Title: Amine neurochemistry and aggression in crayfish

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INTRODUCTION

Identification of neurochemical substrates that underlie behavioral, motivational and affective states forms a principal goal for many disciplines comprising the neurosciences. Research during the past half-century has resulted in an abundant collection of interneuronal-signalling molecules from which our basic understanding has benefited immensely from the existence of functional homologies. For instance, a classic case in point is the pioneering work that elucidated the respective roles of glutamate and gamma-aminobutyric acid as general excitatory and inhibitory neurotransmitters (Iversen et al., 1967; Taraskevich, 1971)—a finding that has been subsequently demonstrated across a majority of taxa. However, our understanding of the interface between molecular biology and animal behavior undoubtedly lacks comparable precision. Due to their role in a broad spectrum of behavioral and motivational states, such uncertainty is particularly true of biogenic amines such as serotonin and dopamine.

The indoleamine serotonin has received a disproportionate amount of attention due to its involvement in aggression (Dyakonova and Schürmann, 1999; Higley et al., 1996; Overli et al., 1999; Kravitz, 2000), social dominance (Raleigh et al, 1991; Winberg et al., 1993; Matter et al., 1998; Bloch et al., 2000), feeding (Heinz et al, 1996; Rozenboim et al., 1998; Meguid et al., 2000) and sexual behavior (Evans et al., 1998; XXX) as well as being implicated in the etiology of affective disorders from depression to schizophrenia (Sivam, 1996; Kroeze and Roth, 1998; Lucki 1998; Blier and de Montigny, 1999; Yadid et al., 2000). As illustrated by such a comprehensive range of behavioral contexts, serotonin systems are inherently difficult to study. Moreover, the anatomical localization and physiological properties of serotonin systems augment this complexity considerably. In both vertebrate and invertebrate species, serotonin neurons are generally situated close to the neuroaxis and have extensive projections that cover large areas of nervous tissue (Azmitia and Segal, 1978; Lidov and Molliver, 1982; Beltz and Kravitz, 1987). To date, fifteen receptor sub-types have been characterized in vertebrate systems with several homologues present in invertebrates (Hen XXX; Kroeze and Roth, 1998; Tierney, 2001). Electrophysiological studies confirm that many serotonergic neurons cycle as slow-rhythm oscillators (Wang and Aghajanian, 1977; Ma et al., 1992) and become autoinhibited after periods of transient excitation (Wang and

Aghajanian, 1977; Heinrich et al, 1999). Furthermore, depending on the context, serotonin has several modes of action: [1] a classical neurotransmitter, [2] a neuromodulator and [3] a neurohormone.

A notably large body of evidence has implicated serotonin systems as key physiological mechanisms for the causation of agonistic behavior and social dominance across a phylogenetic landscape ranging from ants (Kostowski et al., 1975) to humans (Lesch and Merschdorf, 2000) with few species missing in between (Maler and Ellis, 1987; Blanchard et al., 1991; Raleigh et al., 1991; Reisner et al., 1996; Dyakonova and Schürmann, 1999; Korzan et al., 2000; Doernberg et al., 2001). In different systems, investigators have made use of a variety of experimental approaches for examining such relationships by studying the physiological correlates of social status or aggressive state (Blanchard et al., 1991; Higley et al., 1996; Eloffson et al., 2000), utilizing pharmacological interventions (Olivier et al., 1989; Raleigh et al., 1991; Fuller, 1996; Larson and Summers, 2001) or adopting gene-knockout techniques (Chen et al., 1994; Saudou et al., 1994; Cases et al., 1995). However, the particulars of these relationships have remained unclear and controversy has come to typify seemingly incompatible interpretations of such work. Previous attempts at resolving these issues have focused on phylogenetic differences where increased serotonin function is often considered to lower aggression in vertebrates while the inverse scenario seems to hold true for invertebrate taxa (Edwards and Kravitz, 1997; Weiger, 1997). It is possible that the role of serotonin systems in aggression underwent such a "polarity" change during early vertebrate evolution, but a source of such controversy also arises from the use of different, often unstated definitions when dealing with the overarching concept of aggression. When addressed as an intervening variable, authors have considered a variety of disparate concepts at the individual or paired level, including aggressive state, risk taking, lack of control for impulsiveness or violence, negative effects of stress, an ability to win agonistic encounters or social dominance. If the rules that govern the behavioral intricacies of social relations are to be elucidated, it is essential that the precise phenomena under study be stated carefully. Moreover, it is imperative to consider how the structure of a particular social system has been shaped during its evolutionary history. In invertebrates, dominance is primarily a result of physical superiority while in

vertebrates it is determined additionally by the ability to form coalitions and recognize kin. However, even when comparing vertebrate systems there are unique differences. For instance, overt aggression may be a main route to high social rank in some taxa (Blanchard et al., 1991; Winberg and Nilsson 1993; Larson and Summers, 2001), but it most certainly is not in others (Raleigh et al., 1991). Since the utility and expression of agonistic behavior varies greatly as a function of the particular social system under study, it is indisputable that general theories subsuming aggression must be invoked with great care.

Crayfish species offer a number of unique advantages for studying serotonergic links to aggression and social dominance. Unlike many vertebrate social systems, determining social rank in crayfish is not complicated by social coalitions or pair bonds (Issa et al., 1999; Goessmann et al., 2000). Individuals primarily maintain a solitary existence, where conflict between conspecifics centers on obtaining or defending resources such as shelter (Ranta and Lindstrom, 1992), food (Ranta and Lindstrom, 1992; Stocker and Huber, in press) or mates (XXX). However, even in the absence of a resource, crayfish exhibit an inherently aggressive predisposition (Bruski and Dunham, 1987; Issa et al., 1999; Goessmann et al., 2000) that may be partially accounted for by the selective pressure of living in densely populated habitats (Stewart and Haynes, 1994). Agonistic meetings between crayfish are characterized by a series of highly structured behavioral acts with escalation governed by strict rules (Huber et al., 2001). Typically, fights progress through several conspicuous stages including ritualized visual displays, antennae whipping, claw lock and wrestling as well as brief periods of unbridled claw use if size asymmetries are small (Bovbjerg, 1953; Rubenstein and Hazlett, 1973; Bruski and Dunham, 1987). Consistent with predictions derived from game theory models of behavioral ecology (Maynard-Smith and Price, 1973; Parker and Rubenstein, 1981; Enquist and Leimar, 1983; Austad, 1989), such behavioral characteristics are ideal for assigning discrete, quantitative measures to a set of acts and decisions that constitute individual agonistic encounters (Huber et al., 2001).

The presence of a highly structured behavioral framework presents unique vistas to explore the interface between behavior and its underlying physiological mechanisms. Serotonin systems play a likely role in the causation of aggression between clawed-

crustaceans like crayfish (Kravitz, 2000). For instance, a small population of serotonin-containing neurons (Beltz and Kravitz, 1983l; Real and Czternasty, 1990) is involved in "dominant-like" posturing (Harris-Warrick and Kravitz, 1984; Ma et al., 1992) and the generation of tail flips, a common form of retreat (Glanzman and Krasne, 1983; Yeh et al., 1997; Edwards et al., 1999). Moreover, work from several labs indicates a strong association between serotonin systems and aggression in clawed-crustacean species (Antonsen and Paul, 1997; Sneddon et al., 2000; Tierney 2000; Doernberg et al., 2001). Our lab has employed several approaches for exploring the relationships between serotonin and fighting behavior in crayfish. Specifically, we have [1] developed a behavioral framework for identifying sources of behavioral variation in need of physiological explanation, [2] explored the behavioral consequences of acute and chronic pharmacological interventions of the crayfish serotonin system and [3] attempted to relate natural variation in amine levels to the acquisition and reinforcement of social status. Much like any experimental paradigm that relies on pharmacology as a foundation, we have become increasingly cognizant of the need to empirically verify the effects of amine manipulations rather than to assume success based on the description of a molecule's purported mechanism. Towards this goal, our lab has optimized chromatographic techniques that allow highly sensitive detection of biogenic amines in crustacean nervous tissue. In this article, we review our efforts to relate pharmacologically induced, as well as naturally occurring changes in crayfish aggression to quantitative neurochemical analysis using high performance liquid chromatography with electrochemical detection (HPLC/ED).

I. GENERAL OVERVIEW OF QUANTITATIVE NEUROCHEMISTRY

-HPLC Instrumentation

-conditions for separation: mobile phase and column properties

-conditions for detection: electrochemical detectors – potentials

-sample preparation and application

II. EFFECTS OF ACUTE SEROTONIN TREATMENT IN CRAYFISH

Numerous experiments have demonstrated that experimental alteration of central nervous system serotonin has profound effects on aggressive motivation (Maler and Ellis, 1987; Raleigh et al, 1991; Fuller, 1996; Dyakonova and Schürmann, 1999; Doernberg et

al., 2001; Larson and Summers, 2001). In particular, a strong line of evidence indicates a close association between activation of serotonin systems and aggressive state in clawed, decapod crustaceans (Antonsen and Paul, 1997; Kravitz, 2000; Sneddon et al., 2000; Tierney, 2000). Our first attempt to examine the effects of serotonin manipulations on crayfish fighting included pairing conspecifics with large size asymmetries (>30%) and securing a fine-bore, fused silica cannula into the pericardial sinus of the smaller individual (Huber et al., 1997; Huber and Delago, 1998). Initially, pairs established a dominance relationship that invariably resulted in continued retreat of the smaller opponent. Following a control infusion, the pump was switched to a low concentration of serotonin. The amine-infusion produced marked changes in the behavior of the subordinate crayfish that continued well after the pump was turned off. A series of multivariate techniques (e.g., discriminant function analysis) revealed that serotonin treatment had specifically altered the decision to retreat from an encounter, without affecting which animal had initiated, how individual fights progressed or the eventual acquisition of social rank. Furthermore, it was likely that this behavioral change was associated with serotonin re-uptake mechanisms, as the same effect was not observed with infusion of fluoxetine, the active ingredient in Prozac, but was greatly reduced when both compounds were infused concurrently.

To further explore the physiological time course of the experimental treatment, a second group of animals were injected with a bolus of serotonin and its half-life in the haemolymph was determined. Haemolymph samples were collected over a range of latencies following injection and assayed for serotonin with HPLC/ED. The half-life of serotonin was estimated at 10.1 minutes (Fig. 1). This was surprising, since the peak behavioral effects were observed during that later part of the one-hour infusion period. Taken together, the behavioral and neurochemical data suggested that serotonin continued to have effects in the crayfish nervous system even after a large amount of the amine had been cleared. These findings were also indicative of the action of G-protein coupled receptors that characteristically exert a protracted effect on neuronal activity.

The behavioral specificity of acute serotonin treatment in crayfish was unique. Without affecting any other aspect of the crayfish aggressive repertoire, treated animals continued to engage their larger opponent in prolonged bouts of fighting even in

instances that carried substantial risk of injury. We postulated that the treatment had specifically altered the properties and/or activity of neurons that were key sites for "decision making" in the crayfish central nervous system.

III. EFFECTS OF CHRONIC SEROTONIN PHARMACOLOGY IN CRAYFISH

Interest in chronic pharmacological treatment of biogenic amine systems has increasingly become a cornerstone of behavioral pharmacology. Since the serendipitous discovery of the tricyclic antidepressants () and monoamine oxidase inhibitors (), a host of therapeutic, neuroactive molecules with complex physiological and behavioral effects have been developed (Rossby et al., 1995; Berry et al., 1996; Kalsner, 2000; Silva and Brandão, 2000). More recently, it has been suggested that amine-modulatory systems may serve as initiating mechanisms for a complex cascade of large-scale neurochemical, hormonal and anatomical modifications (Kudryavtseva and Avgustinovich, 1998; Nestler, 1998; Jacobs et al., 2000). Irrespective of whether biogenic amine systems are directly linked to psychological and mood disorders, or rather serve as an indirect target for their treatment, amines unquestionably play a critical role in the motivational elements of behavioral systems such as feeding and aggression. In these contexts, the need to carefully examine the time course of amine function will be essential for understanding their modes of action.

We began this task by examining the agonistic behavior patterns of crayfish continuously treated with fluoxetine via osmotic mini-pumps over three weeks (Delago et al., under review). The behavioral findings were in general agreement with the data from acute serotonin infusions: treated animals exhibited a behavioral profile characterized by a decreased willingness to retreat that resulted in more intense and prolonged fighting, especially during the initial days of treatment. However, the behavioral effects accompanying chronic fluoxetine treatment were less pronounced than those of acute serotonin infusion, possibly because the treatment had produced a more uniform baseline as well as generally lower concentrations of serotonin in the central nervous system.

To explore the biochemical effects of chronic disruption of the crayfish central serotonin system, we used silastic tube implants containing the serotonin-depleting compounds 5,7-dihydroxytryptamine and alpha-methyltryptophan as well as the amine itself (Panksepp and Huber, under review). Following behavioral trials that were carried

out across a range of exposure periods, the entire nervous system was assayed for serotonin with HPLC/ED (Fig. 2). 5,7-dihydroxytryptamine treatment resulted in successful reduction of serotonin levels in all central nervous system tissues except brain, despite finding appreciable amounts of 5,7-dihydroxytryptamine were present in all brain tissues. Counter to our predictions, alpha-methyltryptophan treatment was of little utility in altering nervous system serotonin content as were two different rates of chronic serotonin treatment. (5-HT "slow"= XXX; 5-HT "fast"= XXX). Our findings appeared even more counter-intuitive once the agonistic behavior patterns of treated individuals were included. The behavior of serotonin-depleted animals was indistinguishable from that of controls whereas individuals treated with serotonin exhibited different rates of escalation (i.e., "slow"-treated animals escalated significantly faster and "fast"-treated animals escalated significantly slower despite unaltered absolute serotonin levels in both groups).

The results reported above were indicative of the action of powerful compensatory mechanisms that appeared to counteract our pharmacological interventions. Moreover, the findings suggested the existence of neuronal changes that had been manifested far outside the constraints of our experimental hypotheses. More??……..

IV. AMINE CORRELATES OF SOCIAL STATUS IN CRAYFISH

Several studies have shown that serotonin and its metabolites are closely associated with aggressive state and social rank (Higley et al., 1989; Blanchard et al., 1991; Winberg and Nilsson, 1993; Matter et al., 1998). Moreover, in clawed-crustaceans the concentration of serotonin in the haemolymph (Sneddon et al., 2000) and its efficacy for receptors on neuronal circuits controlling tail flip (Yeh et al., 1997) vary as a function of social status. Recently, we have examined whether natural changes in central nervous system concentration of biogenic amines are reflected in crayfish social status (Yue and Huber, 2000).

In a series of experiments, experimental conditions were selected to maximize the behavioral polarities associated with social dominance. Pairs of crayfish were closely matched for body weight, carapace length and claw size. Following the initial

establishment of social rank, a small shelter was provided and the animals remained together continuously for 24 hours.

Early interactions were characteristic of the stereotyped and escalating nature of crayfish fighting. Soon after, a dominant animal emerged and subsequent fights were typically brief, ending with little challenge of the subordinate toward the advance of the dominant. Moreover, no reversals in dominance occurred during the 24 hours after initial pairing. Following the 24 hour-period of continued behavioral reinforcement, central nervous system levels for serotonin and dopamine were analyzed for both individuals and a size-matched control using HPLC/ED. No differences in serotonin or dopamine were detected in any nervous system tissues of dominant or subordinate crayfish (Fig 3). As has been demonstrated by the serotonin/5-hydroxyinoleacetic acid index in vertebrate systems, the possibility remains that changes in amine metabolism rather than absolute amine levels accompany changes in crayfish social status. However, due to the novel structure of many predominant crustacean serotonin metabolites (Kennedy xxx) metabolic turnover ratios remain difficult to attain for the crayfish nervous system.

Nevertheless, these findings were still unexpected. In vertebrate systems, the most dramatic changes in serotonin synthesis are observed during the early days of hierarchy formation (XXX), and serotonin synthesis and serotonin neuron firing are positively correlated (XXX). Coupled to the work of others, these data indicate that the serotonergic basis of crayfish social dominance is organized dynamically, at many levels, including post-synaptic mechanisms (Yeh et al., 1997) and amine release (Sneddon et al., 2000).

CONCLUSIONS

The very characteristics that make biogenic amine systems so intriguing to study is also the exact source of what makes them seem, at times, impenetrable: their behavioral breadth and phylogenetic ubiquity. A common misunderstanding is the notion that a particular amine system *is* the physiological underpinning of a specific behavioral state. All too often, experimenter bewilderment is the product of neurotoxic lesions, for instance, that do not completely augment or extinguish the behavior in question. However, this scenario underscores the very nature of the misconception.

Neuromodulatory systems, like many of the biogenic amine systems, do not produce behavior *per se*, they fine-tune ongoing activity, i.e., they modulate.

A main goal to more fully understand biogenic amine systems is to uncover their contextual specificity. Recent studies in crayfish indicate a surprising, and yet, distinct social specificity for the action of serotonin at a known synapse (Yeh et al., 1997) Such findings require that we reevaluate the orthodox assumption of a one-way causal route from brain to behavior. It is likely that many of the reported behavioral effects associated with biogenic amines hinge on the precise social (Ison et al., 1996; Berton et al., 1999) as well as genetic (Rilke et al., 1998) context in which they are expressed. Likewise, many amine-modulated behavioral states may obtain specificity through interactions with other aminergic and peptidergic systems (Ferris and Delville, 1994; Sivam, 1996; Jacobs and Fornal, 1999; Meguid et al., 2000) Thus, it is essential that our understanding of such systems be culled from a multi-disciplinary and integrated approach to their study. Nothing can substitute for combining techniques of quantitative neurochemistry, neuropharmacology and molecular biology with quantitative behavioral analyses.

Indeed, merging two extremes like molecular biology and behavioral ecology may sound like an audacious goal. However, towards this goal work from our lab has found that serotonin re-uptake mechanisms are specifically involved in decisions to retreat during agonistic encounters between crayfish. Moreover, in accordance with studies in vertebrates (Ase et al., 2000, Gingrich and Hen, 2000, Pan et al., 2001), we have identified the concomitant presence of powerful compensatory mechanisms that are ultimately manifested at the behavioral level following chronic amine manipulations. Our studies and others that have identified the biochemical changes induced by chronic pharmacological disruption of serotonin systems, coupled with experiments addressing social status acquisition in crayfish, support a view of biogenic amines as individual elements embedded in a dynamically-orchestrated system rather than simply as static, neurochemical characteristics.

In large part, it has been HPLC/ED that has aided our lab in identifying and partially resolving both the questions and assumptions that have been outlined in this article. In a much more general sense, inextricably linked to our experimental paradigm is the understanding that pharmacological approaches to the study and therapy of

motivational states have become a staple of the 21st century. To truly understand how behavioral and neuronal processes are modified with this cornucopia of molecules, it will be essential to not only characterize the induction of transient and persistent alterations in nervous system biochemistry, but to identify how the brain responds to the altered state that we, as experimenters, produce.

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FIGURE LEGENDS

Figure 1.

Figure 2. Serotonin (5-HT) content is plotted for individual segments of the crayfish nervous system following chronic treatment with various pharmacological compounds. Silastic tubes (15.0mm length, 0.635mm inner diameter) were loaded with crystals of 5,7-dihydroxytryptamine (5,7-DHT), alpha-methyltryptophan (AMTP), 5-HT ('fast'= XXXpg/hr or 'slow'=XXXpg/hr) or left empty (control). Tubes were implanted into the crayfish thoracic body cavity and individuals remained in social isolation for varying periods of time (e.g., 1-5 weeks) after treatment and before fighting a randomly selected, size-matched conspecific. Following behavioral trials, experimental animals were anaestisized on ice and the entire ventral nerve cord was dissected out. Individual segments were assayed for serotonin content with HPLC/ED **[a]** supraesophageal

ganglion; $F_{(4,52)}$=1.54, P=0.205 **[b]** subesophageal and circumesophogeal ganglia (SEG/CEG); $F_{(4,52)}$=69.94, P<0.001 **[c]** thoracic ganglia (1-5); $F_{(4,51)}$=34.95, P<0.001 **[d]** abdominal ganglia (1-6); $F_{(4,53)}$=10.46, P<0.001. Measures of serotonin were log-transformed to reduce heteroscedasticity of treatment variances. Post-hoc differences between individual groups were identified with Tukey's HSD test and are indicated with an asterisk.

Figure 3.