingh et al., 2007; reviewed by Lakin-Thomas, 2006).

True clock cells in insects have generally been defined in the literature as those cells which possess circadian cycling of the canonical clock proteins PER and TIM. Though *Drosophila* is the only insect in which the molecular oscillator has been

identified, sequence analysis shows a remarkable conservation of its molecular components across insect species (Helfrich-Förster, 1998). *Rhodnius* and *Drosophila* are the only two insect species in which all of their identified brain clock cells have been shown to possess cycling of both PER and TIM (Siwicki et al., 1988; Helfrich-Förster, 1995; Hunter-Ensor et al., 1996; Kaneko et al., 1997; Vafopoulou et al., 2010).

1.3.2. Neuroarchitecture of the insect brain clock

The brain clock of *Rhodnius* is strikingly similar to that of *Drosophila*, with some exceptions. The neuroarchitecture of the larval *Rhodnius* clock is described in detail in Vafopoulou et al. (2010) and will be re-examined later (summarized in Fig. T1; from Vafopoulou et al., 2010). Briefly, it consists of two clusters of clock cells in each brain hemisphere: first, the lateral neurons (LNs) located at the base of the optic lobe near the junction between the optic lobe and central protocerebrum and second, the dorsal neurons (DNs) located just posterior to the mushroom body in an arch configuration. The LNs project axons ventrally where they arborize in a relatively small ovoid neuropil known as the accessory medulla (aMe). The aMe has historically been considered vital for the integration of timing information in the insect brain clock. From the aMe, axons extend distally towards the compound eye forming processes in the medulla and lamina neuropils. It is via this pathway that the LNs become entrained to the solar day by light input from the compound eyes. The LNs also extend axons posteriorly before turning medially and arborizing in the central protocerebrum over the dorsal anterior aspect of the central protocerebral neuropil, a region which has been dubbed the principle

protocerebral arborization area (PPA). Recent evidence has implicated the PPA as another important region for the integration of timing information in the insect brain clock. Axonal connectives between the LNs and DNs of ipsilateral hemispheres have been elucidated in both *Rhodnius* (Vafopoulou et al., 2010) and *Drosophila* (Kaneko and Hall, 2000) and the LNs in each brain hemisphere are connected via axons projected from the contralateral LN cluster facilitating synchronization of hemispheres. Thus, the insect brain clock, particularly that of *Rhodnius*, appears to be a spatially diffuse master clock network in the dorsal brain consisting of four anatomically distinct groups of true clock cells connected by axons, and potentially *two* integrative regions: the aMe and the PPA.

1.4. The Accessory Medulla

The aMe is a small ovoid neuropil located at the base of the optic lobe near the junction between the optic lobe and central protocerebrum. Its role as the integrative centre of the neuronal clock of insects has been widely accepted in the literature and most comprehensively studied in the cockroach *L. maderae* (Reischig and Stengl, 1996; Homberg et al., 2003).

1.4.1. First implication of the aMe as an important clock-related structure

The optic lobes were first implicated as home to a circadian clock when complete optic lobe transection rendered cockroaches arrhythmic (Nishiitsutsuji-Uwo and Pittendrigh, 1968). Lesioning of the area near the base of the optic lobe in the region of the medulla and lobula neuropils led to a loss of rhythmicity in locomotor activity in

cockroaches (Roberts, 1974; Sokolove, 1975; Page, 1978) and a loss of both locomotor and stridulation activity in crickets (Sokolove and Loher, 1975). It was also determined that the 24-hour period of locomotor activity in cockroaches is a result of the mutual coupling of these presumed pacemakers at the base of contralateral optic lobes (Page et al., 1977). Further support for an optic lobe clock came when transplantation experiments of optic lobes from rhythmic animals to animals rendered arrhythmic by removal of both optic lobes restored rhythmicity in host animals and did so with a period of locomotor activity matching that of the donor (Page, 1982). Confirmation that the region at the base of the optic lobe between the medulla and lobula neuropils is home to a self-sustained circadian clock came when it was shown that a circadian rhythm in spontaneous neural activity persists when this region was isolated *in vitro* in both cockroaches and crickets (Colwell and Page, 1990; Tomioka and Chiba, 1992).

The culmination of these experiments was the discovery that, within the insect brain, there is a region located at the base of the optic lobe, likely including the aMe, that is required for the maintenance of various circadian rhythms. Indeed, the involvement of the aMe in the proper functioning of the insect brain clock is irrefutable. Anatomically, most LN axons project into the aMe before extending and branching into other brain areas and the aMe is central in the entrainment pathway of insects as it receives light input from the compound eyes (reviewed by Helfrich-Förster, 2004). Behaviourally, its importance was implicated by the experiments described above. However, a neuropil on its own cannot produce rhythmicity. It was not until the precise cellular localization of

the optic lobe clock that the specific involvement of the aMe in the inner workings of the insect brain clock would be confirmed.

1.4.2. Cellular localization of the optic lobe clock

The precise cellular identity of the optic lobe clock was elucidated quite accidentally in a number of insect species via immunohistochemistry using an antibody against pigment-dispersing factor (PDF; Homberg et al., 1991). The significance of PDF to the study of circadian neuroanatomy in insects is discussed in greater detail below (*Section 1.6.3*). PDF has been found in a similar number of neurons (~10) located near the aMe at the base of the optic lobes in all species studied, including *L. maderae* (Reischig and Stengl, 2003), *D. melanogaster* (Helfrich-Förster, 1995), and *R. prolixus* (Vafopoulou et al., 2010). Homberg et al. (1991) noted that these cells possess three important clock-defining criteria: (1) they are located near the base of the optic lobe, a region which had previously been implicated as home to an autonomous brain clock; (2) there is a route for input via axons that extend from the aMe to the La (though evidence for this was minimal), and; (3) there is a channel for output via axons that extend from the aMe into the midbrain as indicated by the varicose arborizations found along their lengths. If these cells did indeed comprise the "pacemaker" portion of the insect brain clock, their many divergent projections are well poised to enable clock control of various physiological processes (reviewed by Page, 1985). These cells were later found to possess circadian cycling of both PER and TIM in both *Rhodnius* (Vafopoulou et al., 2010) and *Drosophila* (Helfrich-Förster, 1995; Kaneko et al., 1997), confirming them to

be true clock cells. Therefore, with the elucidation of clock cells at the base of the optic lobe, it became evident that the aMe was an important component of the circadian clock puzzle in the insect brain.

1.5. The Principle Protocerebral Arborization Area

A vast meshwork of LN arborizations in the dorsal midbrain of *Rhodnius*, the PPA, has also been implicated as a second major site for the integration of circadian timing information within the insect brain. The PPA has been described in great detail only in *Rhodnius* (Vafopoulou et al., 2007; Vafopoulou et al., 2010). An anatomically equivalent region of LN arborization, though significantly less expansive, has also been observed in *Drosophila* (reviewed by Helfrich-Förster, 2004). A region of arborization, much more extensive than that in *Drosophila*, of alleged clock cells (i.e. those cells which have been deemed clock cells, but have not yet been shown to possess circadian cycling of both PER and TIM) coinciding with the location of the PPA in *Rhodnius* has been observed in several other insect species including the cockroaches *L. maderae* and *Periplaneta americana* (Homberg et al., 1991; Nässel et al., 1991; Stengl and Homberg, 1994; Petri et al., 1995), four species of locust including *Locusta migratoria* and *Schistocerca gregaria* (Homberg et al., 1991; Würden and Homberg, 1995), four species of crickets including *Acheta domesticus* and *Gryllus bimaculatus* (Homberg et al., 1991; Okamoto et al., 2001), the phasmid *Extatosoma tiaratum* (Homberg et al., 1991), and the blowfly *Protophormia terraenovae* (Nässel et al, 1991). The presence of this arborizational structure across such a large span of insects speaks to its probable

functional importance and this neuropil is now believed to be involved in the circadian system in the insect brain, at least in *Rhodnius*.

1.5.1. Importance of the PPA

Varicose fibres have been observed within the PPA of many of the aforementioned insect species (Homberg, 1991; Nässel et al, 1991; Stengl and Homberg, 1994; Petri et al., 1995; Okamoto et al., 2001; Vafopoulou et al., 2007; Vafopoulou et al., 2010), the presence of which indicates that neurochemical communication between cells is occurring in this region. The majority of output projections of the aMe have been found to arborize in the PPA in *Rhodnius* (Vafopoulou et al., 2007; Vafopoulou et al., 2010), *Leucophaea*, and *Drosophila* (reviewed by Helfrich-Föster, 2004). Recently, using an antibody against PDF, Yasuyama and Meinertzhagen (2010) showed that the PPA of *Drosophila* contains numerous output sites as well as some regions of axonal input. Assuming these axons are related to the clock, the authors inferred that the PPA is an important region involved in the integration of timing information within the insect brain.

The PPA appears to be important for the production of rhythmic outputs of the circadian clock from the insect brain, including neuroendocrine (Vafopoulou et al., 2007) and behavioural rhythms (Stengl and Homberg, 1994). Some varicosities along axons within the PPA are closely associated with the axons of cells from which neurohormones are rhythmically released (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b), supporting the conclusion that at least these particular varicosities within the PPA are acting as output sites of the clock. Though there is mounting evidence, especially from

neuroanatomical studies, to suggest that the PPA may well be a very important region of the brain clock in insects, it has received relatively little attention in the literature compared with the aMe.

1.6. The Role of Neurochemicals in the Brain Clock

Endogenous circadian clocks employ neurochemicals as signaling molecules in both input and output pathways. These molecules act as "messengers of time," conveying information about time of day to other brain and body regions in order to effectively synchronize physiological events. In this way, neurochemicals, acting locally as neurotransmitters and/or neuromodulators or distantly as neurohormones, may orchestrate temporal harmony within the organism. This is the case in both mammals and insects.

1.6.1. Neurochemicals associated with the mammalian brain clock

In mammals, the SCN is necessary for synchronization of peripheral clocks, thus it is responsible for distributing the circadian message to the rest of the body (reviewed by Kalsbeek et al., 2006). SCN neurons have been segregated into functional clusters, comprised of neurons that contain different combinations of neuropeptides and potential neurotransmitter communicators of circadian time; these peptides include vasopressin, vasoactive intestinal peptide (VIP), gastrin-releasing peptide, and somatostatin (reviewed by Buijs and Kalsbeek, 2001). Many SCN neurons have also been found to contain γ-aminobutyric acid (GABA) and/or glutamate, both of which act in input pathways of the

clock. GABA has many roles, including potential involvement in the coupling of the bilateral SCN (Honma et al., 2000; Liu and Reppert, 2000; Yamaguchi et al., 2003) and as a modulator in the melatonin-rhythm-generating-system (reviewed by Kalsbeek et al., 2006). Glutamate is involved in the photic entrainment pathway of the SCN – its release from retinal ganglion cells upon their exposure to light causes membrane depolarization of target SCN neurons (Liou et al., 1986; reviewed by Bujis and Kalsbeek, 2001). Melatonin is perhaps the most well known output of the mammalian brain clock and was the first neuroendocrine output to be clearly linked to rhythmic activity from the SCN (reviewed by Kalsbeek et al., 2006). Melatonin is synthesized by the pineal gland with a circadian rhythm, with peak synthesis occurring during the night. It is a neurohormone messenger of time that likely facilitates communication about time of day to a vast number of effector cells distributed throughout the organism. Melatonin receptors have been found within the SCN (reviewed by Pévet et al., 2006) indicating that it likely acts directly on clock cells themselves, perhaps as part of a feedback mechanism.

1.6.2. Neurochemicals associated with the insect brain clock

In insects, the biogenic amine GABA appears to be involved in clock input, acting on a subset of LNs in *Drosophila* as a slow inhibitory neurotransmitter to mediate the response of the LNs to light input (Hamasaka et al., 2005). The peptide prothoracicotropic hormone (PTTH) is a known hormonal output of the clock in *Rhodnius* and acts as a messenger of time – it is released with a circadian rhythm to induce rhythmic steroidogenesis in the prothoracic glands (PGs) (Vafopoulou and Steel,

2001). In previous immunohistochemical studies, authors have inferred the involvement of various neurochemicals in the clock system of cockroaches (Petri et al., 1995; Hofer and Homberg, 2006; Soehler et al., 2008; Soehler et al., 2011), locusts (Würden and Homberg, 1995), and crickets (Sehadová et al., 2007) if they appeared to be associated with the aMe. Particularly in *Drosophila,* a number of neurotransmitters and neuromodulators have been identified that facilitate communication of the clock with the rest of the body (reviewed by Nässel and Winther, 2010).

The neuronal distributions of all neurochemicals presently studied (allatostatin-7, crustacean cardioactive peptide, FMRFamide, and serotonin) have been observed previously in *Rhodnius* (see Materials and Methods) as well as an assortment of other insects and they have all been preliminarily implicated as neurotransmitters and/or neuromodulators of the insect brain clock based on their presence in local and/or projection neurons of the aMe (Dircksen and Homberg, 1995; Würden and Homberg, 1995). Previous functional studies have provided further support that some of these neurochemicals may act as inputs and/or outputs of the clock (see General Discussion).

1.6.3. The pigment-dispersing factor

The neuropeptide PDF plays a particularly important role within the insect brain clock and is central to the present study. PDF was the first neurochemical to be identified as a messenger of time in *Drosophila* (reviewed by Nässel and Winther, 2010). It was first isolated and sequenced from the eyestalks of the fiddler crab *Uca pugilator* (Rao et al., 1985). It is an octadecapeptide that is part of a large family of peptides responsible for

chromatophoral pigment dispersion and the circadian migration of retinal screening pigment in crustaceans (Rao and Riehm, 1989). PDFs with 78-83% sequence similarity with the pigment-dispersing hormone (β-PDH) of *U. pugilator* were isolated from head extracts of the grasshopper *Romalea microptera* (Rao et al., 1987) and the cricket *A. domesticus* (Rao and Riehm, 1988, 1989). The peptide is referred to as a hormone (PDH) in crustaceans as its function as a hormone in these animals is well-established; its function in insects, however, appears manifold and it is thus referred to as a factor (or PDF).

PDF has proven invaluable for studying the neuroanatomy of the insect brain clock, as it very specifically stains the cell bodies and axons of the LNs (see Fig. T2). A role for PDF in the insect brain clock was originally inferred when immunohistochemical studies revealed that PDF stains the LNs and their projections (Homberg, 1991) and it has since been employed extensively to study the neuroanatomy of the insect brain clock in a multitude of insect species. Functional studies were later carried out which indicated that PDF plays a pivotal role as both an input and output of the insect circadian clock (reviewed by Helfrich-Förster, 2009). PDF has been shown to be involved in coupling of the bilateral optic lobe clocks (i.e. it acts as an input) since the destruction of one or more commissural fibres containing PDF that connect contralateral cell clusters results in the disruption of the circadian period (Reischig et al., 2004; reviewed by Helfrich-Förster, 2004). PDF also appears to be involved in maintaining a circadian rhythm of locomotor activity (i.e. it acts as an output), as when PDF-immunoreactive fibres connecting the aMe to the PPA are severed, disruption in locomotor activity rhythms have been observed

and regeneration of these fibres after optic lobe transplantation results in reappearance of the rhythms (Stengl and Homberg, 1994; Reischig and Stengl, 2003; reviewed by Helfrich-Förster, 2004). There is also evidence that PDF plays a role in modulating the period of the clock and that it may be involved in the light input pathway (reviewed by Helfrich-Förster, 2009).

1.6.4. Parallels between insects and mammals regarding the facilitation of communication by neurochemicals in the brain clock

The brain clocks of insects and mammals are functionally analogous. Input pathways from the visual system to the SCN (in mammals) and to the aMe (in insects) allow entrainment of these brain clocks to the 24-hour day and output pathways provide a means by which biological processes (including endocrine secretion, behavioural and activity rhythms) may be controlled by the clock (reviewed by Helfrich-Förster, 2004). Neurochemicals are employed in all of these pathways.

Two increasingly apt examples of inter-phylum homology greatly support the use of insects as models for the mammalian clock system: (1) that VIP in mammals is widely considered to be the functional homologue of PDF in insects and (2) the strong analogy between the insect PTTH-ecdysteroid axis and the mammalian hypothalamo-pituitary-adrenal axis. VIP is to the SCN (at least in part) as PDF is to the insect brain clock. VIP is important for the synchronization of SCN neurons, coupling of the bilateral SCN, and the maintenance of behavioural rhythms (Harmar et al., 2002; Colwell et al., 2003; reviewed by Helfrich-Förster, 2005, Maywood et al., 2006; Michel et al., 2013). In insects, the

peptide PTTH is released rhythmically from neurosecretory cells in the lateral brain and acts on the PGs culminating in the rhythmic release of ecdysteroids into the haemolymph (reviewed by Steel and Vafopoulou, 2006). In mammals, the SCN indirectly regulates rhythmic release of corticotrophin-releasing hormone (CRH) by hypothalamic neurons to signal the rhythmic release of adrenocorticotropic hormone (ACTH) from the neurohypophysis which acts on the adrenal glands leading to the rhythmic release of glucocorticoids into the blood (reviewed by Buijs and Kalsbeek, 2001; Dickmeis and Foulkes, 2011). Ecdysteroids in insects and glucocorticoids in mammals subsequently act as messengers of time to effector cells throughout the organism. Indeed, there is a wealth of evidence that neurochemicals are an indispensible tool used by the brain clock in both insects and mammals to communicate time of day information to various regions in the brain and body.

1.7. The Study of Circadian Neuroanatomy Using Rhodnius prolixus (Hemiptera)

The brain clock of some insects is relatively simpler in structure than that of mammals, with individual cells that are larger in size and whose axons can be traced across comparatively longer distances, making particular insect models appealing for such studies.

1.7.1. Why use Rhodnius for structure-based studies of the insect brain clock?

Rhodnius is particularly attractive for research on the insect brain clock primarily for four reasons. First, the structure of the *Rhodnius* brain clock is simpler than that in

other insects – they have LNs and DNs which have been found to be true clock cells. *Rhodnius* cells are relatively large compared to those of *Drosophila* and they possess highly traceable axons. Second, the structure of the *Rhodnius* brain clock has been studied extensively using an antibody against PDF, with which the LNs and their axons stain intensely (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a), making immunohistochemical study of the system relatively simple. Third, this cumulatively makes *Rhodnius* particularly useful for observing the interaction between various neurochemical-containing cells and their axons. *Rhodnius* is, in fact, the only insect for which a clear structure-function relationship with respect to the brain clock system and a rhythmic output (i.e. the rhythmic release of neurohormones) has been deduced – axons in the PPA interact with axons of neurosecretory cells in the lateral protocerebrum leading to the rhythmic release of neurohormones (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b). Fourth, *Rhodnius* larvae exist in a state of developmental arrest. Engorging on a blood meal triggers stretch receptors in the abdominal body wall which initiates synchronous development to the next larval instar (Chiang and Davey, 1988). In this way, the development of large groups of insects can be easily synchronized thus reducing the variability in neurochemical staining often observed during insect development.

1.7.2. Why not use other insect models?

Historically, *Drosophila* has been utilized to study the insect circadian system, primarily for genetic and molecular convenience – the functional machinery of the

molecular oscillator was elucidated using these animals. The brain clock system of *Drosophila* is comparatively more complex than that of *Rhodnius,* as it contains multiple interconnected groups of clock cells (reviewed by Helfrich-Förster, 2004), unlike the four discrete groups of clock cells in the *Rhodnius* brain. The small size of *Drosophila* cells is also a hindrance for studying the neuroanatomical relations between cells. Circadian rhythms of activity have been shown previously in *Drosophila,* but the neural pathways leading to these rhythms have not yet been elucidated. Thus, *Drosophila* is not ideally suited to this type of research.

Cockroaches and crickets are larger than *Drosophila* and can undergo invasive surgical procedures with a relatively high survival rate. These properties made them important tools for studying behavioural and activity rhythms. However, true clock cells have yet to be identified in either cockroaches or crickets. Though these insects possess a number of rhythmic activity patterns that are easily measurable (e.g. locomotor activity in cockroaches and stridulation activity in crickets), as in *Drosophila* a structure-function relationship has not yet been elucidated between the brain clock and their rhythmic outputs. Additionally, alleged clock cell arborizations in these animals are more complex than in *Rhodnius*, rendering cockroaches and crickets similarly inconvenient for detailed study of the neuroanatomy of the insect brain clock.

Thus, *Rhodnius* is a more appropriate model organism than *Drosophila*, cockroaches and crickets at least when: (1) synchronization of experimental animals is helpful (i.e. when mapping the neural pathways underlying rhythmic outputs, which may

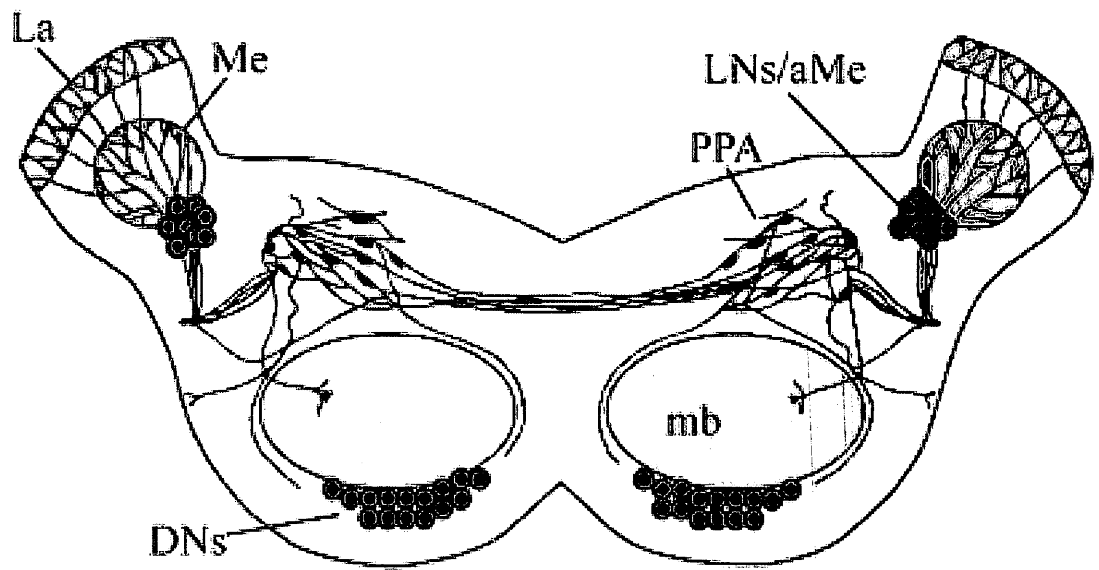
change over the course of development); and (2) simplicity of the clock system is an asset (i.e. when tracing axonal interactions between the brain clock and other neurons).

1.8. Objectives

The literature has put much emphasis on the aMe as the be-all-end-all of the insect brain clock system. The PPA has received substantially less attention, but its extensive nature hints at it having a potentially important role within the insect brain clock system. Indeed it appears that this system is far more complex than previously imagined and comprises a spatially distributed network in the insect brain.

The present study was undertaken to further explore the potential role of the PPA in the integration of circadian timing information in the brain clock of *Rhodnius.* I employed antibodies to four different neurochemicals that have previously been implicated as having roles as neurotransmitters, neuromodulators and/or neurohormones in the clock system of other insects. By double-staining *Rhodnius* brains with antibodies to these four neurochemicals and PDF, I have looked for regions of close (axonal) associations between PDF-immunoreactive (i.e. clock-related) cells and the cells immunoreactive to these neurochemicals. Axons are deemed to be in close association if they are found within 1μm of each another. One would not expect to find additional neuronal and/or glial processes in a space equal to or less than 1μm, thus interaction between closely associated axons is highly likely. I aimed to investigate the potential involvement of these neurochemicals as components in input and/or output pathways of the brain clock system with the expectation that a majority of interactions will occur in

the PPA, thus implicating this region, in addition to the aMe, as a potential integrative centre of the *Rhodnius* brain clock system.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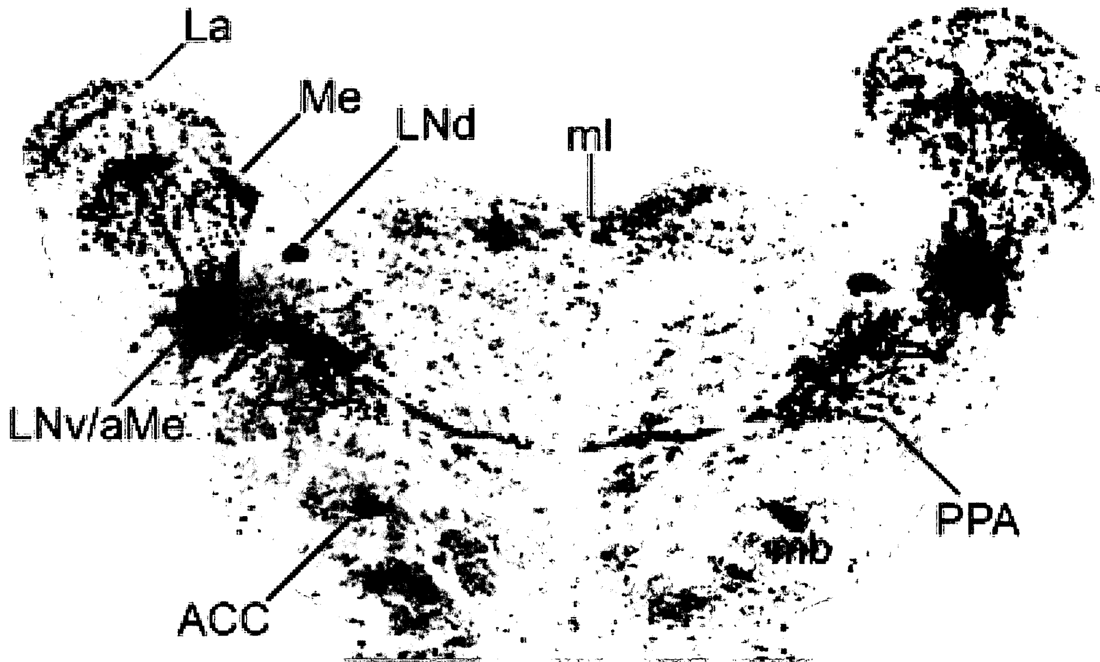
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CHAPTER II:

MANUSCRIPT

Putative involvement of the principle protocerebral arborization area as an integrative centre in the brain clock system of the insect *Rhodnius prolixus* (Hemiptera)

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Running Title:
Putative inputs and outputs of the circadian clock in an insect brain

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ABSTRACT

The brain of *Rhodnius prolixus* contains two bilaterally symmetrical groups of clock cells, the lateral neurons (LNs) and the dorsal neurons (DNs) which are connected via axonal fibres to form a network. In *Rhodnius* the LNs arborize in: (1) the accessory medulla (aMe) and (2) the principle protocerebral arborization area (PPA). The aMe has been considered in other insects as the primary integrator of the brain clock. However, recent studies using *Rhodnius* have exposed the intimate associations of PPA axons and those of neurosecretory cells in the lateral brain which release neurohormones rhythmically, thus implicating the PPA as potentially important for the production of these rhythmic hormonal outputs. Double label immunohistochemistry and laser scanning confocal microscopy were used in the present study to observe axonal interactions of neurons staining with antibodies against allatostatin-7 (AST-7), crustacean cardioactive peptide, FMRFamide, and serotonin – and LN axons labelled with an antibody against pigment-dispersing factor (PDF). Presence of an AST-7-like peptide in retinula cell axons terminating in the aMe suggests a role for this peptide in the photic entrainment pathway. All neurochemicals intensely stained axons densely innervating the PPA, much more so than in the aMe, and making intimate associations with PDF-filled varicosities. Cycling of fluorescence intensity in cell bodies and axons of all neurochemicals studied respectively indicates that the production and release of these neurochemicals occurs with a daily rhythm, which implies clock control, likely via axons in the PPA, and supports a role for these neurochemicals as clock outputs. Numerous neurochemical-filled

varicosities identify the PPA as a potentially important site of input to the clock. These results implicate four neurochemicals as neurotransmitters, neuromodulators and/or neurohormones in the insect brain clock system and suggest that the PPA is important in the integration of circadian timing information in the *Rhodnius* brain, perhaps as important as the aMe, if not more so.

INTRODUCTION

Circadian rhythms have been documented at all levels of biological organization with the exception of viruses. These rhythms are maintained by internal timers known as clock cells, and bona fide clock cells (i.e. those that possess circadian cycling of the canonical clock proteins period (PER) and timeless (TIM)) have only been demonstrated in two insect species, *Drosophila melanogaster* and *Rhodnius prolixus* (Siwicki et al., 1988; Helfrich-Förster, 1995; Hunter-Ensor et al., 1996; Kaneko et al., 1997; Vafopoulou et al., 2010). In mammals and insects, neuronal clock cells control rhythmic outputs including neuroendocrine secretion, behaviour, and activity rhythms (reviewed by Helfrich-Förster, 2004).

Numerous studies of the insect circadian system have made it clear that the insect brain clock consists of a spatially diffuse network of these clock cells (Vafopoulou et al., 2010; Kaneko and Hall, 2000). In all insects studied, one bilaterally symmetrical cluster of clock cells known as the lateral clock neurons (LNs), projects axons ventrally into a small ovoid neuropil at the base of the optic lobe, known as the accessory medulla (aMe). The insect brain clock was localized to this region (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Roberts, 1974; Sokolove, 1975; Sokolove and Loher, 1975; Page et al., 1977; Page, 1978; Page, 1982; Colwell and Page, 1990; Tomioka and Chiba, 1992) and the aMe has since been widely accepted in the literature as being the integrative centre of the neuronal clock of insects (Reischig and Stengl, 1996; Homberg et al., 2003). The aMe has been functionally compared to the central neuronal clock in mammals, the suprachiasmatic

nucleus (SCN; see Helfrich-Förster, 2004). Indeed the aMe is integral in the entrainment pathway of insects by light, but its anatomical isolation and compact structure makes it unlikely that it is responsible for all rhythmic outputs from the brain.

From the aMe, the LNs project in two directions: distally, towards the compound eye, forming a pathway for photic input; and medially into the central protocerebrum, where they arborize over the dorsal anterior aspect of the central protocerebral neuropil in an area now dubbed the principle protocerebral arborization area (PPA; Vafopoulou et al., 2010). The PPA has been observed in several insect species including the cockroaches *Leucophaea maderae* and *Periplaneta americana* (Homberg et al., 1991; Nässel et al., 1991; Stengl and Homberg, 1994; Petri et al., 1995), four species of locust including *Locusta migratoria* and *Schistocerca gregaria* (Homberg et al., 1991; Würden and Homberg, 1995), four species of crickets including *Acheta domesticus* and *Gryllus bimaculatus* (Homberg et al., 1991; Okamoto et al., 2001), the phasmid *Extatosoma tiaratum* (Homberg et al., 1991), and the blowfly *Phormia terraenovae* (Nässel et al, 1991), but has been described in great detail only in *Rhodnius* (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a). All output projections of the aMe have been found to arborize in the PPA of *Rhodnius* (Vafopoulou et al., 2010), *Leucophaea*, and *Drosophila* (reviewed by Helfrich-Förster, 2004), and it appears to be involved in nearly all rhythmic outputs of the neuronal clock of insects, including neuroendocrine (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b) and behavioural rhythms (Stengl and Homberg, 1994). Though there is mounting evidence to suggest that the PPA comprises a second

major site for the integration in the insect brain clock, it has received relatively little attention in the literature when compared with the aMe.

Endogenous clocks employ neurochemicals as signaling molecules in both input and output pathways. These molecules act as "messengers of time," conveying information about time of day to other brain and body regions in order to effectively synchronize physiological events. In this way, neurochemicals, acting locally as neurotransmitters and/or neuromodulators or distally as neurohormones, may orchestrate temporal harmony within the organism. This is the case in both mammals (reviewed by Kalsbeek et al., 2006) and insects (reviewed by Nässel and Winther, 2010). A peptide known as pigment-dispersing factor (PDF) very specifically labels the LNs and their axons and it has been used extensively to trace the neuronal clock system of the insect *Rhodnius prolixus* (Vafopoulou et al., 2010; Vafopoulou and Steel., 2012b). The present paper examines the intimate associations between clock-related axons in the PPA and axons staining with antibodies against allatostatin-7, crustacean cardioactive peptide, FMRFamide, and serotonin. These neurochemicals are all presumed neurotransmitters and/or neuromodulators of the neuronal insect clock based on their presence in local and/or projection neurons of the aMe (Dircksen and Homberg, 1995; Würden and Homberg, 1995). As such, visualization of potential associations between LN axons and the axons of other neurochemical-containing neurons can be carried out with great ease using the method of immunohistochemical double-labeling. The present paper reports that the PPA is likely a significant site for the integration of circadian timing information, at least as important as the aMe, in the insect brain clock.

MATERIALS AND METHODS

2.1. Animals

Fifth (last) instar *Rhodnius* were reared in a 12h light: 12h dark cycle at 28 ± 0.5°C and constant humidity. Only male animals were used. Unfed larvae exist in a state of developmental arrest until they are given a blood meal. Engorging on rabbit's blood stimulates development from one larval stage to the next. Ecdysis to the adult occurs at about 21 days after feeding fifth instar larvae. Day 0 is the designation given to the day of feeding. Brain complexes were excised from larvae on day 13 of larval-adult development. For double-label experiments, dissections were carried out 7h after lights off (mid-scotophase). For single-label experiments, dissections were carried out mid-scotophase and 7h after lights on (mid-photophase). A minimum of four animals were examined at each time point for each antibody.

2.2. Antibodies

The present study examines the distribution of neurons and axons throughout the brain complex which were double-labelled with antisera against four different neurochemicals: allatostatin-7 (AST-7), crustacean cardioactive peptide (CCAP), FMRFamide, or serotonin (5HT) and pigment-dispersing factor (PDF).

A mouse monoclonal antibody against AST-7 was purchased from the Developmental Studies Hybridoma Bank (5F10 supernatant; University of Iowa, IA) and was used at a dilution of 1:20. This antibody was raised against *Diploptera punctata*

AST-7 (APSGAQRKYGFGL; Woodhead et al., 1989) and has been used in previous immunohistochemical studies of various other insects (Stay et al., 1992; Yoon and Stay, 1995). The distribution of AST-7 in the central brain complex of *Rhodnius* seen in the present study was nearly identical to that seen in a previous study (in which the same antibody was used) by Sarkar et al. (2003) in the same species with minor differences in cell counts. The staining seen presently with anti-AST-7 was considerably different from that seen previously when authors stained the brains of *S. gregaria, L. migratoria,* and *Neobellieria bullata* with anti-AST-5 (Veelaert et al., 1995). The specificity of the anti-AST-7 used presently was tested previously by enzyme-linked immunosorbent assay (ELISA; Stay et al., 1992). Cross-reactivity was low (~10%) when tested with other AST peptides (Yoon and Stay, 1995). Furthermore, the trace archive online database of the *Rhodnius* genome contains sequences corresponding exactly to the coding sequence used to produce this AST-7 antibody (http://blast.ncbi.nlm.nih.gov/).

Anti-CCAP was a rabbit polyclonal antibody produced against synthetic CCAP (PFGNAFTGC; Stangier et al., 1987) coupled to glutaraldehyde/polylysine and was purchased from JenaBioscience (ABD-033 supernatant; Jena, Germany). It was used at a dilution of 1:80. Lee et al. (2011) previously observed the distribution of CCAP in the central nervous system of *Rhodnius* using a custom-made antibody against CCAP, which revealed a staining pattern nearly identical to that seen in the present study. This anti-CCAP was tested for cross-reactivity with other similar peptides by ELISA (JenaBioscience). Furthermore, the trace archive online database of the *Rhodnius* genome

contains sequences corresponding exactly to the coding sequence used to produce this CCAP antibody (http://blast.ncbi.nlm.nih.gov/).

A rabbit polyclonal antibody against FMRFamide was purchased from ImmunoStar (catalogue no. 20091; Hudson, WI) and was used at a dilution of 1:500. This antibody was raised against synthetic FMRFamide coupled to bovine thyroglobulin (BTG) to produce immunogenicity. The distribution of FMRFamide-like immunoreactivity in the central brain complex of *Rhodnius* seen in the present study was strikingly similar to that seen in a previous study using a different antibody in the same species (Tsang and Orchard, 1991). The specificity of FMRFamide-like immunoreactivity in the present study was verified by pre-adsorption of the antibody with excess FMRFamide (1mg/mL; Sigma-Aldrich) which completely abolished staining (data not shown). Cross-reactivity of the antibody was tested by pre-adsorption with excess BTG (100μg/mL; Sigma-Aldrich), after which staining remained (data not shown).

A rat polyclonal antibody against 5HT was purchased from MediCorp (1019-003 supernatant; Mtl, QC) and was used at a dilution of 1:500. This antibody was produced against synthetic 5HT conjugated to bovine serum albumin (BSA) to produce immunogenicity. The distribution of 5HT in the central brain complex of *Rhodnius* seen in the present study was strikingly similar to that seen in a previous study (which used a different antibody) by Lange et al. (1988) in the same species. The specificity of 5HT-like immunoreactivity in the present study was verified by pre-adsorption of the antibody with excess 5HT (1mg/mL; Sigma-Aldrich) which completely abolished staining (data not shown). Cross-reactivity of the antibody was tested by pre-adsorption with excess

BSA (100μg/mL; Sigma-Aldrich), after which staining remained (data not shown). Specificity of the antiserum was further verified by immunodot blot (see *Section 2.3*), which showed this particular anti-5HT binds the formaldehyde conjugate of 5HT in *Rhodnius.* These results coincide with those of a previous verification study of the same antibody (Milstein et al., 1983).

The anti-PDF employed for double-labelling in the present study was a guinea pig polyclonal antibody produced against a custom synthesized peptide of the whole pigment-dispersing hormone (PDH) found in *Uca pugilator* (NSELINSILGLPKVMDA; Rao et al., 1985). It was purchased from GenScript (Piscataway, NJ) and used at a dilution of 1:50. The specificity of this antibody was indicated by comparing its distribution pattern with that of a second anti-PDF which has been used extensively by this lab (Vafopoulou et al., 2007; Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a). Distribution patterns were identical (see Fig. 1). The second anti-PDF was a rabbit polyclonal antibody made against synthetic β-PDH of *U. pugilator* (same sequence as above) and has been used extensively in this lab to trace the axonal projections of the LNs in the brain of *Rhodnius* in both larvae (Vafopoulou et al., 2010) and adults (Vafopoulou and Steel, 2012a). This antibody was a generous gift from Dr. K. Rango Rao (University of West Florida, Florida) and was used at a dilution of 1:1000. It recognizes the insect homologues of PDH (PDF) and has been used to trace the axonal projections of putative clock cells in various other insects (Závodská et al., 2003). PDF has previously been cloned from seven insect species (Matsushima et al., 2004) and insect PDFs share an average 83% sequence identity with *Uca* PDH (Vafopoulou and

Steel, 2012a). The specificity of this antibody for *Rhodnius* PDF has been demonstrated previously in this lab (Vafopoulou et al., 2010). Further, the trace archive of the *Rhodnius* genome contains sequences corresponding exactly to the coding sequence used to produce these PDF antibodies. It should be noted that the present study uses the anti-guinea pig PDF antibody simply as a tool to trace axons. No claims are being made as to its identity, thus it is unnecessary to demonstrate its recognition of the native PDF of *Rhodnius*.

Fluorescein isothiocyanate (FITC) goat anti-guinea pig IgG, FITC goat anti-rat IgG, tetramethylrhodamine isothiocyanate (TRITC) goat anti-guinea pig IgG, TRITC goat anti-rabbit IgG and TRITC goat anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO) and used at a dilution of 1:200.

2.3. Dot Blots

The anti-5HT used presently has been tested previously for cross-reactivity with similar molecules (Consolazione et al., 1981) and has been shown to bind specifically to the formaldehyde-5HT conjugate, particularly to 5HT following fixation in paraformaldehyde (PFA; Milstein et al., 1983). Dot blots were presently carried out in order to confirm this. Two brains were dissected. Following incubation of one brain in 4% PFA for 2h, brains were processed separately as outlined in Vafopoulou and Steel, 2002. One brain was used for each blot (two brain blots total). Protein extracts were dotted individually onto nitrocellulose filters. Stock solutions of L-dopamine, melatonin, L-tryptophan, 5HT and BSA were made to a concentration of 0.1g/mL. From this stock,

solutions were diluted further (either in distilled water or 4% PFA) by a factor of 2, bringing them to final concentrations of 0.05g/mL. Solutions diluted in 4% PFA were allowed to incubate for 2h at room temperature. A volume of 15μl of each 0.05g/mL solution was dotted onto nitrocellulose filters. Filters were processed according to protocol outlined in Vafopoulou and Steel, 2002. Filters were submerged in primary antibody solution (anti-5HT conjugated to BSA, produced in rat) for 2h. The anti-5HT was used at a concentration of 1:500. Secondary antibody was an anti-rat IgG conjugated to horseradish peroxidase. It was used at a concentration of 1:200. Immunoreactive material was visualized with 2,2-diaminobenzidine hydrochloride as a peroxidase substrate and hydrogen peroxide. Indeed, after fixation with 4% PFA, 5HT was the only chemical that reacted with the anti-5HT antibody (see Fig. 2).

2.4. Immunohistochemistry and imaging

Brains were dissected under *Rhodnius* saline (129mM NaCl, 8.6mM KCl, 8.5mM $MgCl_2.6H_2O$, 2mM $CaCl_2.H_2O$, 34mM glucose, 15mM Tris.HCl; pH 7.2; Lane et al., 1975) and then immediately fixed in freshly prepared 4% PFA in phosphate buffered saline (PBS; 2.4mM $NaH_2PO_4.H_2O$, 8.3mM Na_2HPO_4, 150mM NaCl; pH 7.2) for 2h at room temperature. Brains were thoroughly washed in PBS (pH 7.2) and then pre-incubated for 1h in 5% control serum containing 1% Triton X-100 as a permeabilizing agent. Brains were then incubated in primary antisera for 24h at 4°C followed by a thorough washing in PBS prior to incubation in secondary antibody solutions for 2h at room temperature on a flatbed shaker. For double-labeling with anti-AST-7 and anti-PDF

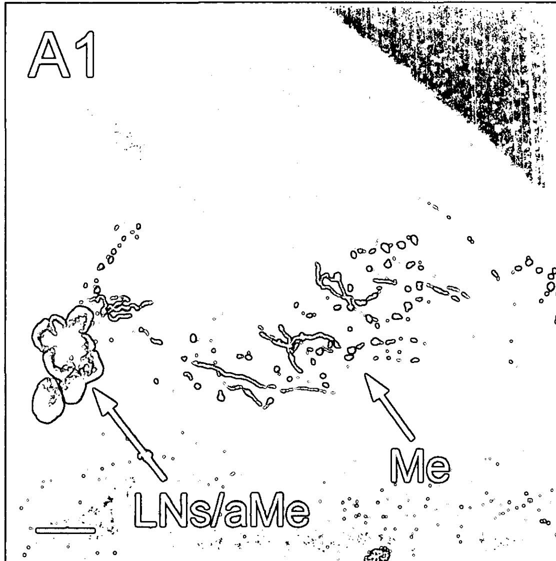
(GenScript), TRITC goat anti-mouse IgG and FITC goat anti-guinea pig were used as the secondary antibodies, respectively. For double labeling with anti-CCAP and anti-PDF (GenScript), TRITC goat anti-rabbit IgG and FITC goat anti-guinea pig IgG were used as the secondary antibodies, respectively. For double labeling with anti-FMRFamide and anti-PDF (GenScript), TRITC goat anti-rabbit IgG and FITC goat anti-guinea pig were used as the secondary antibodies, respectively. For double labeling with anti-5HT and anti-PDF (GenScript), FITC goat anti-rat IgG and TRITC goat anti-guinea pig were used as the secondary antibodies, respectively. After thorough washing in PBS, brains were mounted in 1,4-diazabicyclo[2.2.2]octane mounting medium (Sigma-Aldrich). In controls, the primary antibodies were replaced with non-immune serum or the secondary antibodies were replaced with PBS. In the case of the anti-FMRFamide and anti-5HT, antibodies were also employed after preadsorption. Fluorescence levels in controls were indistinguishable from the background and autofluorescence was not detected (see Fig. 3).

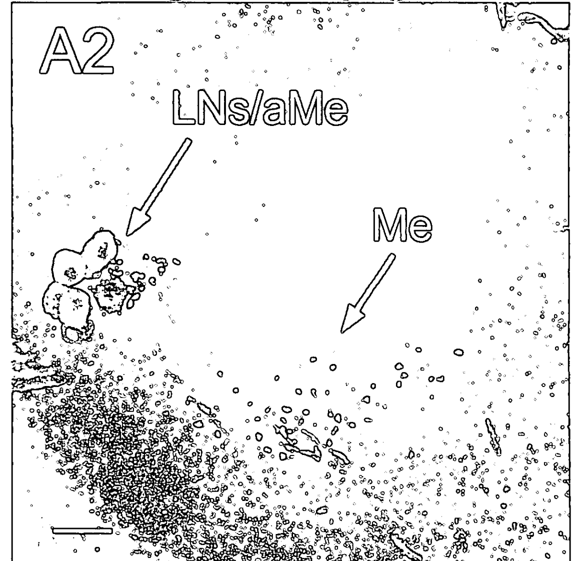
Digital optical sections were viewed using an Olympus FV300 confocal laser scanning microscope at intervals of 1-µm. Parameters of the microscope were kept constant for all image capture. Images were processed using Image J (1.37; NIH, Bethesda, MD). Images were merged using Confocal Assistant (4.2; University of Minnesota, MN). Adobe Photoshop 7.0 (San Jose, CA) was used to create panels and to make minor contrast adjustments. Visual enhancement was achieved in some images by inversion of the gray scale (see Results) so that intense fluorescence appears black on a light background.

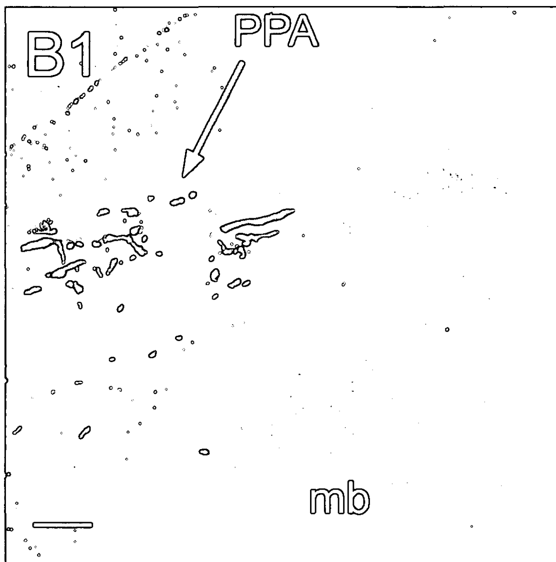
For rhythmicity experiments, mean pixel intensity (±SEM) from 10 cells staining with each of the four antibodies selected randomly from 4 animals per time point was calculated using the line tool of the Image J (1.45) software. The length of the line was kept constant in all measurements. The Student's "*t*" test was used to make comparisons between time points. Background fluorescence levels were obtained from an equal number of adjacent cells which lacked fluorescence in the same preparations. Mean pixel intensity was calculated for each of the cells represented in Fig. 13.

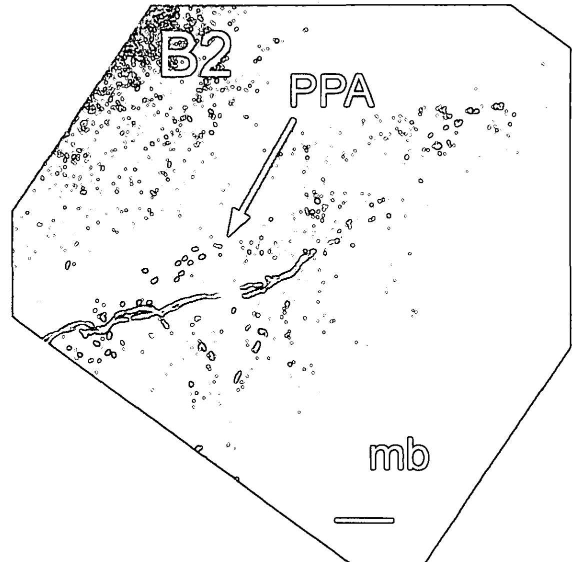
anti-rabbit PDF

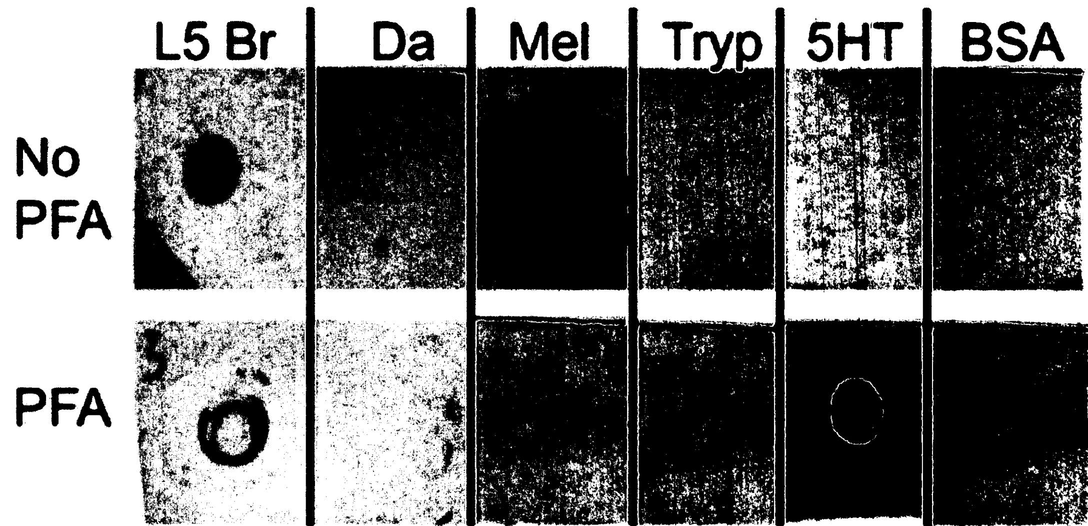
anti-guinea pig PDF










L5 Br
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Mel
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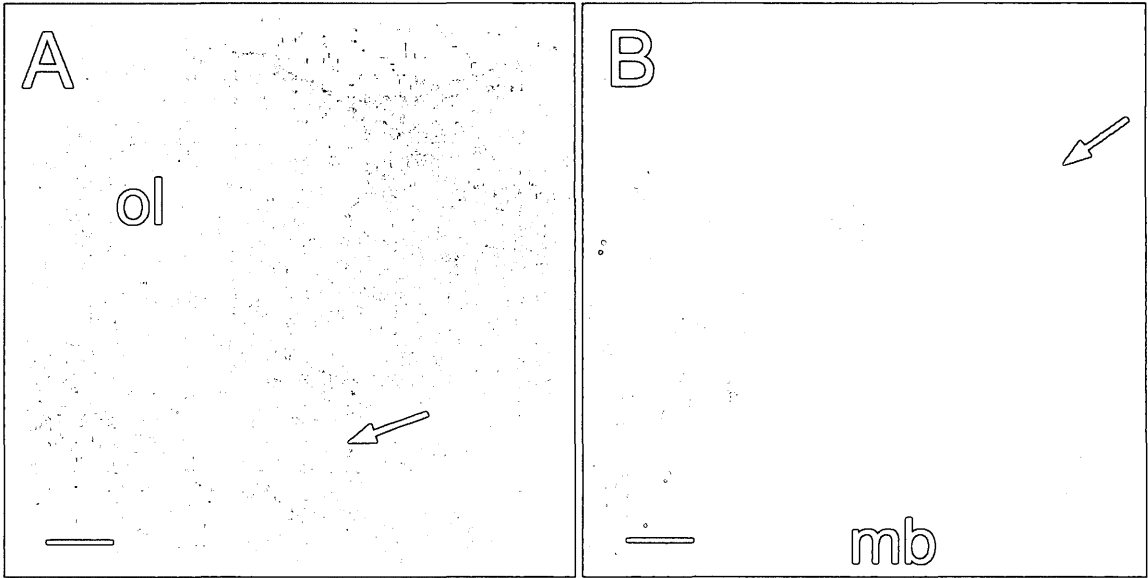
5HT
BSA
No PFA
PFA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RESULTS

3.1. Localization of brain neurons with projections to clock-related areas

Somata immunoreactive to four different neurochemicals were localized in the compound eyes, optic lobes, and dorsal protocerebra of fifth instar larval insects. Fluorescence resulting from binding of anti-allatostatin-7 is presently referred to as AST-7-like-immunoreactivity (AST-7-lir); from binding of anti-crustacean cardioactive peptide as CCAP-like immunoreactivity (CCAP-lir); from binding of anti-FMRFamide as FMRFa-like immunoreactivity (FMRFa-lir); and from binding of anti-serotonin as 5HT-like immunoreactivity (5HT-lir). Binding of anti-PDF will be referred to as PDF-like-immunoreactivity (PDF-lir).

3.1.1. AST-7-lir in the compound eyes

Anti-AST-7 was the only antibody presently studied to stain the compound eyes (Fig. 4). In *Rhodnius,* the compound eyes consist of hundreds of self-contained visual units known as ommatidia, each of which possesses eight photoreceptor units called retinula cells. The cytoplasm and axons of one retinula cell per ommatidium were filled with AST-7-lir (Fig. 4B; shown at higher magnification in Fig. 4B1, B2). These retinula cell axons project medially from the compound eye into the optic lobe, through the lamina (La) and into the medulla (Me) wherein they arborize extensively (Fig. 4C-E). These retinula cells arborize entirely within the optic lobe. There were no cell bodies staining with anti-AST-7 in the optic lobe. Thus, it is probable that the arborizations in

the Me derive solely from retinula cells staining with anti-AST-7, which likely synapse with cells that are devoid of AST-7-lir. A few axons showing AST-7-lir originating from a small number of retinula cells project postero-medially, beyond the Me and into the accessory medulla (aMe) wherein they form terminal varicosities giving some indication that the anti-AST-7 may be binding an allatostatin-like peptide that is involved in a photic entrainment pathway of the insect brain clock. AST-7-like staining of the compound eyes was always specific; autofluorescence of ommatidia is shown in Fig. 4A. It should be noted that this is not real staining.

3.1.2. AST-7-lir in brain neurons

Intense AST-7-lir was observed in a limited number of cells with projections to clock-related brain regions (a total of ~9 cells), particularly the PPA. These cells can be subdivided into two anatomically distinct and contralaterally symmetric groups and they are responsible for the intense and extensive AST-7-lir observed in arborizations in the PPA. All of these cells were intensely labeled with anti-AST-7 and staining was always cytoplasmic. A panoramic image of AST-7-lir throughout the central brain complex is shown in Fig. 5A (white lined boxes outline the two cell groups). The first group, called here A-G1 (Fig. 5B), consists of seven cells. Six of these were roughly 12-μm in diameter and projected axons ventro-medially for a short distance before arborizing in the PPA. The seventh (indicated with a short arrow in Fig. 5B) was roughly double this size and possessed a second branch that projected medially and then posteriorly to terminate in the retrocerebral complex just posterior to the brain (vertical axon traced with arrowheads in

Fig. 5B). The second group, called here A-G2 (indicated by arrow in Fig. 5C), consists of two cells ~12-μm in diameter and projected axons postero-medially for a short distance before curving sharply towards the anterior brain to arborize in the PPA. Several other axons showing AST-7-lir crossed the midline, but the cells from which they originate could not be determined and they could be traced no further (not shown).

3.1.3. CCAP-lir in brain neurons

CCAP-lir was also observed in a limited number of cells in four distinct groups in each brain hemisphere (a total of ~160 cells). All of these cells were intensely labeled with anti-CCAP and staining was always cytoplasmic. A panoramic image of CCAP-lir throughout the central brain complex is shown in Fig. 6A (black lined boxes outline three of four cell groups). Two of these groups were located in the optic lobe and two in the central protocerebrum. The first group, called here C-G1 (Fig. 6B-D), consisted of ~150 cells (~8-μm in diameter) located mainly in the distal optic lobe in the region of the La and Me, from the dorsal brain surface to a depth of approximately 100-μm. The majority of these cells did not have traceable axons, but likely arborized primarily within the Me. Arrow in Figs. B, C, and D point to C-G1 cells. The second group, called here C-G2 (not shown), consisted of 4 cells located very near the dorsal brain surface, dorsal to the Me at the base of the optic lobe, near the junction between the optic lobe and lateral protocerebrum. Three of these cells (~16-μm in diameter) had axons which could be followed for a short distance before entering the Me near its base and branching. The fourth cell (~12-μm in diameter) was located just posterior and slightly medial to the base

of the Me and projected an axon anteriorly for a short distance before curving distally, apparently through the aMe to branch within the Me (shown in Fig. 9A). The third group, called here C-G3, consisted of only one cell (~16-μm in diameter). This cell is indicated with an arrow in Fig. 6F. It was situated in the lateral protocerebrum and projected posteriorly for a short distance before curving medially and anteriorly to divide into two branches which extended into two distinct brain regions: the first into the PPA in the dorsal anterior protocerebrum (branching pattern shown in fig. 6F), and the second just ventral to this (branching pattern not shown). The fourth group, called here C-G4, consisted of two cells (~16-μm in diameter) that were located in the postero-medial protocerebrum. These cells are indicated with an arrow in Fig. 6E. The axons of these cells intertwine as they project towards the anterior to branch in the PPA. Several axons showing CCAP-lir crossed the midline, but the cells from which they originate could not be determined and they could be traced no further (not shown).

3.1.4. FMRFamide-lir in brain neurons

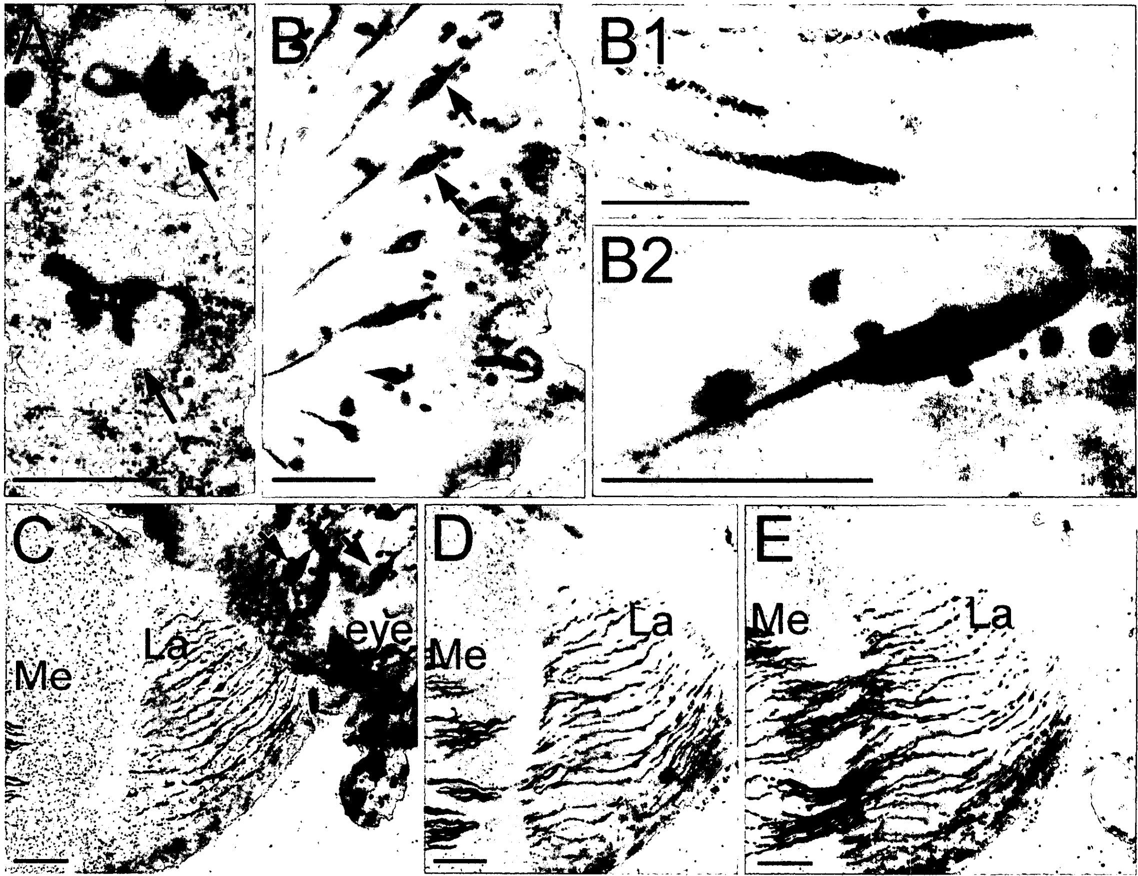
Intense FMRFa-lir was observed in cells in every dorsal brain region and staining was always cytoplasmic (Fig. 10A). Four cells showing FMRFa-lir (~12-μm in diameter) were observed at the base of contralateral optic lobes, near the junction between the optic lobe and lateral protocerebrum, apparently just dorsal to the aMe in the region of the lateral neurons (LNs). The projections of these cells followed a strikingly similar path to that of the LNs – they projected ventrally to arborize in a small ovoid neuropil from which they projected in two directions: distally, towards the compound eye through the

Me and La neuropils; and medially, into the central protocerebrum where they arborize extensively in the PPA. Several other FMRFa-ir cells (~12-16-µm in diameter) situated in the central protocerebrum also appeared to contribute to the extensive branching pattern of axons in the PPA. Strong FMRFa-lir was observed in several large medial neurosecretory cells (and their axons) on either side of the midline (fig. 10A). Fluorescence in these cells has previously been shown in *Rhodnius* (Tsang and Orchard, 1991). Several axons containing FMRFa-lir crossed the midline, but the cells from which they originate could not be determined and they could be traced no further (not shown).

3.1.5. 5HT-lir in brain neurons

5HT-lir was observed in a limited number of cells in five distinct groups in each brain hemisphere (a total of ~20 cells). All of these cells were intensely labeled with anti-5HT and staining was always cytoplasmic. A panoramic image of 5HT-lir throughout the central brain complex is shown in Fig. 7A (the five cell groups are outlined by white lined boxes). Two of these groups were located in the optic lobe and three in the central protocerebrum. The first group, called here S-G1 (Fig. 7F), consisted of four cells (~12-µm in diameter) located just dorsal to the Me. These cells projected axons ventrally into the Me within which they branched extensively. The second group, called here S-G2 (indicated with an arrow in Fig. 7G), consisted of five cells (~12-µm in diameter) located just posterior to the base of the Me near the junction between the optic lobe and lateral protocerebrum. The axons of these cells projected anteriorly to branch within the Me. Some of these branches likely terminated within the Me, while others extended axons

which were detected with faint 5HT-fluorescence in the aMe. The third group, called here S-G3 (indicated with arrows in Fig. 7B), consisted of three cells (~12-μm in diameter) situated in the anterior protocerebrum just anterior to the PPA. These cells had axons which intertwined as they projected a short distance medially before curving posteriorly to branch in the PPA. The fourth group, called here S-G4 (indicated with arrows in Fig. 7C), consisted of two cells (~12-μm in diameter) located in the lateral protocerebrum which projected axons that intertwined as they travelled postero-medially before curving anteriorly to branch in the PPA. The fifth group, called here S-G5 (indicated with arrows in Fig. 7D), consisted of five cells (~12-μm in diameter) situated in the postero-medial protocerebrum which projected axons that intertwined as they travelled anteriorly to branch in the PPA. Several axons possessing 5HT-lir crossed the midline, but the cells from which they originate could not be determined and they could be traced no further (not shown).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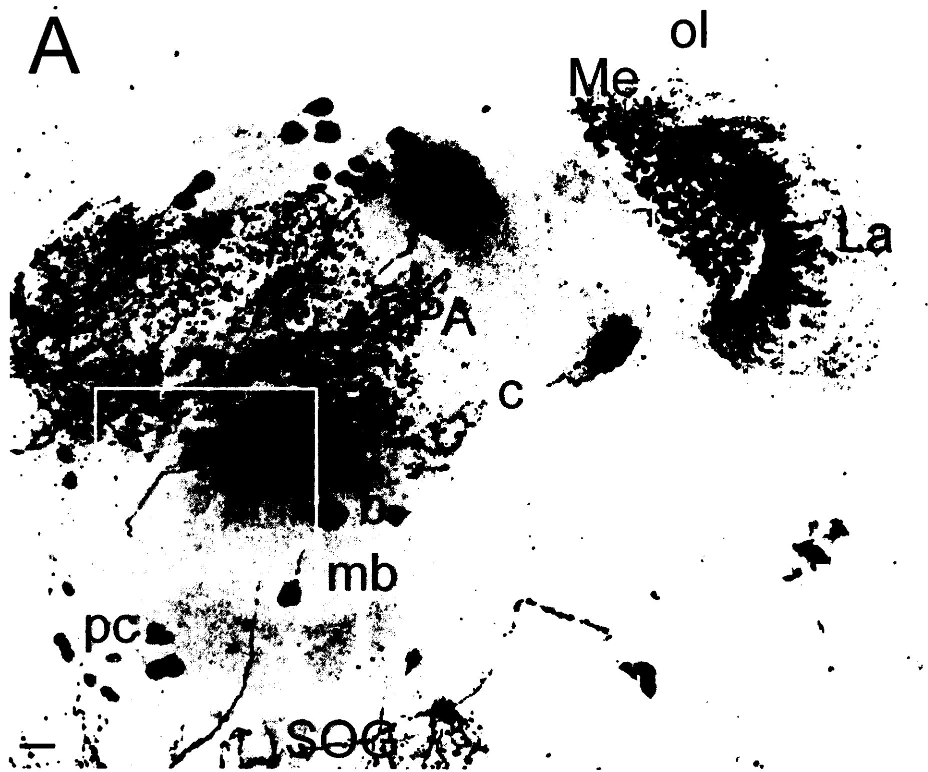
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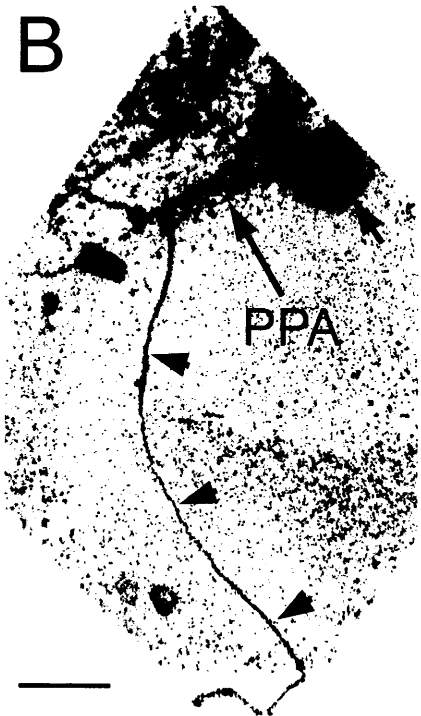
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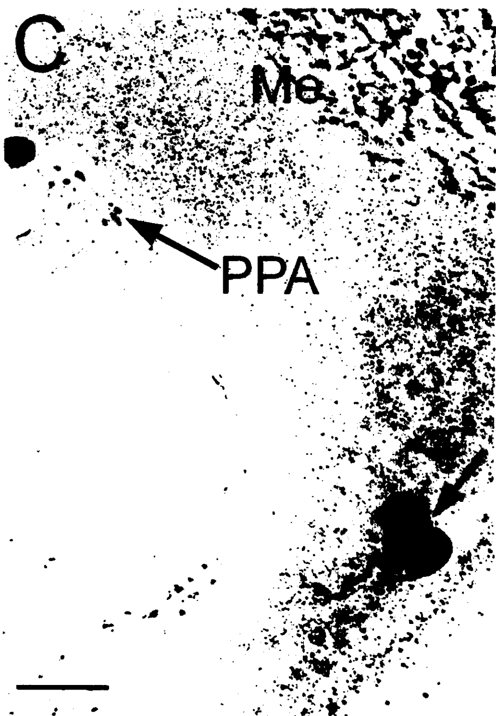
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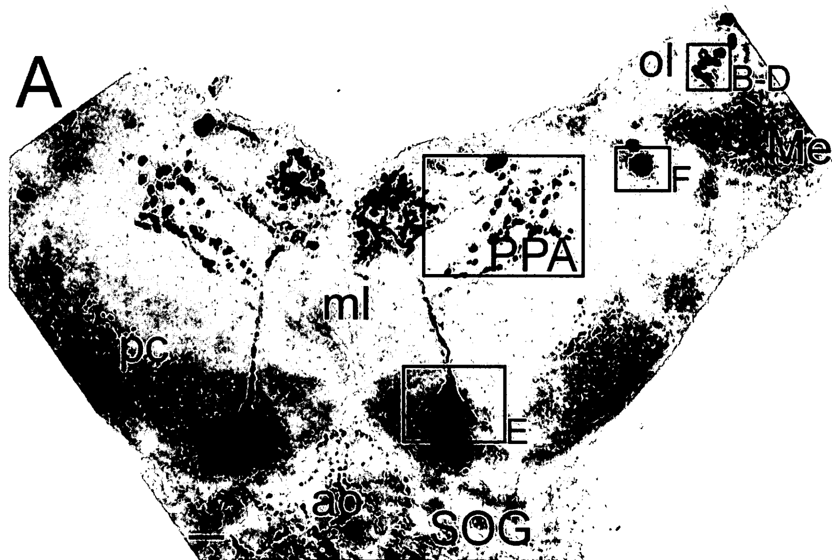
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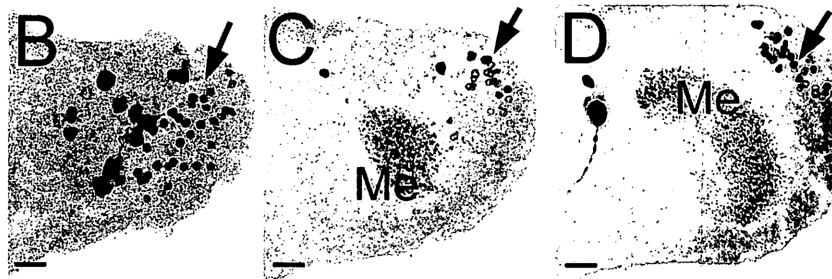
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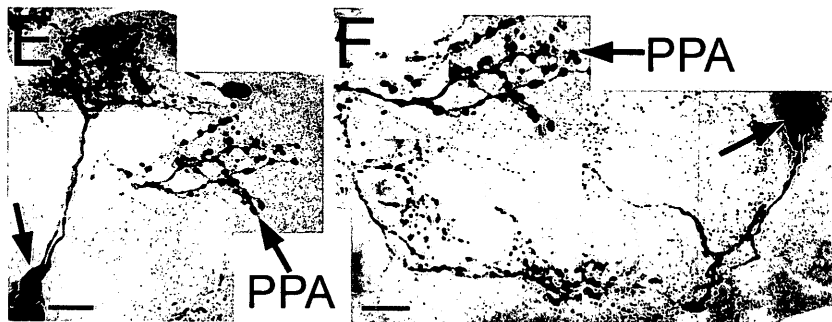
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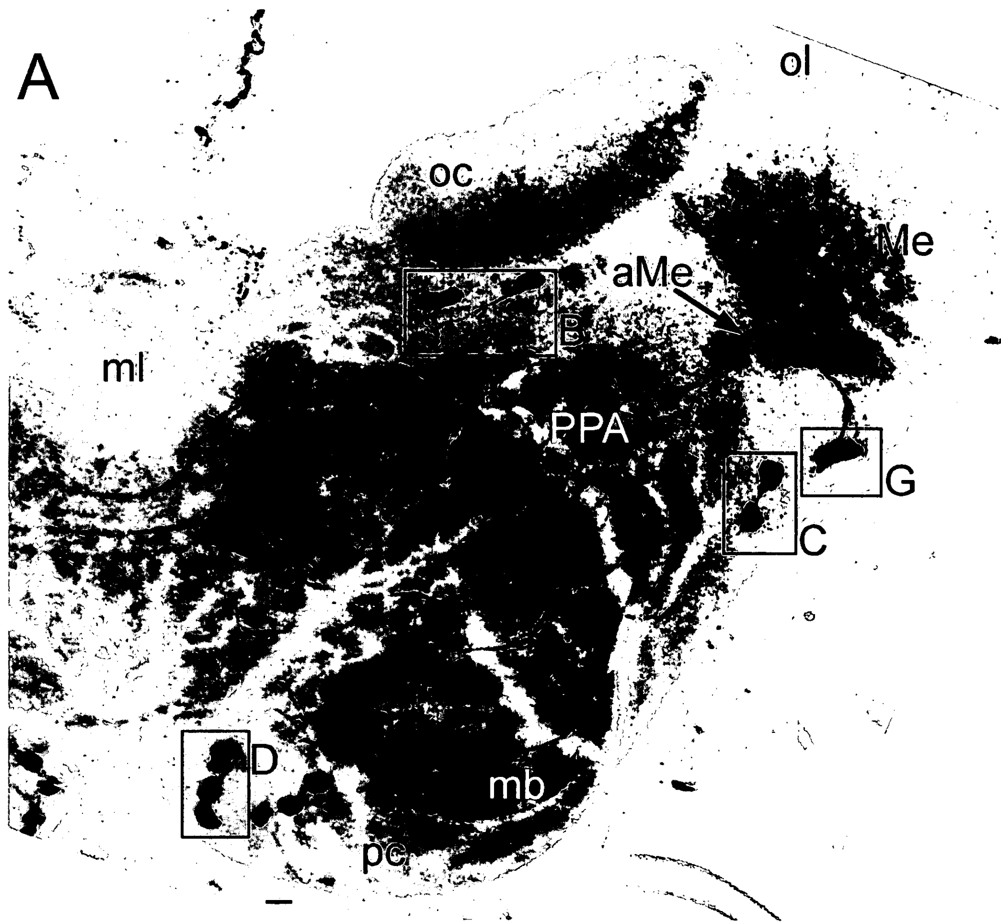
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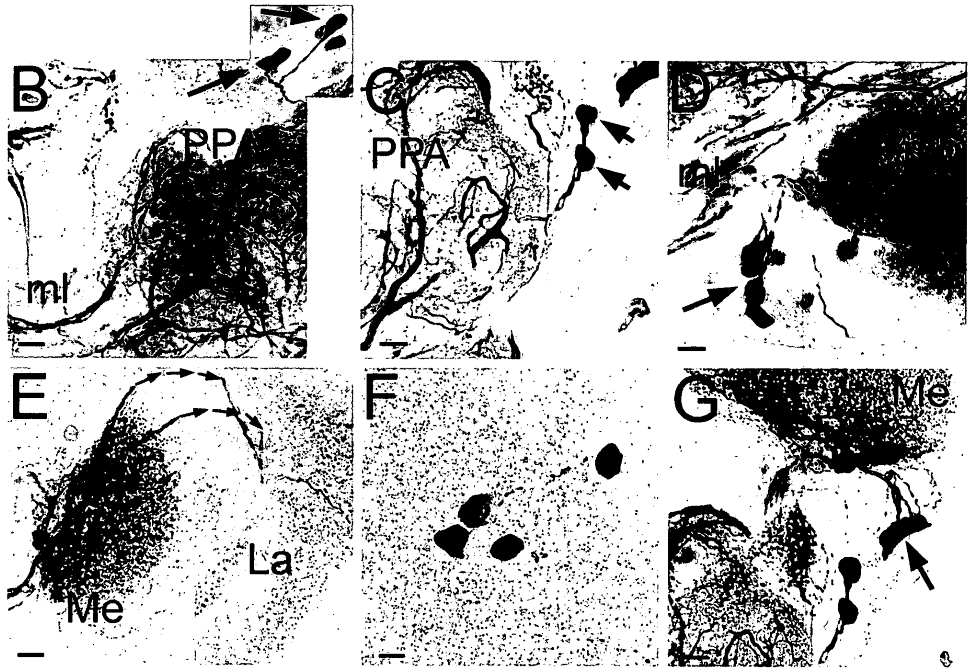
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3.2. Neuroanatomical relationships of clock cells and neurochemicals in the brain

All four antibodies presently used stained neurons that arborize in clock-related regions, including: the aMe, Me and La neuropils located in the optic lobes; and the PPA located in the dorsal anterior protocerebra. This introduced the possibility that these neurons may be interacting with the circadian clock system in the *Rhodnius* brain. Therefore, brains were double-labeled with anti-PDF and antibodies raised against each of the four neurochemicals described above and the projections of these neurons were examined, looking specifically for regions of potential interaction with LN axons. PDF has long been used as a tool to trace the axonal projections of the LNs. In *Rhodnius,* the LNs arborize in the aMe, from which they project into two brain regions: (1) towards the compound eyes, through the Me and La neuropils; and (2) towards the central protocerebrum within which they arborize in the PPA.

3.2.1. Double-labeling of anti-AST-7 with anti-PDF

Brains double-labeled with anti-AST-7 (green channel; left) and anti-PDF (red channel; right) are shown in Fig. 8. The second column shows the merged images of left and right. Multiple slices of 1-μm thickness were taken from the same z-series showing proximity of axons with AST-7- and PDF-lir in clock-related regions and selected slices of each region are represented: the aMe (Fig. 8A-C), the Me (Fig. 8D-F), and the PPA (Fig. 8G-I). The third column shows a selected area of potential interaction (outlined by a white box in images in the second column) at higher magnification. Here, yellow

signifies extreme closeness of, and thus high potential for interaction between, axons and/or varicosities showing AST-7- and PDF-lir.

In the optic lobe, there are no cell bodies staining with anti-AST-7. All arborizations observed therein derive from the retinula cells. These cells extend axons through the La and arborize primarily in the Me in close proximity to arborizations showing PDF-lir (merged image in Fig. 8E). Varicosities are common in both axon types implying potential for synaptic contacts between them (arrows and arrowheads in Fig. 8E1 respectively point to AST-7- and PDF-lir in varicosities). Few retinula cell axons extend into the aMe in close proximity to axons that show PDF-lir (merged image in Fig. 8B), within which they form terminal varicosities (arrow in Fig. 8B1 points to one), indicating that these may be supplying the clock with input. Projections of the A-G1 and A-G2 cells comprise the intense AST-7-lir observed in the PPA, many of which occur in close proximity to axons with PDF-lir (merged image in Fig. 8H). In this region, axons filled with AST-7- and PDF-lir form a tangled mass and both axon types possess varicosities along their lengths (arrows and arrowheads in Fig. 5H1 respectively point to AST-7- and PDF-lir in varicosities), indicating potential for communication of A-G1 and A-G2 cells with the LNs.

3.2.2. Double-labeling of anti-CCAP with anti-PDF

Brains double-labeled with anti-CCAP (green channel; left) and anti-PDF (red channel; right) are shown in Fig. 9. The second column shows the merged images of left and right. Multiple slices of 1-μm thickness were taken from the same z-series showing

proximity of axons filled with CCAP- and PDF-lir in clock-related regions and selected slices of each region are represented: the aMe (Fig. 9D-F), the Me (Fig. 9G-I), and the PPA (Fig. 9J-L). The third column shows a selected area of potential interaction (outlined by a white box in images in the second column) at higher magnification. Here, yellow signifies extreme closeness of, and thus high potential for interaction between, axons and/or varicosities showing CCAP- and PDF-lir. The top row shows the position of one C-G2 cell with respect to the LNs (Fig. 9B) and points to its axon which projects through the aMe (arrowhead in Fig. 9B).

Two groups of cells displaying CCAP-lir, C-G1 and C-G2, contribute to the intense staining observed in the Me. The axons of these cells are situated in close proximity to axons with PDF-lir (merged image in Fig. 9H) and both axon types possess varicosities along their lengths (arrows and arrowheads in Fig. 9H1 respectively point to varicosities filled with CCAP- and PDF-lir). Axons with faint CCAP-lir detected in the aMe (merged image in Fig. 9E) were likely branches derived from the C-G2 cells. These axons possessed no varicosities, but were engulfed by varicose fibres showing PDF-lir (areas of close contact emphasized in higher magnification merged image in Fig. 9E1 in which arrowheads point to varicosities filled with PDF-lir and arrows point to axons showing CCAP-lir). Projections of the C-G3 and C-G4 cells comprise the arborizations showing CCAP-lir in the PPA, many of which occur in close proximity to axons showing PDF-lir (merged image in Fig. 9K). In this region, axons filled with CCAP- and PDF-lir intertwine with one another and possess varicosities along their lengths (arrows and

arrowheads in Fig. 9K1 respectively point to varicosities filled with CCAP- and PDF-lir), indicating potential for communication between C-G3 and C-G4 cells and the LNs.

3.2.3. Double-labeling of anti-FMRFa with anti-PDF

Brains double-labeled with anti-FMRFamide (green channel; left) and anti-PDF (red channel; right) are shown in Fig. 10. The second column shows the merged images of left and right. Multiple slices of 1-μm thickness were taken from the same z-series showing proximity of axons showing FMRFa- and PDF-lir in clock-related regions and selected slices of each region are represented: the aMe (Fig. 10B-D), the Me (Fig. 10E-G), the La (Fig. 10H-J), and the PPA (Fig. 10K-M). The third column shows a selected area of potential interaction (outlined by a white box in images in the second column) at higher magnification. Here, yellow signifies extreme closeness of, and thus high potential for interaction between, axons and/or varicosities filled with FMRFa- and PDF-lir.

Four cells displaying FMRFa-lir were observed at the base of the optic lobe in the region known to house the LNs. Double-labeling confirmed the position of these cells, nestled in amongst the LNs (merged image in Fig. 10C). The axonal projections of these cells follow a strikingly similar path to that of the LNs – the axons of both cell types intertwined as they projected ventrally into the aMe (merged image in Fig. 10C). From the aMe, they projected distally towards the compound eye through the Me (merged image in Fig. 10F) and the La (merged image in Fig. 10I). In each of these regions (the aMe, the Me, and the La) varicosities filled with FMRFa-lir were observed in close proximity to axons showing PDF-lir, and vice versa (arrows and arrowheads in Fig.

10C1, 10F1, and 10I1 respectively point to varicosities filled with FMRFa- and PDF-lir). Several cells showing FMRFa-lir gave rise to the extensive arborizations seen in the region of the PPA. The axons of these cells were observed in close proximity to axons showing PDF-lir (merged image in Fig. 10L). Both axon types had varicosities along their lengths (arrows and arrowheads in Fig. 10L1 respectively point to varicosities filled with FMRFa- and PDF-lir). In this region, axons filled with FMRFa- and PDF-lir form a messy jumble of processes and the presence of varicosities on both axon types implies potential for communication between some cells showing FMRFa-lir and the LNs.

3.2.4. Double-labeling of anti-5HT with anti-PDF

Brains double-labeled with anti-5HT (green channel; left) and anti-PDF (red channel; right) are shown in Fig. 11. The second column shows the merged images of left and right. Multiple slices of 1-μm thickness were taken from the same z-series showing proximity of axons filled with 5HT- and PDF-lir in clock-related regions and selected slices of each region are represented: the aMe (Fig. 11A-C), the Me (Fig. 11D-F), the La (Fig. 11G-I), and the PPA (Fig. 11J-L). The third column shows a selected area of potential interaction (outlined by a white box in images in the second column) at higher magnification. Here, yellow signifies extreme closeness of, and thus high potential for interaction between, axons and/or varicosities showing 5HT- and PDF-lir.

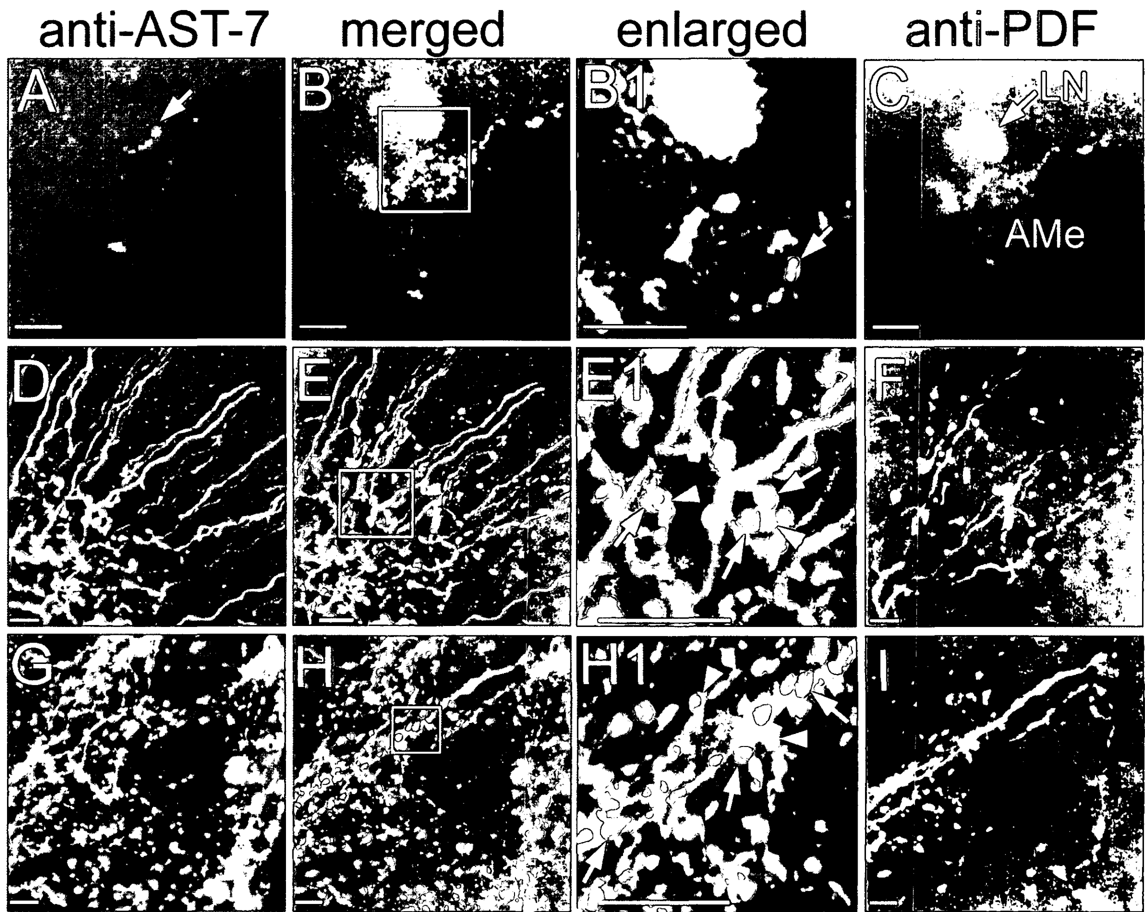
Two groups of cells staining with anti-5HT, S-G1 and S-G2, reside in the optic lobe and contribute to the intense arborizations observed therein. Both groups arborize extensively in the Me with axons in close proximity to axons staining with anti-PDF

(merged image in Fig. 11E), and extend branches into the La also in close proximity to axons displaying PDF-lir (merged image in Fig. 11H). These axons intertwined in both the Me and La, and varicosities were observed along the lengths of both axon types. Arrows and arrowheads in Fig. 11E1 respectively point to varicosities filled with 5HT- and PDF-lir in the Me. Arrows in Fig. 11H1 point to regions in the La where varicosities filled with 5HT- and PDF-lir come into such close proximity that they appear yellow. Areas of extreme closeness between axons staining with anti-5HT- and anti-PDF were particularly evident in the La (yellow areas in Fig. 11H1). Some axons, likely from S-G2 cells, projected into the aMe within which they were engulfed by varicose fibres filled with PDF-lir (Fig. 11B). Arrows in Fig. 11B1 point to an axon filled with 5HT-lir and arrowheads point to varicosities filled with PDF-lir. One large axon displaying intense 5HT-lir projects through the PPA and comes into very close proximity to varicosities filled with PDF-lir in the region (Fig. 11K). The vast number of these varicosities potentially making synaptic contacts with this axon is highlighted in Fig. 11K1, 11K2, and 11K3 (yellow colour). The origin of this axon could not be determined, but it is likely derived from one of the S-G3, S-G4, or S-G5 cells, as these are the cells which project into that region and arborize in the PPA.

3.2.5. In summary

In the optic lobe, axons staining with each of the four antibodies under study were observed to be in close proximity to axons filled with PDF-lir in the aMe and Me neuropils. Axons displaying FMRFa- and 5HT-lir further extended into the La and were

seen in close proximity to axons showing PDF-lir therein. In the PPA, axons filled with AST-7-, CCAP-, and FMRFamide-lir were seen in close proximity to axons possessing PDF-lir and varicosities were observed along the lengths of all axons staining with each of anti-AST-7, anti-CCAP, anti-FMRFa, and anti-PDF. An axon displaying 5HT-lir was observed in close proximity to varicosities filled with PDF-lir. A summary diagram of the distribution of ALP-, CLP-, FLP-, and SLC-containing neuronal cell bodies with projections to clock-related regions in the brain is shown in Fig. 12.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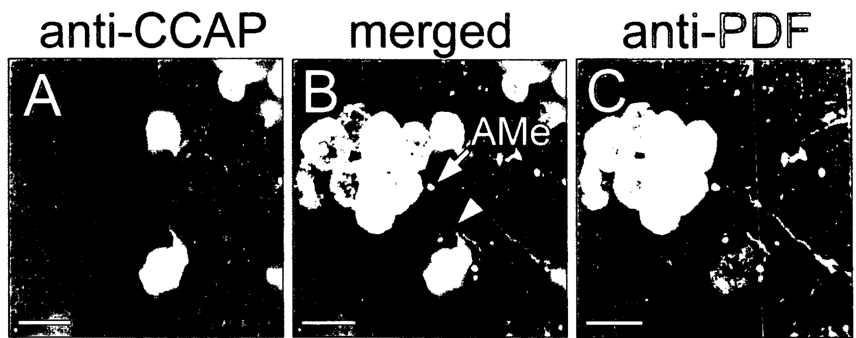
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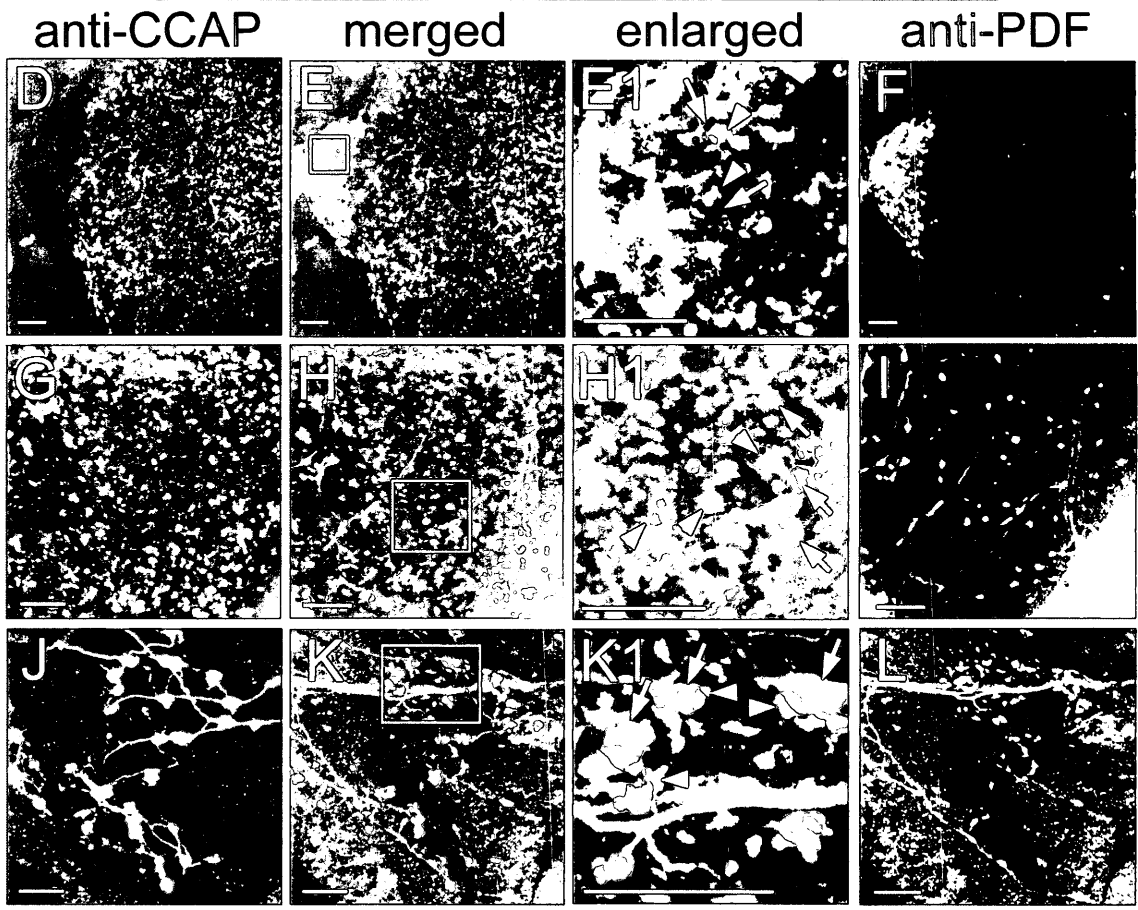
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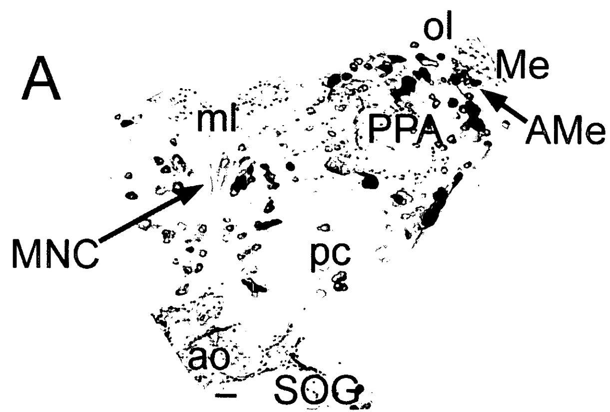
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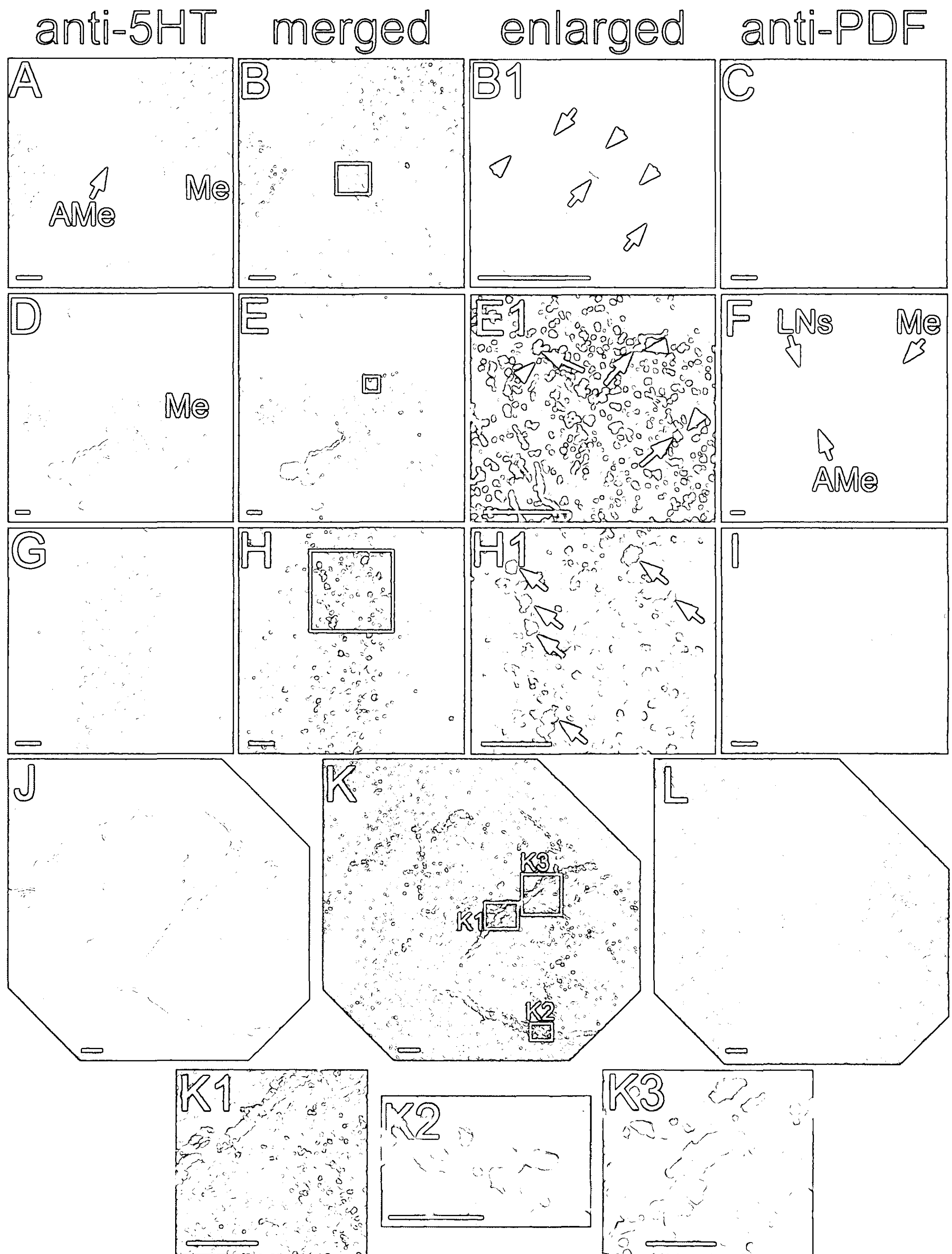
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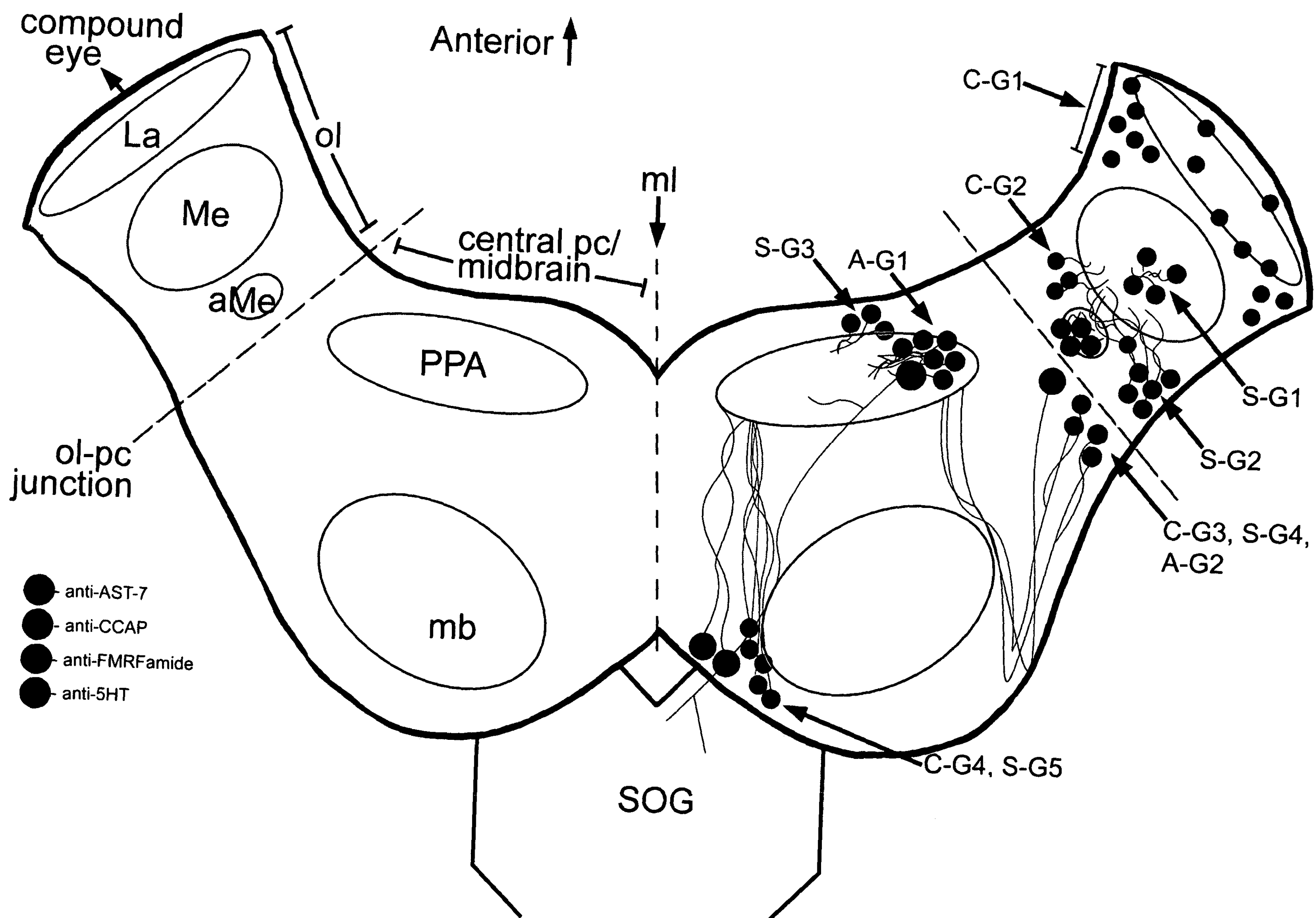

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PPA
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A-G1
S-G1
S-G2
C-G3, S-G4, A-G2
C-G4, S-G5
anti-AST-7
anti-CCAP
anti-FMRFamide
anti-5HT

3.3. Daily rhythmicity in cells and axons immunoreactive to anti-AST-7, -CCAP, -FMRFamide, and -5HT

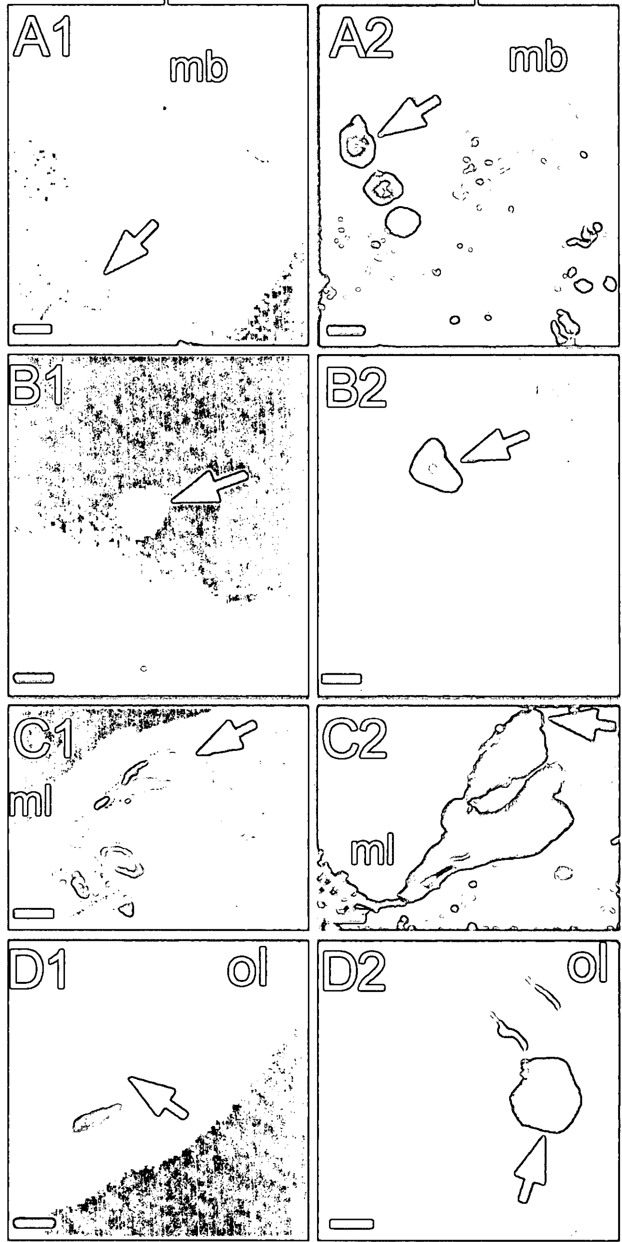
If the neurochemicals presently studied are, in fact, outputs of the brain clock system, it is likely that they have a circadian rhythm of release. Their rhythmic release, in addition to their proximity to varicose fibres associated with the LNs, would cumulatively support their potential role as clock outputs.

3.3.1. Daily rhythmicity of staining in cells

Fluorescence resulting from the binding of anti-AST-7, anti-CCAP, anti-FMRFamide, and anti-5HT was markedly lower in cells during photophase (Fig. 13A1, 13B1, 13C1, and 13D1, respectively) than during scotophase (Fig. 13A2, 13B2, 13C2, and 13D2, respectively). Cells staining with anti-AST-7 were taken from the posterior protocerebrum; cells staining with anti-CCAP were taken from the posterior protocerebrum (C-G4 cell); cells staining with anti-FMRFamide were taken from the antero-medial protocerebrum; and cells staining with anti-5HT were taken from the base of the optic lobe (S-G2 cell). Statistical analysis of mean pixel intensities (±SEM) confirmed that the visible reduction in fluorescence intensity between scotophase and photophase is significant ($P<0.05$ when cells in scotophase are compared with the corresponding cells in photophase) in all cells presently examined. This information is summarized in Table 1.

3.3.2. Daily rhythmicity of staining in axons

The presence of neurochemicals in axons followed exactly the same pattern as in cells. Axons stained intensely during the scotophase, but were visibly depleted of fluorescence during the photophase. Relative intensity of fluorescence was observed in two dense regions of axonal arborizations: the Me and the PPA. No quantitative measurements were taken for these regions. Fluorescence was judged subjectively. Axons in the Me staining with anti-AST-7, anti-CCAP, anti-FMRFa, and anti-5HT were depleted of fluorescence during the photophase (Fig. 14A1, 14B1, 14C1, 14D1, respectively), but were visibly replete with staining during the scotophase (Fig. 14A2, 14B2, 14C2, and 14D2, respectively). This pattern is subjectively much clearer for axons in the PPA: staining of anti-AST-7, anti-CCAP, anti-FMRFa, and anti-5HT was much lower during the photophase (Fig. 14A3, 14B3, 14C3, and 14D3, respectively) and substantially higher during the scotophase (Fig. 14A4, 14B4, 14C4, and 14D4, respectively).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Scotophase
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A2
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B1
B2
C1
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D1
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D2
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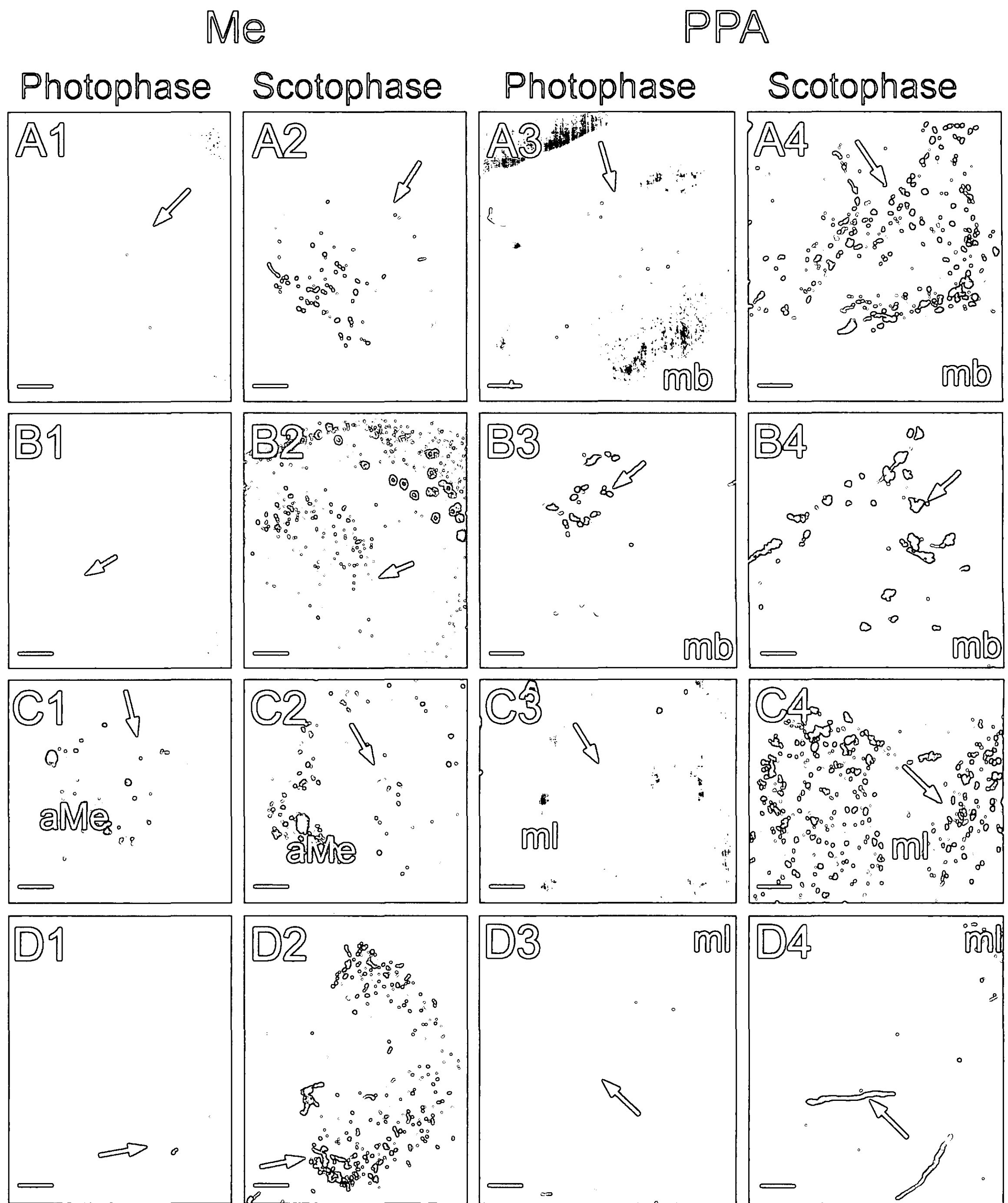
Table 1
Mean pixel intensity ±SEM of immunofluorescence in cells

	Photophase			Scotophase		
	Mean	±SEM	BF	Mean	±SEM	BF
AST-7	867	34	9 ± 0.8	3579	327	15 ± 1.0
CCAP	813	37	2 ± 0.2	3603	92	7 ± 0.5
FMRFa	1366	35	8 ± 0.7	2664	77	76 ± 1.1
5HT	2330	103	15 ± 0.2	4027	32	30 ± 1.5

BF = background fluorescence, N = 40


Me
PPA
Photophase
Scotophase
Photophase
Scotophase
A1
A2
A3
A4
mb
mb
B1
B2
B3
B4
mb
mb
C1
C2
C3
C4
aMe
aMe
ml
ml
D1
D2
D3
D4
ml
ml

DISCUSSION

Within the insect brain, there are two main groups of clock cells – the LNs located at the base of the optic lobe and the DNs located in the posterior brain region. Axonal connections between these groups have been elucidated in two insect species, *D. melanogaster* (Kaneko and Hall, 2000) and *R. prolixus* (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a), indicating the existence of a clock cell network. The aMe, a small ovoid neuropil located ventral to the LNs, was implicated in *Drosophila* as the primary site for integration of circadian timing information, as all synaptic inputs appeared to lead there. However, recent studies in *Rhodnius* have revealed a much larger area of clock cell arborizations located atop the dorsal anterior aspect of the central protocerebral neuropil known as the PPA (first described by Vafopoulou et al., 2007). This area seems to be more highly developed in hemi- than holo-metabolous insects (Helfrich-Förster, 1998). In *Rhodnius*, axons from the PPA to neuroendocrine cells within the brain have been documented, as has the circadian release of peptides from these cells (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b). The circadian control of behaviour in *L. maderae* has also been linked to a region in these insects which may be equivalent to the PPA of *Rhodnius* (Stengl and Homberg, 1994). Thus, it appears that the PPA is central to the production of circadian outputs, at least in hemimetabolous insects. However, pathways by which the PPA receives and integrates circadian information have not been studied previously. The present study examines these pathways and supports a role for the PPA as an important integrative part of the clock system in *Rhodnius*.

4.1. Morphology of neurons with projections to clock-related brain regions

4.1.1. Projections of photoreceptor cells

Anti-AST-7 was the only antibody used that stained the cytoplasm of retinula cells. No other cells in the optic lobe were observed to contain allatostatin-7-like peptide (ALP). Two axons staining with anti-AST-7 were traced from their origin at the retinula cells to the aMe. A previous study of AST-7-like distribution in *Rhodnius* (utilizing the same antibody) also showed staining of the retinula cells (Sarkar et al., 2003), but the authors did not trace the axons of these cells. The axons seen here possessed terminal varicosities located near clock cell axons that stained with anti-PDF, making it likely that ALP is released into the aMe to act as a neurotransmitter and/or neuromodulator input affecting the clock. The aMe has been implicated in the photic entrainment of the insect brain clock (reviewed by Helfrich-Förster, 2004). The ALP-positive axons seen here are well placed to conduct information from the external environment to the aMe and ultimately to the brain clock. Therefore, ALPs may be involved in the process of photic entrainment in *Rhodnius.*

There is evidence that the ALP observed presently may play another role within the optic lobe of *Rhodnius.* A far greater number of ALP-positive retinula cell axons were observed to arborize in the medulla than in the aMe, an observation not anomalous to *Rhodnius.* This phenomenon has been observed previously in cockroaches (Petri et al., 1995), locusts (Würden and Homberg, 1995), and *Drosophila* (Yoon and Stay, 1995). Thus, a second role for ALP in the optic lobe may be to enable communication between

retinula cells and post-synaptic neurons to affect a process which may not be directly linked to the functioning of the clock.

4.1.2. Local neurons of the optic lobe

Other neuron types, including those staining with anti-CCAP or anti-serotonin, were similarly anatomically restricted to the optic lobe with arborizations in the aMe, the medulla, and/or the lamina. These neurons will be referred to here as local neurons of the optic lobe, as they do not possess axons connecting any of the optic lobe neuropils to the midbrain. Anatomically similar neurons that possessed arborizations restricted specifically to the aMe with no more than faint branches in the medulla have been described previously in cockroaches as "local neurons" of the aMe (Petri et al., 1995; Loesel and Homberg, 2001), thus there is precedence for calling these "local neurons" of the optic lobe. There is evidence, based on the observed potential for communication pathways between the axons of local neurons of the optic lobe and those staining with anti-PDF, that these neurons may be involved in the functioning of the brain clock.

From an immunohistochemical standpoint, within the optic lobe alone, the medulla and lamina seem to be more important than the aMe as potential sites for neurochemical communication. C-G1 and S-G1 cells are located entirely within the optic lobe and arborize intensely within the medulla. A group of ~170 cells was observed in the optic lobe of *L. migratoria* which was similar in morphology to the C-G1 cells seen here and were dubbed amacrine neurons of the medulla and/or tangential neurons of the medulla-lobula (Dircksen and Homberg, 1995). C-G2 and S-G2 cells are located at the

base of the optic lobe, near the junction between the optic lobe and central protocerebrum (midbrain) very near the base of the medulla. These cells form clusters that are located relatively near to the LNs, but are separate from them. These cells arborize primarily within the medulla, but also extend faint branches into the aMe before entering the medulla and arborizing therein. Cells containing CCAP-like peptides (CLPs) and serotonin-like chemicals (SLCs) were observed previously in *S. gregaria* and *L. migratoria* in an anatomically similar location to those seen presently and similarly connect the aMe to the medulla via fine fibres (Dircksen and Homberg, 1995; Würden and Homberg, 1995). Branches that could not be immunohistochemically traced back to any SLC-positive cells extend from the medulla to the lamina to arborize extensively. A handful of ALP-positive retinula cells are the only cells presently studied which appear to terminate within the aMe neuropil, while FMRFamide-like peptide (FLP) is the only other neurochemical present in axonal varicosities in the aMe. In *Rhodnius,* most local neurons that we identified in the optic lobe arborize entirely within the medulla and send no branches into the aMe. Very few neurons send axons through the aMe prior to arborizing intensely in other optic lobe areas, particularly the medulla and lamina. Thus, the medulla and lamina are implicated as integrative regions which appear to be substantially more active than the aMe.

4.1.3. Local and projection neurons of the midbrain

Two clusters of cells, S-G3 and A-G1, are presently considered to be local neurons of the midbrain. These groups are located in the anterior protocerebrum and

project short axons relatively directly into the PPA before branching. Within the midbrain, there were five groups of cells located in two anatomically distinct regions which projected axons relatively long distances before arborizing extensively in the PPA. These cells are referred to here as projection neurons (PNs) of the midbrain. PNs have been described previously as neurons with processes connecting them with other anatomically distinct brain regions (Petri et al., 1995; Loesel and Homberg, 2001). C-G3, S-G4, and A-G2 are located in the lateral protocerebrum and all have projections that follow a strikingly similar trajectory: they extend towards the posterior brain, angled slightly medially all the way, before curving sharply towards the anterior brain to arborize within the PPA. C-G3 bifurcates and extends branches ventral to and distinct from the PPA. C-G4 and S-G5 are located in the postero-medial protocerebrum and project axons which similarly intertwine with one another as they shoot directly towards the anterior, weaving in a repetitive wave-like ventral-to-dorsal pattern, before arborizing in the PPA. All neurochemicals identified here were seen in axons in the PC bridge which is known to be involved in the bilateral integration of information in the brain (Popov et al., 2003). The largest of the A-G1 cells also possesses a bifurcated projection, one branch from which extends into the retrocerebral complex. This cell was observed by Sarkar et al. (2003) to send one of its processes out of the brain via the paired nervi corpora cardiac II and into the aorta. Sarkar et al. (2003) noted that this cell branches extensively in the aorta and forms neurohaemal sites. In the present study, the aorta of *Rhodnius* was observed to contain a rich population of axons positive for ALP, CLP, FLP, and SLC (data not shown), indicating the potential release of all the neurochemicals

presently studied from this neurohaemal organ to act as neurohormones. Many cells containing FLP likely also contribute to the staining seen in axons in the PPA.

4.2. Putative inputs and outputs of the insect brain clock system

Neurochemical-filled varicosities found along axonal branches represent release (i.e. output) sites, but it is also possible for single branches to possess both input and output sites (reviewed by Nässel, 2009). Santos et al. (2007) used the strength of axonal staining along with the presence of varicosities to define input and output sites along axons – strongly stained axons generally possess varicosities that likely represent output sites, while weakly stained axons may represent input sites containing fewer neurochemical-filled vesicles. In the present study, varicosities along the length of axons are presumed to be sites of neurochemical output, but the possibility that these axons also possess input sites is not discounted.

4.2.1. Putative functional roles within the aMe and PPA

In the optic lobe, the neurochemicals presently studied appear to be of minimal influence to the aMe and their putative functional roles in the brain clock with respect to the aMe seem clear: ALP appears to act as an input to the clock as it is present in terminal varicosities in the aMe; both CLP and SLC appear to be outputs from the clock, as there were no observable varicosities positive for either of these neurochemicals in the aMe, but there were axons in close proximity to PDF-filled varicosities; and FLP may act as both input to and an output from the clock, as indicated by the presence of FLP(s) in

varicosities as well as in a huge number of axons, both faintly and strongly stained, in the aMe. Within the optic lobe alone, the presence of these neurochemicals is far more substantial in axons of the medulla and lamina neuropils than in the aMe.

In stark contrast to the limited immunoreactivity of axons innervating the aMe, all neurons presently identified densely innervated the PPA. Axons positive for ALP, CLP, FLP, and SLC that were observed in the PPA are engulfed by varicose PDF-ir (i.e. clock-related) axons, but only those axons containing ALP, CLP, and FLP possessed varicosities. This suggests a bidirectional flow of information between cells containing these neuropeptides and the LNs. No SLC-filled varicosities were observed within the PPA, but large axons were present. In other words, ALP, CLP, and FLP may be facilitating the transmission of information from other brain regions to the clock (i.e. ALP, CLP, and FLP may act in input pathways to the clock) and all neurochemicals presently studied, including ALP, CLP, FLP, and SLC, are likely receiving rhythmic inputs from the clock via PDF-filled varicosities (i.e. ALP, CLP, FLP, and SLC may act in output pathways from the clock), indicating a possible role for all of these neurochemicals as neurotransmitters and/or neuromodulators within the brain clock system.

Further support for the roles of these neurochemical-containing cells as outputs of the brain clock can be provided by their morphology. The cells seen presently in the midbrain of *Rhodnius* staining with anti-AST-7, anti-CCAP, anti-FMRFamide, and anti-serotonin appear to have a similar morphological appearance to those deemed "projection neurons" by Hayashi and Hildebrand (1990) in the brain of the moth *Manduca sexta.*

Projection neurons are known to be involved in information output (Homberg et al., 1989) and therefore it seems likely that the neurochemical-immunoreactive cells observed presently in the midbrain of *Rhodnius* function as clock outputs. The anatomical placement of the cells and projections staining with these antisera with respect to PDF-ir axons in the midbrain, in combination with their likely identity as PNs indicates that these cells may be involved in the circadian output of timing information. The elaborate nature of ALP-, CLP-, FLP-, and SLC-ir arborizations observed in the *Rhodnius* PPA compared with the aMe is, on its own, indicative of the PPA being a more prominent site for integration within the brain than the aMe. The extensive intermingling of these varicose arborizations with varicose PDF-ir fibres suggests that neurons staining with these neurochemicals likely interact with the clock via arborizations in the PPA.

4.2.2. Daily rhythmicity

All neurochemicals seen here appear to cycle with a daily rhythm in both cells and axons, indicating they are all produced and transported with a synchronous daily rhythm and may all act as messengers of time (i.e. outputs) within the insect brain clock. The apparent daily rhythm of neurochemical staining in cells and axons appears to follow the same pattern: staining in both is much higher during mid-scotophase and much lower during mid-photophase. This gives an indication that the production and release of neurochemicals in and from these cells may be tightly coupled (i.e. release occurs as the neurochemicals are produced). The existence of a daily rhythm in all neurochemicals seen here implies communication of timing information from the clock to these

neurochemical-containing cells; outputs from the clock must be driving this rhythmicity. Since all cells containing neurochemicals exhibit a daily rhythm and all have extensive arborizations in the PPA, these clock outputs are very likely coming from axons in the PPA. This conclusion is supported by recent work from this lab which drew a similar functional link between axons from the PPA and the rhythmic release of neuropeptides from nearby cells (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b). A peptide known as eclosion hormone (EH) has been shown to have a gated rhythm of release in the silkmoth *Antheraea pernyi* (Truman, 1972). In a variety of insects, EH-positive cells are located largely in the lateral protocerebrum and, particularly within the hemipteran species studied, these cells have ample opportunity to interact with the clock via PDF-positive fibres in the central protocerebrum (Závodská et al., 2003), which may be equivalent to the PPA, thus highlighting the importance of this region across a range of insect species. The PPA is implicated as an important integrative center of the brain clock system and the neural pathways responsible for the apparent rhythmic production of these neurochemicals are likely derived from the PPA.

4.3. Potential function of the principle protocerebral arborization area

Circadian systems require three essential components: a rhythm-generating clock, input pathways, and output pathways by which this clock can convey timing information to the rest of the organism (Helfrich-Förster, 1998). Yet, this third component, the pathways by which the brain clock regulates overt circadian rhythms in insects, remains relatively poorly understood (Tomioka and Matsumoto, 2010). Recent evidence has

implicated the PPA as integral to the clock output pathways which culminate in overt circadian rhythms. In fact, it appears that the region of the PPA is responsible for controlling behavioural rhythms (Stengl and Homberg, 1994) and more recently, it has been proposed that it regulates rhythmic outputs from the neuroendocrine system (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b). This lab has shown that PDF-positive axons in the PPA region of *Rhodnius* engulf the axons of neurosecretory cells containing PTTH (Vafopoulou et al., 2007) as well as insulin-like and testis-ecdysiotropin neuropeptides (Vafopoulou and Steel, 2012b). Timing input is conveyed to these neurosecretory cells via PDF-ir axons in the PPA that originate from the LNs, leading to the circadian rhythmic production and release of the neuropeptides. These studies provide novel insight into the structure-function relationship of the clock in the brain and its local effector cells. They provide by far the most convincing evidence that the clock in the insect brain directly causes the rhythmic production and release of neurochemicals and this, in combination with the present study, is the only work in *any* insect system to do so.

Preliminary evidence seen here indicates that ALP, CLP, FLP, and SLC are produced and released with a daily rhythm. All four neurochemicals were observed in the present study to stain significantly more axons far more intensely in the PPA than they did anywhere else in the brain and the majority of interactions between these axons and those which are PDF-positive occur in the PPA. Thus, it is likely that the potential rhythmicity observed in the production and release of these neurochemicals, particularly those with the opportunity to make synaptic contacts with PDF-positive axons in the

PPA, is caused by clock input from these PDF-positive axons. This raises the expectation that, at least in *Rhodnius,* the PPA is in fact a site of indispensible importance with respect to the integration of timing information within the insect brain.

An anatomically similar region to the PPA in *Rhodnius* exists in *Drosophila* and is known as the superior protocerebrum. While it was posited early on that the aMe was the central clock in *Drosophila*, this second region was suggested to have some potential importance with respect to distributing the circadian message to the rest of the organism (Helfrich-Förster, 1995; Helfrich-Förster, 2004). A comparable region to the PPA containing extensive PDF-ir arborizations has also been observed in the central brain of a multitude of other insect species (reviewed by Helfrich-Förster, 2005), supporting the importance of this region. When compared to the local neurons of the optic lobe, it is apparent that the projection neurons of the midbrain produce far more substantial axonal staining in the PPA than the local neurons of the optic lobe do in the aMe. This lends support to the conclusion that the PPA appears to be a hub for the integration of information in the *Rhodnius* brain, not the aMe alone as has been suggested for other insect species.

4.4. Conclusions

All neurochemicals seen here were observed to stain far more intensely in the PPA than the aMe indicating that the PPA may be a more important integrative region, at least in *Rhodnius*. This region has previously been associated with the production of behavioural (Stengl and Homberg, 1994) and neuroendocrine (Vafopoulou et al., 2007;

Vafopoulou and Steel, 2012b) rhythms. Whereas the aMe was generally seen as the primary site for integration in the insect brain clock, the present study indicates it is just a part of a far more extensive system that is comprised of multiple groups of interconnected clock cells and at least two integrative regions.

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CHAPTER III:

GENERAL DISCUSSION

3.1. Neurochemicals putatively associated with the insect brain clock

The neurochemicals presently studied for potential interaction with the brain clock system in *Rhodnius* were selected based on evidence from previous studies showing that they have been immunohistochemically localized within the brains of several other insect species (detailed below) and, in many of these species, their involvement within the brain clock was deduced from their staining of cells and axons associated with the putative aMe. Whereas ALP, FLP, and SLC have all previously been observed in the aorta of *Rhodnius* (Lange et al., 1988; Tsang and Orchard, 1991; Sarkar et al., 2003), this is the first observation of CLP in the aorta (see Fig. 6A). All neurochemicals have previously been linked to functions outside of the brain (discussed in more detail below) and throughout the body, thereby corresponding to the classic definition of neurohormones, which may explain their presence in the aorta.

3.1.1. Allatostatin-7

The allatostatin (AST) peptides comprise a large family of peptides, the most well-known subfamily being the AST-A peptides which are known for their role as endocrine modulators in the inhibition of juvenile hormone (JH) synthesis (reviewed by Stay and Tobe, 2007). However, the role of ASTs in the inhibition of JH synthesis does not appear to be universal (Duve et al., 1993; Duve and Thorpe, 1994; Yoon and Stay, 1995; Rankin

et al., 1998) and these peptides are thought to be involved in many other interneuronal and neuromodulatory functions in insects (reviewed by Bendena et al., 1999; Carlsson et al., 2010). The ASTs have also been shown to have inhibitory effects on gut contraction in cockroaches and in *Rhodnius* (Lange et al., 1993; Lange et al., 1995; Sarkar et al., 2003). In cockroaches, 13 AST peptides are cleaved from the same precursor (reviewed by Bendena et al., 1997). All members of the AST-A subfamily share a highly conserved C-terminal pentapeptide consensus sequence (Tyr/Phe-Xxx-Phe-Gly-Leu/Ile-NH_2) which is referred to as the "core" region and is responsible for receptor interaction (reviewed by Bendena et al., 1999). There is a comparably high degree of variation in the N-terminal amino acid ("address") sequence which is responsible primarily for functional potency with respect to the inhibition of JH synthesis (reviewed by Stay et al., 1994). It has been suggested that there may be a different receptor for each member of the AST-A subfamily (Gäde et al., 1997).

AST-7 is a tridecapeptide that was first isolated from brain extracts of the cockroach *D. punctata* (Woodhead et al., 1989; referred to as AST-1). Donly et al. (1993) called the tridecapeptide "AST-7" after cloning its cDNA and deducing its amino acid sequence. The neuronal distribution of anti-AST-7 staining observed here in *Rhodnius* is very similar to that seen previously by Sarkar et al. (2003), though minor differences were observed in precise cell counts. ALPs have been observed in the brains of several other insect species, including the cockroaches *D. punctata* and *L. maderae* (Stay et al., 1992; Petri et al., 1995), the locusts *Locusta migratoria* and *S. gregaria* (Veelaert et al., 1995; Würden and Homberg, 1995; Vitzthum et al., 1996), the cricket *G. bimaculatus*

(Schildberger and Agricola, 1992; Neuhaüser et al., 1994), the flies *Calliphora vomitoria, D. melanogaster* and *N. bullata* (Duve and Thorpe, 1994; Veelaert et al., 1995; Yoon and Stay, 1995), and the moth *M. sexta* (Žitňan et al., 1995). The presence of two putative receptors for Dip-AST-7 was detected in the brain of *D. punctata* (Cusson et al., 1991) making it likely that AST-7 indeed acts as a neurotransmitter and/or neuromodulator in the insect brain. ALPs have also been immunohistochemically detected in several peripheral tissues (reviewed by Gäde et al., 1997), which is consistent with present findings of ALP in the aorta of *Rhodnius.* AST-like-ir seen previously in interneurons associated with the aMe has been invoked as evidence for a role for these peptides as neurotransmitters and/or neuromodulators in the insect clock (Petri et al., 1995; Würden and Homberg, 1995). Evidence presented here showing associations between ALP-positive projections and PDF axons in the PPA as well as findings of daily rhythmicity in ALP production and release further supports a functional role for ALP in the brain clock system.

3.1.2. Crustacean Cardioactive Peptide

CCAP is a cyclic nonapeptide first isolated from the pericardial organs of the shore crab *Carcinus maenas* in which it was shown to have a cardioexcitatory function (Stangier et al., 1987). It has been shown to modulate heart rate in insects (Lehman et al., 1993; Dulcis et al., 2005) as well as to contribute to organizing the ecdydsis motor pattern (Ewer and Truman, 1996; 1997; Dircksen, 1998) and stimulate hindgut and oviduct contraction (reviewed by Homberg, 2002). The presence of CCAP in brain neurons of other insect species, as well as in *Rhodnius,* as was seen previously and in the present study, implies an

additional function for the peptide as a neurotransmitter, neuromodulator, and/or neurohormone. The immunohistochemical distribution of CCAP has been observed in previous studies of *Rhodnius* (Lee et al., 2011) and other insects including *L. migratoria* and *S. gregaria* (Dircksen et al., 1991; Dircksen and Homberg, 1995; Würden and Homberg, 1995), the beetle *Tenebrio molitor* (Breidbach and Dircksen, 1991), the moth *M. sexta* (Davis et al., 1993), and the stick insect *Baculum extradentatum* (Lange and Patel, 2005). CCAP was found not to cycle in head extracts of the insect *Dianemobius nigrofasciatus* (Sehadová et al., 2007), in contrast to the present work indicating that CLP cycles with a daily rhythm. Park et al. (2003) noted that projections of DN2 neurons of *Drosophila* overlap with projections of CCAP-positive cells in the dorsal brain; the DN2 cells are thought to be involved in temperature entrainment in *Drosophila* (Picot et al., 2009). The CCAP-positive cells observed in *Drosophila* by Park et al. (2003) and the C-G4 cells observed presently in *Rhodnius* (see Results) appear to be located in the same general region (the postero-medial protocerebrum) and they are similarly paired and possess axons that project into the dorsal midbrain in both insects. Just as in *Rhodnius,* it appears that these CCAP-positive cells contribute to the CCAP-ir branching in the PC bridge in *Drosophila*. Faintly CLP-positive axons were observed to cross the region of the DNs in *Rhodnius* (not shown). In combination with the evidence seen here that CLP cycles with a daily rhythm and many of its axons are enmeshed by PDF-ir axons in the PPA, the observation that these cells likely interact with the clock in *Drosophila* provides further support for their interaction with the clock in *Rhodnius*.

3.1.3. FMRFamide

FMRFamide is part of a larger family of comparatively small peptides known as the FMRFamide-related peptides (FaRPs; Price and Greenberg, 1989). FMRFamide, a cardioexcitatory tetrapeptide, was first isolated from the clam *Macrocallista nimbosa* and was the first of the FaRP family to be fully characterized (Price and Greenburg, 1977). The presence of FaRPs in brain neurons of a multitude of insect species implies a role for the peptide family as neurotransmitters, neuromodulators, and/or neurohormones. The immunohistochemical distribution of FLPs has previously been observed in the brains of two hemipterans, *Rhodnius* (Tsang and Orchard, 1991) and *Triatoma infestans* (Settembrini and Villar, 2005), using two different antibodies and the staining seen in both was strikingly similar to that seen presently in *Rhodnius* using yet another antibody against FMRFamide. Since FMRFamide is so small, it is likely that its amino acid sequence is contained within many different proteins and these antibodies are likely binding a number of proteins containing this short sequence. However, Tsang and Orchard (1991) claim their anti-FMRFamide recognizes only C-terminal RF-amide peptides. Contrary to the lack of co-localization of FLPs and PDF observed presently in *Rhodnius*, Settembrini and Villar (2005) observed co-localization in a handful of cells near the base of the optic lobe and from this the authors posited that FMRFamide therefore likely plays a role in the clock system of *T. infestans*, though more evidence is required to define these PDF-positive cells as clock cells. Co-localization of FLP and PDF mRNA was notably absent in *Musca domestica* and *D. melanogaster* (Matsushima et al., 2007). FLP immunoreactivity has also been observed in the brains of the cockroach *L. maderae* (Petri et al., 1995), the locusts *L.*

migratoria and *S. gregaria* (Rémy et al., 1988; Würden and Homberg, 1995), the Colorado potato beetle *Leptinotarsa decemlineata* (Veenstra and Schooneveld, 1984), the fruit fly *D. melanogaster* (White et al., 1986; Nichols, 2003), the moth *M. sexta* (Carroll et al., 1986; Homberg et al., 1990), and the blowfly *Calliphora erythrocephala* (Nässel et al., 1988).

Members of the FaRP family were suggested to be involved in the visual system of *Drosophila* (White et al., 1986) and have since been functionally linked to circadian rhythms of the visual system in both *Drosophila* and *M. domestica* (Pyza and Meinertzhagen, 2003). In the lamina of flies, circadian rhythms have been found to exist in axonal diameter (Pyza and Meinertzhagen, 1995). In this region, communication of time of day information is thought to be the result of neuropeptide signalling; specifically, PDF causes an increase in axonal diameter mimicking the effect of light and it appears that one or more FLPs mimic the effect of dark, causing diametric shrinkage (Pyza and Meinertzhagen, 2003). FLP was also seen presently in axons in the lamina of *Rhodnius,* and perhaps FLP is playing a role in the circadian regulation of the visual system in *Rhodnius*. Functional studies have also implicated FaRPs in the modulation of locomotor activity rhythms and in the coupling of contralateral brain clocks in *L. maderae* (Soehler et al., 2008; 2011). FLP was presently observed in the largest medial neurosecretory cells (MNC) on either side of the midline in the *Rhodnius* brain. FaRPs have been sighted previously in the MNCs of *Rhodnius* (Tsang and Orchard, 1991) and in *T. infestans* (Settembrini and Villar, 2005), *D. melanogaster* (White et al., 1986), the blowfly *P. terraenovae* (Hamanaka et al., 2007), the mosquito *Aedes aegypti* (Brown and Lea, 1988), and the honeybee *Apis mellifera* (Eichmüller et al., 1991). It has been shown that FaRPs

are indeed released into the haemolymph in *Rhodnius* (Elia et al., 1993), showing it can act as a neurohormone.

3.1.4. Serotonin

Serotonin is a biogenic amine that has long been studied in mammals due to its widespread physiological significance. It is produced via the same biochemical pathways in both vertebrates and invertebrates (reviewed by Blenau and Baumann, 2001) and just as in mammals, its function in insects is manifold, affecting sleep (Yuan et al., 2006), circadian behaviours (Nichols, 2007), sensory processing (Nichols et al., 2002), feeding (Novak and Rowley, 1994), heart rate (Dasari and Cooper, 2006), and locomotion (Kamyshev et al., 1983). It has been implicated as having an important role in the photic entrainment pathway and/or coupling of bilateral clocks of multiple insect species including: the cockroach *P. americana* (Page, 1987), the cricket *G. bimaculatus* (Saifullah and Tomioka, 2002; 2003) and the flies *Calliphora erythrocephala, Calliphora vicina,* and *M. domestica* (Pyza and Meinertzhagen, 1996; Chen et al., 1999; reviewed by Meinertzhagen and Pyza, 1999; Cymborowski, 2003). Further, it has been suggested that serotonin in the laminar axons of *M. domestica*, similar to FaRPs observed in the same region, functions as a modulator of circadian rhythms in the visual system: specifically, it may act on photoreceptor cells of the compound eyes via axons in the lamina to modulate the effect of light on these cells at different times throughout the day (Pyza and Meinertzhagen, 1996). Perhaps the SLC presently observed in the optic lobe of *Rhodnius* plays a similar role. Similar to our present findings, previous studies have reported a

rhythm of serotonin levels in the olfactory and accessory lobes of the American lobster, *Homarus americanus*, with levels in the accessory lobes peaking at dusk and remaining high through the night (Wildt et al., 2004).

The immunocytochemical distribution of SLC has previously been observed in the brains of a multitude of insect species indicating a role as a neurotransmitter, neuromodulator, and/or neurohormone in each of these species, including: *R. prolixus* (Lange et al., 1988) and *T. infestans* (Settembrini and Villar, 2004), within which the staining was very similar to that seen here; the cockroaches *L. maderae* and *P. americana* (Bishop and O'Shea, 1983; Klemm et al., 1983; Petri et al., 1995), the locusts *L. migratoria, Schistocerca americana, S. gregaria* (Klemm and Sundler, 1983; Konings et al., 1988; Homberg, 1991; Würden and Homberg, 1995), the fruit fly *D. melanogaster* (Vallés and White, 1988), the honeybee *A. mellifera* (Schürmann and Klemm, 1984) and the moth *M. sexta* (Homberg and Hildebrand, 1989). Serotonin receptors have been identified throughout the brain of *Drosophila* (Yuan et al., 2005, 2006; Nichols, 2007; Johnson et al., 2009) some of which are believed to be homologs of those found in mammals (Saudou et al., 1992; reviewed by Blenau and Baumann, 2001), indicating that serotonin is binding and affecting cells in the brain of *Drosophila* and lending support to a putative role for 5HT as a neurotransmitter and/or neuromodulator in *Rhodnius*.

3.2. The spatially diffuse circadian network of the insect brain

Recent studies describe the insect brain clock as a bilaterally symmetrical network comprised of numerous groups of clock cells and at least two integrative regions, including

the aMe and the PPA (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a). Such a network consisting of clock cells, each possessing circadian cycling of the canonical clock proteins, PER and TIM, has only been demonstrated in two insect species, *D. melanogaster* and *R. prolixus* (Kaneko and Hall, 2000; Vafopoulou et al., 2010). However, putative clock cells existing within a presumed brain clock network have been identified in numerous insect species representing at least eleven different orders, including: the bristletail *Lepismachilis y-signata*, the mayfly *Siphonurus armatus,* the damselfly *Ischnura elegans,* the locusts *S. gregaria* and *L. migratoria,* the stonefly *Perla burmeisteriana,* the backswimmer *Notonecta glauca,* the waterstrider *Gerris palludum,* the goldsmith and Indian tiger beetles *Pachnoda marginata* and *Pachymorpha sexguttata*, the honeybee *A. mellifera,* the caddisfly *Hydropsyche contubernalis,* the blowflies *N. bullata* and *Phormia regina,* and the silkmoth *A. pernyi* (Frisch et al., 1996; Sauman and Reppert, 1996; Bloch et al., 2003; Závodská et al., 2003). The majority of these insects possess at least two groups of PER-positive cells in each brain hemisphere – one in the lateral protocerebrum and one in the dorsal protocerebrum (Helfrich-Förster, 2005), which are similar in location to the LNs and DNs of *D. melanogaster* and *R. prolixus* (for summary images see Helfrich-Förster, 2004; also Vafopoulou et al., 2010). Most of these insects also possess PDF-positive fibres that send branches into the optic lobe and into the central protocerebrum, into an area that may be equivalent to the PPA of *Rhodnius*. The arborizations of the PDF-ir fibres in the midbrain of these insects are generally quite extensive, indicating the potential importance of this region within the clock network in a number of diverse insect species.

3.3. Analogy with the mammalian brain clock system

The neuroendocrine system is under circadian control, which is the case for both insects (Vafopoulou and Steel, 2009) and mammals (Buijs and Kalsbeek, 2001; Buijs et al., 2003). The structural and functional parallels between the vertebrate and insect neuroendocrine systems (the hypothalamo-hypophyseal system and the intercerebralis-cardiacum-allatum system, respectively) have long been known (Scharrer and Scharrer, 1944). This similarity can now be extended to the circadian system. The SCN (in mammals) and the accessory medulla (in insects) are responsible for the entrainment of the brain clock via light input from the eyes (reviewed by Helfrich-Förster, 2004). In mammals, neuroendocrine rhythms are regulated by the SCN and there has been speculation that the accessory medulla is analogous to the SCN (Helfrich-Förster, 2004). However, neural pathways have recently been elucidated that show close associations between axons from the PPA and axons of neurosecretory cells that produce and release neuropeptides with a circadian rhythm (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b); no such pathways have been elucidated between the aMe and neurosecretory cells anywhere in the insect brain. Thus, the aMe appears to be insufficient on its own to comprise a system which is functionally equivalent to the mammalian SCN. The source of neuroendocrine regulation appears to be the PPA, not the aMe, and present findings strongly indicate that the PPA is an important site for the integration of timing information, perhaps even more so than the aMe. It therefore appears that, at least in *Rhodnius,* the PPA in concert with the aMe, *not* the aMe alone, is the functional equivalent of the mammalian SCN.

3.4. Conclusions

The present study supports the ever-advancing notion that the insect brain clock is not restricted to an isolated region in the brain (i.e. the aMe), but rather comprises a spatially distributed network with many potential sites for input to and output from the system, particularly within the region of the PPA. Just as has been seen for the aMe in the past, many neurochemicals have been found within the PPA which may potentially facilitate interaction between the clock and cells in other brain regions. Using double labelling immunohistochemistry, I observed potential interactions between chemicals binding to antibodies against AST-7, CCAP, FMRFamide, or serotonin and the clock, as labelled with an antibody against PDF. By observing the distribution of a variety of neurochemicals in the insect brain with respect to clock axons, three conclusions can be derived from present study: 1) ALP may be a component in a photic entrainment pathway of the *Rhodnius* brain clock; 2) the aMe appears to be involved in the integration of timing information in the *Rhodnius* brain, perhaps significantly less so than in other insects, and; 3) the PPA appears to be an essential component in the brain clock system of *Rhodnius*. This latter region of clock cell arborizations is rich with neurochemical-filled axons and varicosities, indicating that all neurochemicals presently studied behave as inputs and/or outputs therein. Both the aMe and the PPA, rather than the aMe on its own, appear to be important integrative regions within the circadian clock system and likely act in concert to coordinate and maintain the internal synchrony of circadian events.

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