Hypocretin (Orexin), Dopamine, and Goal-Directed Behavior

John W. (John Whitney) Muschamp
The members of the Committee approve the dissertation of John W. Muschamp defended on 27 March 2007.

Elaine Hull
Professor Directing Dissertation

David Quadagno
Outside Committee Member

Thomas Joiner
Committee Member

Mohamed Kabbaj
Committee Member

Zuoxin Wang
Committee Member

The Office of Graduate Studies has verified and approved the above named committee members.
To the memory of
Margaret Atwood Muschamp
(1943-2003)

“And whether it is Thursday, or the day is stormy,
With thunder and rain, or the birds attack each other,
We have rolled into another dream.
No use charging the barriers of that other:
It no longer exists.”

-John Ashbery, from Spring Day, in The Double Dream of Spring.
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ABSTRACT

The role of hypocretin (orexin, hcrt/orx) neurons in regulation of arousal is well established. Recently, hcrt/orx has been implicated in food reward and drug-seeking behavior. I report here that in male rats, Fos-immunoreactivity (ir) in hcrt/orx neurons increases markedly during copulation and with estrous female cues, while castration produces decreases in hcrt/orx neuron cell counts and protein levels in a time course consistent with post-castration impairments in copulatory behavior. This effect was reversed by estradiol replacement. Immunolabeling for androgen (AR) and estrogen (ERα) receptors revealed no colocalization of hcrt/orx with AR and few hcrt/orx neurons expressing ERα, suggesting that hormonal regulation of hcrt/orx expression is via afferents from neurons containing those receptors. Double-immunolabeling for ERα and melanin concentrating hormone (MCH) showed no expression of this receptor in MCH cells adjacent to hcrt/orx neurons. I also demonstrate that systemic administration of the orexin-1 receptor (OX1) antagonist SB 334867 impairs copulatory behavior. One locus for hcrt/orx’s pro-sexual effects may be the ventral tegmental area (VTA). I show that hcrt-1/orx-A produces dose-dependent increases in firing rate and population activity of VTA dopamine (DA) neurons in vivo. Activation of hcrt/orx during copulation, and in turn, excitation of VTA DA neurons by hcrt/orx may contribute to the robust increases in nucleus accumbens DA previously observed during male sexual behavior. Subsequent triple-immunolabeling in anterior VTA showed that Fos-ir in tyrosine hydroxylase positive neurons apposed to hcrt/orx fibers increases during copulation. Together these data support the view that hcrt/orx peptides may act in a steroid-sensitive manner to facilitate the energized pursuit of natural rewards like sex via activation of the mesolimbic DA system.
1. INTRODUCTION

With its origins in the work of early theorists like B.F. Skinner and Clark Hull, as well as experimentalists like James Olds who were ready to enter the “black box” of the brain, the study of motivation is a major discipline of behavioral neuroscience. For a generation of postwar physiological psychologists, the seat of motivation lay in “centers” within hypothalamic tissue, each charged with enacting a discrete behavior, be it feeding, or fighting, or sexual behavior. As circuitry models replaced hypothalamic center hypotheses and the role of dopamine in movement and motivated behavior became clear, experimental work on motivation diverged – even balkanized – into various subdisciplines. It became strangely uncommon to consider oneself a researcher on the problem of “motivation” generically. Speaking glibly, investigators concerned with feeding and reproduction continued to work in the hypothalamus. Meanwhile, the field of motivation broadly was “hijacked” by researchers interested in addiction in much the same way drugs of abuse are said to hijack the natural motivation circuitry. Apart from nodding citations in review articles, these various strains of motivation research have been divorced, with only a handful of scientists attempting to bridge the gap.

One glimmer of hope for a unified, mechanistic understanding of the neurobiology of goal directed behavior came with the discovery of the hypocretin (orexin) peptides. The neurons containing these molecules provide a literal link between the hypothalamus and mesolimbic dopamine system, and thus a figurative link between the vast literature that arose around each structure’s putative contribution to motivated behavior. The purpose of this dissertation and the work it describes is to explore this link as it relates to a single, well-characterized motivated behavior: male reproductive behavior. It is hoped that this will give a glimpse of the diverse neural architecture that evolved to reliably engage this critical behavior, and perhaps to suggest a template that may apply to motivation broadly. To come to grips with this circuitry, each major component will be treated in separate chapters. Their likely contributions to the behavior in question will be discussed in light of the data presented, and installed in an informal (non-quantitative) theoretical model that is proposed in the final chapter.
2. THE BASAL GANGLIA AND THEIR ROLE IN ADAPTIVE BEHAVIOR

The view that the ventral striatum is a “final common pathway” for goal-directed behavior was crystallized in a review by Gordon Mogenson (Mogenson et al., 1980). The literature on the nucleus accumbens (NAc) since that time has become truly vast. In the decade preceding Mogenson’s review, about 300 papers explicitly concerned with the NAc reached print (Figure 1). In the decade after, the number increased eightfold. At the time of this writing, searching the National Library of Medicine’s PubMed database under the term “nucleus accumbens” returns about 10,000 articles published since 1980 – a rate of almost one article a day. It is true that these figures must to some extent mirror the rapid expansion of the multidisciplinary venture of neuroscience itself, but there is no question that for the last quarter century, the NAc has been foremost in the minds of investigators seeking to explain goal-directed behavior.

Despite its extraordinary role in determining behavioral, as well as scientific output, we should recall that the NAc is one piece of the striatum, and in turn, the basal ganglia. Medium spiny neurons (MSN) of the dorsal and ventral striatum have a common ontogeny in the embryonic neuroepithelium (Heimer et al., 1997). To dichotomize them strictly as separate dorsal and ventral compartments, with the former governing only the mechanistic aspect of motor programming, while the latter imbues the process with affective ‘color,’ is to ignore a certain amount of functional, anatomical, and neurochemical data to the contrary (Voorn et al., 2004; Vanderschuren et al., 2005). These authors note the relative homogeneity of cyto- and chemoarchitecture across dorsal and ventral domains, and the difficulty with which a clear border between the two is
drawn. In addition, they cite findings of “ventral-typical effects” (i.e. reward-related neurons and psychomotor stimulant reinforcement) in the dorsal striatum (see Vanderschuren et al., 2002; Vanderschuren et al., 2005).

While it does seem clear that the NAc has a privileged position in regulating behavioral output, particularly where affectively-valenced reinforcers are concerned, it must ultimately be viewed as a portion of the striatum which came to be specialized for this purpose over the course of evolution (Marin et al., 1998; Smeets et al., 2000). As such, a consideration of basic structure and function of the greater striatum and basal ganglia may serve as a heuristic for understanding the specialized role of the NAc in goal-directed behavior.

**Overview of Basal Ganglia Circuitry**

The striatum has been associated with motoric behavior at least since it was given that name by Oxford physician Thomas Willis in his *Cerebri anatome* of 1664 (for a biographical sketch, see Molnar, 2004). Named for the streaked appearance lent by corticofugal projections, Willis noted that these fiber bundles in the striata of puppies were “rude” (undeveloped), and that this must account for the young animal’s lack of “motion and sense” (Finger, 2000; Bennett and Hacker, 2002). Today we recognize that the gross disturbances of motor control found in Huntington’s and Parkinson’s diseases are, respectively, the result of degenerative lesions of the striatum and areas in the midbrain that furnish it with modulatory input (Elble, 1998). Experimental lesions in animals, together with careful tract-tracing and unit recording experiments, have done a great deal to expand our knowledge of basal ganglia function under both normal and pathological conditions. The enunciation in recent years of
‘biologically realistic’ computational models of basal ganglia function has also added to our understanding of this system (see Gurney et al., 2001a, b; Bar-Gad et al., 2003). For our purposes, however, the familiar “box and stick” diagrams of the basal ganglia can still offer guidance with regard to the functional relationship between individual components and their contributions to coherent behavior (Figure 2).

At the coarsest level of resolution, Swanson (Swanson, 2000, 2003, 2005) has installed the basal ganglia within the framework of his “triple descending projection” model of telencephalic organization and motor control (Figure 3). This elegant model holds that the functional neuroanatomy of the forebrain is arranged around a simple motif that begins with excitatory glutamatergic projections from cortical layer V to the striatum. Striatal GABAergic neurons receiving this cortical input in turn send inhibitory projections to the pallidum, which accounts for the second layer of the model. The third and final layer consists of GABAergic neurons in the pallidum which project directly to elements of the brainstem motor system and thalamus, and ultimately exert a disinhibitory influence over their targets in a feedforward manner. Swanson stresses the massive nature of the projections at each level (i.e. that nearly the entire cortex projects to the striatum, almost all of the striatum to the pallidum, etc.), and that these projections are often topographically organized. He also notes, as have many other investigators, the important collateral, or reentrant loop made by pallidal afferents to near their origins in the cortex as relayed via excitatory projections from the thalamus. Locomotor behavior arises from this arrangement as thalamic targets are released from inhibition by action of striatal projections to the pallidum. This scheme is supported by physiological data that show projection neurons of the striatum and thalamus to be under

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**Figure 3. Triple descending model.**

Excitatory (e) glutamatergic projections from cortical layer V project to brainstem motor nuclei and send collaterals to striatum. Inhibitory (i) GABAergic medium spiny neurons in striatum project to brainstem and pallidum. Pallidal elements also use GABA projections to thalamus which have a net disinhibitory (d) effect on excitatory projections from thalamus to cortex. Adapted from Swanson (2005).
tonic inhibition (Kawaguchi et al., 1989; Surmeier et al., 1991; Wilson, 1993), while those of the pallidum show high spontaneous firing rates (Georgopoulos et al., 1983; Mitchell et al., 1987).

It is easy to become captivated by the byzantine complexity of the basal ganglia, with their multiple feedback loops, recurrent collaterals, and neurons that bear a wide array of chemical markers and signaling molecules. Before we descend further into this complexity, it should be stated simply at the outset that the basal ganglia are a collection of structures tasked principally with modifying locomotor and cognitive output by affecting the excitability of individual neurons of the cortex. The practical and theoretical discussion of how this is achieved will be left until after the following explanation of the basic parts list and wiring plan.

The canonical structures of the basal ganglia are: the striatum, consisting of the caudate and putamen; the globus pallidus, divided into an external (GPe) and internal (GPi) segment, the latter occurring as the homologous entopeduncular nucleus (EP) in rodents; the subthalamic nucleus (STN); and the substantia nigra, in its reticular (SNr) and compact (SNC) parts. The network has an input side in the striatum and an output side in the GPi/EP. These are treated in that order below.

The Striatum: Cortical Inputs, Projections to Pallidum, and Cytology

The MSNs of the striatum receive afferents from the entire cortex. Regional differences exist (e.g. denser projections from somatosensory cortex than from visual cortex), but the striatum is the target of massive projections from functionally diverse areas of cortex that show substantial convergence onto striatal neurons (see Wilson, 2004). The extent of this convergence has been the subject of debate for a number of years, with some investigators suggesting that it is minimal (Alexander et al., 1986; Alexander et al., 1990; Romanelli et al., 2005). This account finds that cortico-striatal afferents from discrete areas of cortex retain their spatially segregated character at each level of the basal ganglia, and indeed all the way back to their sites of origin in the cortex. This view is distinguished from earlier work that found a more complete pattern of convergence where input from cortical areas was increasingly ‘focused/funneled’ and integrated with each
layer of the basal ganglia (Percheron and Filion, 1991; Yelnik et al., 1996). The resulting highly processed information was then returned to areas of association cortex involved in motor planning.

More recently, these opposing models have been synthesized in the “Tripartite Model” of André Parent and others (Parent and Hazrati, 1995a, b; Haber, 2003). This model finds that there are three minimally overlapping areas of cortex that supply the striatum with input: sensorimotor, associative (from association areas in all lobes), and limbic (which also includes input from allocortical structures like hippocampus and amygdala). In this model, convergence occurs only within these separate channels, such that it has become common to refer to striatal domains by their source of cortical afferents: The dorsolateral aspect of the caudate and putamen becomes “sensorimotor striatum,” while “limbic striatum” is found in the ventral most portions of the caudate, putamen, and NAc.

It is notable that within each striatal subdomain, afferents to individual MSNs may come from widely separated, but functionally allied areas of cortex. This is to say that the patterning of cortico-striatal afferents is not a simple transposition of the cortical map onto the striatum, but rather, convergence sees to it that a single MSN may, for example, gather information from across the cortex about processed sensorimotor and primary motor information related to saccades and the orbicularis oculi, as well as relevant information about the visual field from association cortex in the occipital lobe (Flaherty and Graybiel, 1991, 1993).

In light of the physiologic finding noted above that MSNs have a very low tonic firing rate, this pattern of convergent input has moved some investigators to stress the arrangement’s “combinatorial logic,” (Kincaid et al., 1998; Zheng and Wilson, 2002). It is hypothesized that only simultaneous firing of the proper combination of cortical inputs to a single MSN can cause depolarization of that neuron, thus binding spatially disparate areas of cortex in a single neural expression that is then channeled through the basal ganglia (see Wilson, 2004), to eventually effect a particular behavioral element. This notion is borne out by examinations of synapse frequency of corticostriatal afferents. It is clear from comparing estimates of neuronal number in cortex and striatum that MSNs are a major site of cortical convergence. However, it is a convergence of a remarkably
democratic sort, with a very low probability that more than one of a single MSN’s ~11,000 synapses is made with the same cortical afferent (Zheng and Wilson, 2002). In this way, a single MSN can be affected, if only incrementally, by a large pool of potential cortical afferents, but none may exert dominance over the MSN’s membrane potential and probability of firing. This kind of architecture is ideal if, as mentioned above, the basal ganglia is purposed with learning motor output patterns in a Hebbian fashion, where connections underlying successful patterns become heavily weighted, as unsuccessful connections are pruned. At the beginning of task acquisition, a large number of combinatorial patterns are available (behavioral plasticity is high), and these are narrowed by the rigors of the task environment (learning) to select those that enable the most effective pattern of behavioral output (behavioral plasticity is lowered as an efficient ‘habit’ is formed.) It is this feature of basal ganglia organization that appears most relevant to goal-directed behavior.

At first glance, the cytoarchitecture of the striatum appears quite uniform, with a high proportion (~95%) of its ~3 million (rat) neurons being GABAergic MSNs (DiFiglia et al., 1976; Kita and Kitai, 1988; Oorschot, 1996). However, investigations of the organ’s connectivity and chemoanatomy have revealed chemical diversity and detail that appears to have functional significance. The medium spiny projection neurons (MSNs, Figure 4) and striatal interneurons are classified not only by the origin of their cortical afferents (above), but also by which part of the pallidum (GPI/EP vs. GPe) they innervate, as well as their expression of various receptors and signaling molecules.

Perhaps half of striatal MSNs project to the the basal ganglia’s output array in the GPi/EP and the SNr (Parent et al., 1984). This is known as the “direct pathway” and it is thought to facilitate movement by the mechanism discussed above: feedforward inhibition of output structures and thus disinhibition of their targets in the thalamus (see
Smith et al., 1998). MSNs making up this path are identified by their pattern of descending projections, as well as expression of dopamine D_1-family receptors, the tachykinin Substance P, and the endogenous κ opioid receptor ligand dynorphin (see Bolam et al., 2000; Levesque et al., 2003). The “indirect” pathway is made up of the remaining population MSNs that are D_2 receptor- and enkephalin-positive and project to the GPe (Gerfen, 1992). The GPe sends few projections outside the basal ganglia, and within the system, it projects almost exclusively to the glutamatergic neurons of the STN. Neurons of the STN in turn project to the classical output nuclei (GPi/EP, SNr). The interposition of the GPe and the STN is responsible for excitation of the output nuclei and thus inhibition of their targets in the thalamus (see Wilson, 2004). In this way, the indirect path is thought to diminish motor output.

The other major binary division of striatal MSNs is into either the striosomal “patch” compartment, or the surrounding “matrix.” The former is identified by high μ opioid receptor and low acetylcholinesterase levels, while the former labels intensely for calbindin and somatostatin fibers (Figure 5, Gerfen, 1992; Groves et al., 1998). In addition to these neurochemical markers, it is believed that there is some degree of connectional divergence to these compartments, with projections from limbic cortices preferentially innervating patches, while projections from sensorimotor cortex are more likely to synapse within the matrix (Groves et al., 1998). In this way the patch

![Figure 5. Chemical neuroanatomy in striatal compartments. Adapted from Wilson (2004).](image-url)
compartment has been assumed to play a role in reward and reinforcement, while the processing in the matrix is concerned with more mundane issues of muscle group selection, joint angles, and so forth. The differential pattern of patch/matrix connectivity has been advanced as an anatomic explanation for physiologic data showing different populations of reward- and task-responsive striatal neurons. For example, in a recent experiment in rodents (Schmitzer-Torbert and Redish, 2004), the authors were able to differentiate groups of dorsal striatal neurons that would increase their firing selectively based on either an internal spatial representation of a maze task, or food rewards found while running the maze. It has thus been suggested, based on this pattern of afferent connectivity, that it is the striosomal, patch compartment that is responsible for processing reward-related information. Previously, it had been suggested that the patch compartment also sent its own projections preferentially to dopaminergic neurons of the midbrain, and that this further substantiated its role in reward processing (though recently this has been called into question in primate models (Levesque and Parent, 2005)).

It has been shown that communication between the two compartments does not occur via GABAergic recurrent collaterals of the projection neurons (Aosaki et al., 1994; Aosaki et al., 1995), but rather by a small but important population of cholinergic interneurons (Bolam et al., 1984; Phelps et al., 1985; Kawaguchi, 1992). A similar population of GABAergic interneurons imposes a third layer of organization on the striatum. As with the MSN projection cells, the aspiny GABAergic interneurons are differentiated by neurochemical markers. The presence of either parvalbumin, or somatostatin, nitric oxide synthase, and neuropeptide Y mark two separate populations of interneuron (Bolam et al., 2000). Unlike their neighboring MSNs, these cells have high tonic firing rates and often fire synchronously due to the presence of gap junctions and perhaps common sources of afferent drive (see Wilson, 2004). It is believed that these interneurons may play a role in lateral inhibition in the striatum or possibly in increasing the signal-to-noise ratio of striatal output by inhibiting subthreshold responses in weakly stimulated MSNs (Koos and Tepper, 1999). This process would presumably aid in sculpting appropriate goal-directed behavioral responses and motor programs by impairing the expression of task-irrelevant movements by neighboring ensembles of cells.
What the Basal Ganglia Do: Holistic Perspectives on Basal Ganglia Function

We know from the clinical presentation of Parkinson’s and Huntington’s diseases that the basal ganglia are, at a minimum, required for smooth, coherent execution of motoric output in addition to maintaining muscle tone at rest. But symptoms like bradykinesia and tremor are only the grossest manifestations of basal ganglia dysfunction, and beneath these are subsumed more subtle losses of cognitive and volitional faculties (see Nieoullon, 2002). Aside from what we have inferred from observation of movement disorders, what functions did the basal ganglia evolve to perform, and how do these relate to the expression of adaptive behavior?

As has been noted elsewhere (Graybiel and Kimura, 1998; Graybiel, 2005), were the basal ganglia solely responsible for biomechanical aspects of motor output, they might be anatomically and hodologically similar to portions of the midbrain motor strip (cf. Holstege, 1991). However, the basal ganglia appear to have neuroplastic and computational capacities beyond these simpler structures. A number of investigators have attempted to unify the large body of empirical data on basal ganglia function under the rubric of “action selection,” “dimensionality reduction,” or “adaptive network” models. These are given a brief sketch below.

Habit Learning in an Adaptive Network (Graybiel)

The basal ganglia as an adaptive network responsible for “habit learning” of various motor programs has been advocated most notably by Ann Graybiel. Specifically, she argues that the basal ganglia may be the final arbiter of movement and action. Decisions about “when and where” to move are made by the basal ganglia and are ultimately the result of conditional processing of neo- and allocortical inputs to the striatum (Graybiel and Kimura, 1998). Importantly, this processing is modifiable by trial-and-error learning. This model draws on some of her earlier anatomical work that shows the overlap of functionally related parts of cortex, for example, the somatosensory and primary motor representation of a single finger, in the striatum (Flaherty and Graybiel,
As alluded to above, this pattern of convergent input should be able to bind numerous potential contributors to an action program into a nodal point that is modifiable over time by task outcome and environment. One prediction about neuronal physiology made by this model is that changes in striatal unit activity should be observed across task acquisition as the combinatorial logic of task-critical cortico-striatal inputs is resolved at the level of a recorded unit. Recent experiments by Graybiel’s group support this hypothesis.

Using wire electrode arrays implanted in the rat sensorimotor (i.e. dorsolateral) striatum, Graybiel’s group (Barnes et al., 2005) recorded the activity of projection neurons during the acquisition, over-training, extinction, and reinstatement of a simple T-maze task. Beginning as the animal traversed the runway portion of the maze, one of two readily distinguished tones were played to indicate which arm of the maze held a chocolate reward. Animals were recorded for about 40 trials under each condition. Across the different trial conditions, two populations of neurons became apparent: those that showed task-related phasic increases in firing activity, and those that did not.

Amongst the former category, firing was seen throughout the task during early acquisition trials. However during late acquisition and during over-training (when performance was maintained at asymptote) firing shifted to the earliest and latest parts of the task – at or before animals began to run the maze, then again later at goal receipt, or with over-training, at the turn in the maze. During the extinction phase, when the goal was removed from the maze, firing patterns looked once more as they did during acquisition, with activity spaced more evenly across the different components of the task. Reinstatement of the reward caused a rapid return to a late acquisition-like firing pattern in which phasic activity was clustered around the initiation of maze running and the decision point in the maze.

In neurons that did not show firing increases that were task-related, there were activity decreases during acquisition, such that by the end of acquisition, when the task had been mastered, these neurons were completely silent. Conversely, during extinction, these previously silent neurons began firing again at high levels. When the reward contingency was reinstated the neurons once again fell silent.
Together, these data seem to support the model discussed above where early phases of habit learning are marked by a trial-and-error approach where task-critical inputs to certain striatal neurons are eventually selected from a pool of possible contenders. This selection process is mirrored in recordings where a consolidation of previously ‘random’ activity occurs in some task-critical neurons, while in other neurons, presumably those that process information that is task-irrelevant, activity falls off. Another finding that would appear to buttress this notion comes from analysis of firing frequencies in those neurons that continued to fire during acquisition, over-training, and reinstatement. While their neighbors fell silent, these neurons appeared to ‘work harder,’ firing proportionally more spikes per phasic response with successive trials. This may represent a form of consolidation, where the habit is ‘burned in’ to a set of neurons that is in receipt of the proper afferents to effect the habit most efficiently.

**Action Selection (Mink, Gurney, and Redgrave)**

Another systems-level model of basal ganglia function has been derived from the work of Jonathan Mink (Mink, 1996), and Gurney, Prescott, and Redgrave (Gurney et al., 2004). These theorists view the basal ganglia as a switching device that evolved to efficiently allocate finite locomotor output resources in a manner that would enhance organismal fitness, and to resolve ‘arguments’ between different neural systems that make claims on these resources. The theory has a more computational flavor than does that of Graybiel, but the two perspectives may complement each other and so provide a more faithful rendering of what the basal ganglia do.

Put simply, Redgrave (Redgrave et al., 1999) suggests that the circuitry of the basal ganglia, particularly that of the striatum is arranged to “select some actions/motor programs at the expense of others.” This idea hinges on the large and multifarious nature of environmental stimuli (e.g. food, water, receptive mates, and other organisms vying for same) that impinge on the brain and compete not only for attention, but also concrete action. In this model, the basal ganglia become a central selector that evaluates and compares the salience of competing external or internal “competitors” and resolves a cogent behavioral solution from the highest (general course and vector) to the lowest.
frame of analysis. In addition to neurally ‘endorsing’ a pressing and coherent plan of action, the basal ganglia functions in a “winner-take-all” fashion to inhibit possible interference by weaker, less salient competitors.

A number of functional and anatomic features of the basal ganglia are cited by Redgrave et al. to support their claims. The first is the possibility of lateral inhibition within the striatum (see Koos and Tepper, 1999). When integrated with data provided by Barnes et al (2005), we can envision a classic “center surround” pattern of activation-inhibition where, during learning, extraneous, task-irrelevant competitors are quieted by their activated neighbors. The responsiveness of these active, task-relevant neurons is also simultaneously strengthened by feed-forward excitation through the parallel, reentrant, cortico-striato-thalamic loops mentioned in the previous section.

Another feature is the basic functional character of the basal ganglia as a disinhibitory circuit from the striatum to the pallidum. The low basal activity in the striatum contrasted with the high tonic firing of pallido-thalamic neurons lends itself to the model: actions are selected by focal excitation of striatal modules which then supply feed-forward disinhibition, via the pallidum, to thalamo-cortical nodes. Lateral inhibition in the striatum and tonic activity of pallido-thalamic tracts censor weak competitors from the stream of behavioral expression. It is notable that failure of this kind of neural selection may result in the symptoms of movement disorders (e.g. tremor, chorea) where the basal ganglia’s ability to filter such “noise” from the behavioral output stream is compromised by neurodegeneration.

Summary and Ventral Transition

The preceding discussion makes clear that the neostriatum and basal ganglia are organs tasked with refining patterned movement and motoric output. Research on the basal ganglia is commendable for showing roughly how these brain structures achieve their evolved function; however, questions about why remain. This is to say that the role of the basal ganglia, particularly the ventral striatum, in motivational processes continues to be scrutinized. A survey of this literature is made in the following section.
The Nucleus Accumbens and Ventral Striatopallidal System

Origins and Background

Although the nucleus accumbens (NAc) has been called by that name for almost a century, it was only recently that it came to be as we know it – a ventral extension of the striatum (see comment in Heimer et al., 1997). Prior to the anatomical work of Lennart Heimer in the 1970s, the NAc was typically apportioned with the septal-diagonal band complex. Heimer is fond of crediting his use of a variety of silver stains with the realignment in how we view the basal forebrain, but it was as much a product of the anatomist’s shrewdness for homology (de Olmos and Heimer, 1999; Heimer, 2003). One of his co-authors, Scott Zahm (Zahm, 2000), described the process:

“…Heimer and his colleagues observed patterns of chemical neuroanatomical organization in the mammalian mediobasal forebrain that made functional sense only if some of the traditional boundaries between nuclear entities were ignored, while others were emphasized. Thus, they observed that outputs from the primary olfactory cortex terminate not in the hypothalamus, but in ‘striatal-like’ medium size cell territories in the olfactory tubercle, which in turn project to districts in deeper layers of the tubercle that, in cytoarchitectural and ultrastructural appearance, look very much like the globus pallidus.”

The confluence of “limbic” (i.e. hippocampus, basolateral amygdala) inputs to the NAc took on added significance when it was demonstrated that they were being integrated by a striatal element that could potentially affect locomotor output. In Mogenson’s formulation (Mogensen et al., 1980), the NAc exists at the limbic-motor interface and oversees the transformation of “motivation to action.” As noted above, this model has exerted virtual hegemony over the behavioral neuroscience literature on motivated behavior, whether it concerns feeding, sex, drug seeking and addiction, or pair-bonding and other affiliative behaviors. The following section will provide a background and overview of the NAc and why it is believed to play so important a role in such a diverse and seemingly unrelated collection of behaviors.
Basic Organization and Chemoanatomy

Though it consists mainly of GABAergic MSNs like the dorsal striatum, casual macroscopic examination of the NAc reveals that its character is not wholly striatal (Heimer et al., 1997). For example, the NAc is not perforated by the large corticofugal fibers that mark the dorsal striatum (Figure 6). Also, unlike the dorsal striatum, where there is a relatively even distribution of neurons, non-specific histological preparations show clusters of cells, especially in the ventromedial extent of the NAc where overall cell density is lower (Heimer et al., 1997).

Just as the dorsal striatum has been subdivided into discrete compartments based on the presence or absence of various neuroactive substances or chemical markers, the NAc can be viewed as a modified extension of the neurochemical motifs present more dorsally. The picture that has emerged from immunocytochemical and autoradiographic investigations is of at least two major compartments that become prominent as one reaches the more caudal portion of the NAc: a more “striatum-like” dorsolateral “core,” and a more chemically and hodologically diverse ventromedial “shell.” Striatal features of the core include readily discernable calbindin-poor, μ-OR rich “patches” amidst a calbindin-rich “matrix” that is largely absent of μ-OR labeling. Indeed, features such as these blur the border between core and dorsal striatum (Zahm and Brog, 1992).

Over the years, various chemical markers have been used to demarcate core from shell (Zaborszky et al., 1985; Voorn et al., 1989), the most common being the absence of strong calbindin labeling in the more caudal portions of the shell (Jongen-Relo et al., 1994). Rather, the shell shows more
labeling for substance P, acetylcholinesterase, calretinin, dopamine D\(_3\) receptors, and neurotensin (Meredith, 1999). The shell has also been demonstrated to possess higher tissue concentrations of DA (Deutch and Cameron, 1992). On the other hand, the core shows greater labeling for enkephalin and the dopamine transporter (DAT, Zahm, 1999). It should be noted, however, that both compartments typically show non-homogenous distribution of makers, both within a single rostro-caudal level, and across the rostro-caudal extent of the NAc. Expression of prepro-dynorphin, for instance, shows patchy distribution across both structures that at times may appear more in the core and dorsolateral shell (Furuta et al., 2002). The “cone” or “rostral pole” of the NAc has been particularly difficult to fit into the neat “core-shell” dichotomy, due to the intermingling there of substances that in more caudal areas are believed to be specific to one compartment or other (Zahm and Brog, 1992). While the distribution patterns of a few of these substances has enhanced our understanding of NAc anatomy, and also helped to shed light on NAc ontogeny and its relationship to the dorsal striatum (e.g. Song and Harlan, 1994), their functional significance and relationship to behavior continues to be examined (Parkinson et al., 1999).

Substance P is seen to enhance dopaminergic transmission and locomotor output associated with intra-NAc DA administration (Kalivas and Miller, 1984; Boix et al., 1992a; Boix et al., 1992b). The mechanism underlying these effects appears involve the activation by substance P of neurokinin 1 receptors on cholinergic interneurons (Pickel et al., 2000). Some investigators have proposed that increases in extracellular DA facilitate purinergic tone on glutamatergic afferents, thereby reducing overall excitability of MSNs (Kombian et al., 2003). Modulation of MSN activity would then allow for disinhibition of output nuclei in the ventral pallidum.

Neurotensin was originally proposed to facilitate DA release, reverse apomorphine-induced reductions in extracellular DA (Tanganelli et al., 1989), and perhaps to do so preferentially in the NAc (Blaha et al., 1990). Also, like substance P, neurotensin reduces measures of locomotor activation induced by intra-NAc DA (Kalivas et al., 1984). Subsequent reports depict more complex, biphasic effects on DA release, with high perfusate concentrations increasing extracellular DA, and low concentrations having the opposite effect (Tanganelli et al., 1994). A cellular understanding of these
effects is incomplete, but a substantial body of work generated by Charles Nemeroff’s group has explored the neuromodulatory character of neurotensin at a systems level (reviewed in Binder et al., 2001). These investigators and others have argued convincingly that the peptide dampens mesolimbic DA activity (Brun et al., 2001; Caceda et al., 2005).

Without question though, the most important neuroactive substances in the NAc are DA and glutamate. The functional neuroanatomy and physiology of these substances and the way in which they interact with other biomolecules expressed in the ventral striatum will be treated below in a separate section.

**Afferents from Prefrontal Cortex**

Dopamine in the NAc appears to control goal-directed behavioral expression in part by integrating highly processed reward-relevant information from allo- and neocortical sources (Mogenson et al., 1980). One of the most important sources of cortical input to the NAc is from the prefrontal cortex (PFC, Figure 7). Understanding the nature and arrangement of these inputs has been a major topic of study and over the years has produced a number of satisfying experimental results. The results of these studies can seem less concrete than those from fine-grained analyses of the dorsolateral striatum, where convergent inputs can be traced to representations of, say, a single finger in the motor and somatosensory cortices (Graybiel and Kimura, 1995; Parent and Hazrati, 1995a, b; Wilson, 2004). Recent studies along these lines have shown that projections from hippocampus and PFC converge on single neurons of the NAc (French and Totterdell, 2003), but the nature of these

![Figure 7](image_url)
interactions is somewhat unclear (but see Grace, 2000). This relative ambiguity is owed to the nature of the role of PFC in behavior, where it appears less explicitly to do with motor programming, and more to do with its necessary antecedents: Planning why – or why not – a particular behavior should be enacted, and under what circumstances. Given the slightly more illusive nature of PFC compared to motor cortex, particularly in rodents (Uylings et al., 2003), we must at times catalog exquisite anatomical specialization with only a vague sense for how such features might ultimately contribute to behavior. Despite strides made in the psychological characterization of connections between PFC and NAc, our understanding of this relationship is not fully mature, and the behavioral significance of certain anatomical findings remains to be seen. What is known, however, will be detailed here.

A number of studies from the early 1990s mapped projections from various parts of PFC to NAc. Plant lectin injected across layers V and VI of the rostroventral part of the prelimbic division of the mPFC produce anterogradely labeled fibers in both ipsilateral NAc shell and NAc core (Sesack et al., 1989). This is contrasted to injections in the caudal and central prelimbic area, or the dorsal anterior cingulate and medial precentral cortices, which produce labeling in dorsomedial and dorsolateral portions of the striatum, respectively. Interestingly, injections into the prelimbic cortex produced more intense labeling in other traditional ‘limbic’ structures like the central and basolateral amygdala, suggesting some reciprocity amongst structures involved in affect and motivation.

Attempts to further parse cortical afferents to either NAc shell (NAcSh) or NAc core (NAcC) were made by injection of retrograde tracers into these structures (Brog et al., 1993). Injections into the medial and lateral NAcSh extended the above anterograde data by revealing strong labeling in infralimbic PFC, and lighter labeling in the prelimbic area. The latter structure appears to be preferentially innervated by the NAcC, which shows a pattern of labeling that is opposite to that of the NAcSh: Greater labeling from prelimbic and less from the infralimbic cortex.
More exhaustive studies also sought to take into account the variegated features of the NAc (Berendse et al., 1992), by labeling for enkephalin and anterograde tracer injected into parts of the PFC. The pattern of enkephalin labeling, in which highly-labeled, cell-poor areas surround more tightly packed neurons that express μ-opiate receptor (OR), is thought to be analogous to the same pattern observed in the dorsal striatum (Heimer et al., 1997). Data from these experiments revealed a few simple principles about the cortical afferents to NAc. One is that in the more dorsolateral portion of the NAcC, the organization is understandably more striatum-like, with cortical layer, not cortical area, determining which compartment afferents from the mPFC and the more lateral and orbital division innervate. Projections from more superficial layers (II-V) of these areas tend to project to the enkephalin-poor “patch”-like compartment, while those from deep layer V and layer VI synapse outside them. As one moves deeper in the NAcSh, however, particularly its caudal extent, it is cortical area, rather than any particular layer, that determines which cellular compartment is innervated (Figure 8). For example, the prelimbic afferents discussed above are more likely to terminate within the μ-OR-rich cell clusters, while those from the infralimbic area prefer the surrounding neuropil labeled strongly for enkephalin. With these findings, the authors note their previous work that shows the enkephalin-labeled neurons projecting to the VTA, while those in clusters project to the ventral pallidum. Curiously, this compartmentalization of afferents is somewhat reversed as one enters the rostral reach of the NAcSh. Here,
prelimbic fibers terminate within enkephalin-rich zones, while other PFC afferents show more homogenous distribution that is at times punctuated by layer-dependent grouping that is unrelated to any identified marker, but suggestive of the more striatal pattern discussed above.

**Glutamatergic Function in Nucleus Accumbens**

Unsurprisingly, microiontophoresed glutamate has potent excitatory effects on the resident MSNs of NAc (Hu and White, 1996; Kiyatkin and Rebec, 1999). Glutamatergic depolarization of MSNs appears to be mediated chiefly by AMPA receptors, as NMDA excitations require higher ejection currents, are less robust, and occur only following pretreatment with AMPA. Action at metabotropic glutamate receptors (mGluR), on the other hand, appear to have opposite effects, dampening the excitatory effects of applied AMPA and NMDA. It is possible that this effect is mediated by members of the group II subtype, since it was blocked by an mGluR II antagonist, but less so by a ligand with mixed group I/II affinity (Hu and White, 1996; Conn et al., 2005). More recent functional data suggest a novel mechanism by which mGluR II/III receptors may affect glutamatergic tone in NAc (Baker et al., 2002). These authors report that basal extracellular glutamate in NAc originates, not from the vesicular pool, but rather the intracellular metabolic pool, and it is released into the extracellular space by a selective antiporter (system x^-). Antiporter activity is in turn negatively regulated by mGluR II/III receptors, which reduce extracellular glutamate.

**Afferents from Midbrain**

It is now taken for granted that the NAc receives massive dopaminergic input from the ventral tegmental area (VTA), and to a lesser extent the substantia nigra pars compacta (SNpc). This functional anatomical arrangement has been alternately called the mesocortical or mesolimbic system (Moore and Bloom, 1978; Oades and Halliday, 1987), depending on the author’s adherence to traditional notions of the “limbic system”. Typically, mesolimbic is used to designate those fibers that terminate in the ventral
striatum, while mesocortical now refers to the fibers that continue rostrally to the PFC. Beyond this and the nigrostriatal vs. mesolimbic/mesocortical designation, the midbrain DA paths are regarded as somewhat monolithic.

At the level of the striatum, however, a number of distinctions can be made. Unlike the dorsal striatum, where the dense plexus of DA fibers is rather uniform, their pattern in the NAc is patchy. Particularly in the more rostral extreme, immunolabeling for DA itself reveals preferential innervation of cell-poor areas (Voorn et al., 1986). More broadly, it is the NAcSh that shows heaviest DA labeling, and within that structure, DA fibers align with those areas of high enkephalin expression discussed above (Figure 9, Voorn et al., 1989). Thus, it may be possible to trace a polysynaptic recurrent loop in which projection neurons in the enkephalin-rich matrisomal portion of the NAcSh receive DA inputs and, in turn, send return projections to the midbrain. This raises the question about the organization of DA projections from the VTA (Figure 9). It is known that this pattern of innervation arises from the so-called “dorsal tier” of calbindin-positive DA neurons in the VTA (Bentivoglio and Morelli, 2005), and the more medial parts of the SNpc (Hasue and Shammah-Lagnado, 2002). Further it appears that both dopaminergic and non-dopaminergic (GABA-containing) projection neurons of the VTA project together in topographic fashion along medio-lateral lines (Van Bockstaele and Pickel, 1995).

**Figure 9.** Overlap between dopamine fibers and other neuroactive markers in nucleus accumbens. DA, dopamine; ENK, enkephalin; SP, substance P; CABP, calcim binding protein (calbindin); ac, anterior commissure; ICjM, island of Calleja magna; LV, lateral ventricle. Adapted from Voorn et al. (1989).
Synaptic Organization and Signaling Elements

The convergence of projections from cortex and midbrain, and the remarkable density of spines on the GABAergic medium spiny neurons (MSNs) that comprise the NAc (Meredith et al., 1992), provide a rich substrate for neurochemical interactions. The synaptic arrangement thought to have greatest functional significance is the convergence of glutamatergic cortical afferents and midbrain DA terminals on a single MSN (Figure 10).

A few experiments have used plant lectins or electrolytic lesions to identify mPFC projections that are in apposition to those from midbrain DA cells immunopositive for tyrosine hydroxylase (TH, Sesack and Pickel, 1992). Examination by electron microscope revealed that among the 25% of PFC terminals that could be seen to make asymmetric (excitatory) contact in a given thin section, it was almost always at spines or dendrites of NAc MSNs. Almost one quarter of these spines were also seen to receive TH-positive input. Most commonly, TH-containing terminals made symmetric contact near the neck of a spine in receipt of asymmetric cortical input at the head. This has been taken as a mechanism by which DA can modulate excitatory drive to the MSN.

There is little reason to believe that, under normal circumstances, these excitatory synapses are not endowed with the usual molecular machinery associated with the postsynaptic density (e.g. Kim and Sheng, 2004). Mapping studies in primates suggest that striatal synapses are particularly enriched in AMPA receptors containing the GluR1
subunit (Martin et al., 1993). In rodents, the NMDAR1 subunit is frequently found postsynaptically outside the release zone of dendrites receiving excitatory and dopaminergic afferents (Gracy and Pickel, 1996). This report showed that NMDAR1 can also be detected presynaptically in TH-positive axons, where it appears in quantity between varicosities, suggesting that DA terminals themselves may also be shaped by local glutamate.

Localization of DA transporter (DAT) protein has also been studied at the light and electron microscopic level. Consistent with the broader pattern of DA innervation, DAT expression in the dorsal striatum is even and more substantial. As one moves more into the more rostral, ventromedial portions of the NAcSh, however, DAT expression appears stippled, and to obey some of the same rules of compartmentalization outlined above (Ciliax et al., 1995; Freed et al., 1995). It seems probable that patches of high DAT expression coincide with those of high DA and enkephalin labeling. At the ultrastructural level, DAT appears far enough outside the release zone so as to permit some diffusion and presumably a delimited area of high transmitter concentration (Hersch et al., 1997; Wickens and Arbuthnott, 2005). This is contrasted with the dorsal striatum where vesicularly-localized DAT terminates DA transmission more promptly (Marshall et al., 1990).

Of the DA receptors, the D3 class shows an especially dense concentration in the NAc and ventral striatum, actually appearing to demarcate the dorsal boundary of the structure (Diaz et al., 2000). The D1 and D2 subtypes, however, show a broader distribution throughout the NAc and striatum (Mansour et al., 1990). While co-expression of both receptors is common in the NAc, D2 appears preferentially with enkephalin, and D1 with tachykinin (Lu et al., 1997). Given their slightly higher expression in the more medial part of the NAcSh, it has been suggested that D1 regulates the descending return projection to VTA (Meredith, 1999). At an ultrastructural level, both receptor subtypes are found in dendrites and their spines (Yung et al., 1995), with D2 also appearing presynaptically on TH-positive afferents, where it likely functions in an autoreceptor capacity (Delle Donne et al., 1996; Delle Donne et al., 1997).
Dopamine and Dopamine-Glutamate Interactions in Nucleus Accumbens

*In vitro* recording has shown that D₁ activation can be seen to reduce the frequency of EPSPs, suggesting that the receptor somehow modulates excitatory afferent drive (see Nicola et al., 2000). *In vivo* recordings are likely to provide a more faithful picture of how DA regulates MSN activity in the presence of various active inputs. Like their counterparts in the dorsal striatum, MSNs in NAc display a bistable resting potential, with -60 mV “up” state that alternates with a -80 mV “down” state (Figure 11, see O'Donnell et al., 1999). Because this phenomenon is visible *in vivo* only when afferent inputs are left intact, it is believed that the depolarized up state derives from excitatory afferents. It has also been speculated that the bistable phenomenon may be responsible for the D₁ receptor’s complex actions *in vivo*. This receptor has been said to improve the signal-to-noise ratio of convergent inputs by compounding hyperpolarizations during the down-state and facilitating depolarization when the neuron has reached its up-state (O'Donnell et al., 1999; Nicola et al., 2000). More recently, experiments have been performed in which D₁ and D₂ antagonists were perfused directly into NAc near the intracellular recording electrode (West and Grace, 2002). These investigators report that D₁ blockade increases the cumulative time neurons spent in the down-state. Conversely, D₂ blockade increased firing rate by driving neurons into the up-state. Together these findings were taken to mean that *in vivo*, D₁ has an excitatory effect that is tonically opposed by D₂. In this way it is proposed that under basal conditions, D₂ signaling biases MSNs to favor the down-state. This inhibitory control may be overcome, however, during phasic activation by excitatory afferents which drive the neuron into the up-state. Once in this state of relative depolarization, D₁ activation can then push the neuron to the spike threshold. It is notable that these findings echo those of one of the first *in vitro* studies where NMDA-evoked
potentials are enhanced by D₁ agonists, and attenuated by activation of D₂ (Cepeda et al., 1993).

The mechanism underlying this effect has been investigated by several neurochemical studies. One of these suggests that DA receptor-mediated effects on MSN excitability are a presynaptic phenomenon (Kalivas and Duffy, 1997). Measuring extracellular glutamate in NAc, it was found that both amphetamine and the D₂ agonist SKF-82958 reduce levels of this transmitter. This effect proved reversible with D₁ or D₂ blockade. While the systemic route used in these experiments clouds interpretation somewhat, the D₂ effect is aligned both with the anatomic and electrophysiological data described above.

Studies of MSN Activity in Freely Moving Animals: Apparent Role in Reward

As noted in the previous section, the confluence of anatomical inputs from “limbic” structures to the NAc has led most investigators to conclude that the NAc is also set apart from the dorsal striatum in functional terms, and that the MSNs of the NAc should be in some way responsive to affective stimuli, particularly those that are rewarding. Much of the evidence to support this hypothesis has come from the laboratory of Regina Carelli. Using wire electrodes to record from MSNs in the NAc of freely moving rats, Carelli reports several patterns of phasic firing response as animals complete an operant task for different rewards (Carelli, 2000). In these tasks animals are allowed to lever press for food, water (in lightly deprived animals), or cocaine (i.v.) rewards and the availability of each is signaled by a distinct light cue above the lever. After a lever press response is elicited, reward delivery may be paired with the onset of a 10 s tone. The four basic patterns of
responses she has cataloged in different MSN neurons are: i. Increased firing before each lever press for cocaine or water; ii. Phasic increases after completion of each response; iii. Phasic decreases, or momentary pauses in firing, after response completion, and; iv (Figure 12). Phasic increases both before and after the behavioral response, with a brief pause during which the lever press response is emitted. Interestingly, this final category appears only in neurons responsive to cocaine. The major finding, however, comes from recordings made from single neurons across lever pressing for different reinforcers (Carelli and Ijames, 2001; Roop et al., 2002). The responsiveness of MSNs can be “tuned” to a particular reinforcer, showing one of these phasic firing changes only in response to, say, water or cocaine, but not the other. Estimates suggest that the large majority of MSNs (92%) show this kind of specialized, non-overlapping pattern of responsiveness. Notably, the main division in this pattern of specialized responding is between natural rewards and cocaine (Carelli, 2002, 2004). There are apparently two populations of MSN, one responsive to natural rewards, the other to pharmacological rewards, in this case, cocaine. Amongst the former group, when the same MSN is recorded during lever pressing for food, water, or a reward with elevated incentive value like sucrose solution, it is more common to find phasic responses to each of these stimuli. It is estimated that in MSNs that respond to natural reinforcers, about 68% do not discriminate by type of natural reinforcer. Whether they are cocaine- or natural reward-responsive, both types of neuron are able to bind cues associated with reward delivery. For example, after training, cocaine-responsive neurons show phasic responses to the onset of the auditory stimulus previously paired with cocaine injection. Recordings from the same natural reward sensitive neuron in animals given access to both cocaine and water, each previously paired to a separate tone, the water-associated, but not cocaine-associated tone engenders a phasic response.

The role of DA in this process, particularly where the formation of reward associations are concerned, has been investigated by running animals through similar operant paradigms while recording NAc DA release on a subsecond timescale with fast-scan cyclic voltammetry. Such studies show that phasic increases in DA coincide with presentation of audiovisual cues previously associated with delivery of cocaine during self-administration sessions (Roitman et al., 2004; Stuber et al., 2005). These data
complement those from *in vivo* recordings of midbrain DA neurons, and, further, the phasic DA response has been suggested as a means by which reward associations are effected in the NAc. Both of these topics will be treated at greater length below.
3. ANATOMY AND PHYSIOLOGY OF MIDBRAIN DOPAMINE NEURONS

Anatomical Considerations

Though the experiments presented in this dissertation are concerned primarily with VTA function and physiology, some discussion of their anatomy will be valuable in that what we know about DA neuron physiology derives from studies made of cells in both major anatomical divisions of the mesotelencephalic DA system, the substantia nigra pars compacta (SNpc) and the more medially located ventral tegmental area (VTA). It is not uncommon for principles deduced from studies of cells in the substantia nigra to be extrapolated to those of the VTA, and vice-versa. At times, investigators are lax in making such anatomical distinctions, recording from both nigral and tegmental DA units, and pooling data when no difference in cell responses are apparent. Other investigators, however, have been more selective, preferring to study one or other cell group exclusively.

Nowadays it is generally taken for granted that cells of the nigral division affect mechanical aspects of motor behavior via the dorsal striatum and are the province of Parkinson’s disease researchers, while the cells of the (VTA) are responsive to reward and play a role in addiction via their projections to the NAc. However, in light of reward- and salience-responsive neurons in the SNpc, and the absence there of phasic firing that coincides with limb movements (as might be expected of neurons tasked with managing fine motor control), the simple SNpc-VTA dichotomy has been called into question by some investigators (Horvitz, 2000).

What anatomical basis is there for physiological studies of either SNpc or VTA DA neurons? The evidence is more hodological than cytological. This is to say that neurons of the SNpc and VTA differ more in their projection patterns than in their morphology or ultrastructure (see Bentivoglio and Morelli, 2005). Though the SN was
described in the 18\textsuperscript{th} century, and its compact and reticular divisions by 1888, it was only in 1925 that the VTA was suggested to be a distinct nuclear entity (see Bentivoglio and Morelli, 2005). This notion was reaffirmed when Dahlstrom and Fuxe first mapped DA-containing cells in the midbrain, assigning the familiar “A9” and “A10” designations to the SN and VTA, respectively (Figure 13, Dahlstrom and Fuxe, 1964). This nomenclature was later discarded in light of the common prenatal ontogeny of SN and VTA DA cells (Moore and Bloom, 1978), and the fact that: “[t]he cells are continuous from medial to lateral and do not appear to form distinct groupings or nuclei.” In this same paper, the authors go on to argue for differentiating SN from VTA based solely on their afferents to the forebrain (neostriatum vs. NAc, limbic cortex). Ultimately, functional differences and their respective contributions to behavior must emerge from these divergent patterns of connectivity, as return projections from the targets of SN and VTA (e.g. striatum vs. prefrontal cortex) also differentially regulate DA cell physiology (see Lee et al., 2004). For the purposes of this paper, the two cell populations must be regarded separately at a systems level, and research reports concerned with the SN addressed only inasmuch as they might elucidate the character of their neighbors in the VTA.
Early Studies

Not long after anatomical evidence for catecholamines in the CNS was published (Dahlstrom and Fuxe, 1964), physiologists began to use these new maps of chemical neuroanatomy to guide electrodes to the areas of the brain known to be rich in DA-containing cells. The first experiments by Benjamin Bunney’s group stereotactically probed the extent of the SN and VTA with glass electrodes, seeking to identify a pattern of neuronal activity that was unique to these structures (Bunney et al., 1973a; Bunney et al., 1973b). After such a pattern was identified, the challenge was then to demonstrate that cells near the tip of the recording electrode were, in fact, DA neurons. Bunney used a somewhat indirect approach to generate presumptive evidence that his cells contained DA. He found that pretreating his animals with the dopaminergic neurotoxin 6-OH-DA several days before he performed his recordings abolished his ability to detect the putative DA cells in SN and VTA. In separate experiments, he iontophoresed L-DOPA from his recording electrode onto nearby cells presumed to contain DA based on their electrical signature on the oscilloscope. After processing sectioned tissue for DA histofluorescence (a la Dahlstrom and Fuxe), neurons immediately beside the electrode track were found to fluoresce more brightly than those further away that had not been exposed to excess L-DOPA. Since only catecholaminergic neurons can take up L-DOPA, it was reasoned that this was an effective way of tagging DA cells which had been recorded, and thus confirming their unique electrical signature.

In Vivo Extracellular Studies

The earliest experiments mentioned above were significant in that they laid out the fundamental characteristics of DA neuron physiology (Figure 14). Nowadays, these characteristics are regarded as so reliable that in routine extracellular studies, it is rare that cell labeling, or even histology for placement, are used to identify the subjects of recording (but see Lock et al., 2004 for a dissenting view). The most basic identifying features for DA neurons include an action potential or spike with a waveform that is bi- or triphasic (the latter taking the shape of a letter M). The action potentials are of long (2-
5 ms) duration, have an initial positive phase which is said to be “notched,” and is thought to reflect the immediate depolarization of the initial segment, before the wave of depolarization spreads throughout the larger volume of the soma and dendritic arbors. The amplitude of these action potentials, measured from peak-to-peak is typically 0.5-1.5 mV. VTA DA neurons display two main discharge patterns: a single-spike firing mode, which may be either highly regular and pacemaker-like or irregular, and a burst firing mode in which multiple volleys (2-8) of action potentials are fired in rapid succession, followed by a pause. The mechanisms of burst firing will be dealt with in greater depth below, but the temporal criteria used to define a burst can be noted here: when the interval between two or more action potentials (the “interspike interval,” “ISI”) falls below 80 ms, this is recognized as the onset of a burst. As the burst proceeds, the amplitude of each successive spike is slightly reduced producing a ‘tapered’ appearance in the spike train. The ISI also becomes lengthened with each spike in a burst. When the ISI exceeds 160 ms, the burst is regarded to have concluded. Following a burst there is a quiescent “post-burst inhibitory period” of ~350 ms during which no action potentials occur. The question of average frequency and bursting rate of midbrain DA neurons brings methodological issues to the fore, because the type of animal preparation used affects the properties of individual cells and, in turn, the activity of the cell population (Bunney et al., 1973a; Bunney et al., 1973b; German et al., 1980; Chiodo and Bunney, 1983).

The classical method of in vivo extracellular recording is performed using electrolyte-filled (2 M NaCl) glass electrodes in chloral hydrate or urethane anesthetized rats secured in a grounded stereotaxic frame. Supplemental anesthesia is given at regular intervals (~45 min) across a recording session that may last several hours depending on the experimental design. Mean frequency estimates for VTA cells in anesthetized preparations range from 3.5 – 5.4 Hz, with about 50% of these cells firing in burst-mode. Estimates for the SNpc are also within that range, with a mean frequency of 4.5 Hz, and about 50% of those cells firing in burst mode. It is often found that VTA neurons show slightly higher firing frequencies and shorter-duration spikes (Bunney et al., 1973a; Bunney et al., 1973b; Yim and Mogenson, 1980).
From the beginning, concern that the anesthetic was contributing to experimental artifacts moved some investigators to use conscious and paralyzed, but locally-anesthetized animals. However, as the paralyzed preparation is likely to be profoundly stressful, this feature of the preparation itself appears to be confounding, producing conflicting experimental results. Some investigators report few differences in firing parameters between anesthetized and paralyzed preparations (Xu and Shen, 2001), while the original studies by Bunney report an abolition of firing in paralyzed animals (Bunney et al., 1973b). Inasmuch as aversive stimuli reduce VTA cell activity, the latter finding may be expected (Ungless et al., 2004).

In an effort to surmount the ethical and scientific problems with the paralyzed preparation, Gessa’s group developed an unanesthetized preparation in which the animal is conditioned over time to be comfortable in a head restraint and body jacket that suspends the rat in a hammock-like fashion during recording (Fa et al., 2003). The study also provided the most recent account of anesthetic effects on DA neuron firing properties. These authors compared their head-restrained preparation to light or deep choral hydrate anesthesia, as well as to \textit{in vitro} midbrain slice preparations. They found no differences in mean firing rate between preparations (3.14-3.5 Hz), but measures of bursting were changed depending on the preparation. With lighter anesthesia, or in fully conscious animals, there are many more bursting cells, and the number of spikes that are part of a burst also increases markedly. For example, in deeply anesthetized animals (maintained on chloral hydrate at 120 mg/kg/h after the initial 400 mg/kg dose), only 5.3% of cells show bursting activity, compared to 71.6% in the unanesthetized-restrained group. They also found substantial differences in another basic measure of DA cell population activity, the number of cells found in each pass of the recording electrode, or “track.” Mirroring findings for firing rate, more deeply anesthetized animals show half the number of spontaneously active neurons as awake, restrained subjects.

The cells-per-track technique (Chiodo and Bunney, 1983) is the standard means of sampling activity of the midbrain DA neuron population. The technique calls for the repeated passage of a glass electrode throughout the extent of VTA or SNpc at 200 μm intervals. The number of cells found in each pass is then averaged and used as a dependent measure that relates to the activity of the total neuronal population. This
approach is particularly useful for examining the effects of chronic pharmacological manipulations (e.g. repeated cocaine). For example, the number of active DA neurons during withdrawal from chronic ethanol can be seen to decrease using the cells-per-track technique (Shen, 2003). More fundamentally, the technique has shed light on the behavior of DA neurons under basal, non drug-treated conditions. Such studies suggest that about 40-50% of the total DA cell population is maintained in a quiescent, hyperpolarized state, but can be activated by electrical or pharmacologic stimulation. The basally active population typically produces an average of 0.8-1.2 cells-per-track (see Chiodo, 1988).

Figure 14. Traces from identified ventral tegmental area dopamine neurons in vivo.

A. Long, triphasic action potential of VTA DA neuron showing prominent notched rising phase. B. Pacemaker firing mode. C. Burst firing mode. D. Depolarization inactivation. Neurons undergoing depolarization inactivation rarely repolarize sufficiently to spike and are thus undetectable. Occasionally, some do fire weakly and at very low frequencies. This is one such case. Adapted from recordings made by the author in January, 2006.
Intracellular Studies

By 1980, Grace and Bunney had produced the first *in vivo* recordings of SNpc neurons in which the cells were impaled by the recording electrode, thus allowing for the examination of membrane properties (Grace and Bunney, 1980).

A number of basic electrical characteristics were revealed in these experiments. The resting membrane potential of DA neurons is -50 to -60 mV, and action potentials are preceded by a long depolarization (40-120 ms) to a firing threshold of approximately -43 mV. Following action potential generation, there is a 3 mV afterhyperpolarization (AHP) that decays over 1-6 ms. It has been suggested that the variable refractory period accounts for the irregular single-spike firing pattern, and that when the calcium-dependent potassium conductance that is responsible for it is blocked by calcium chelators, a regular pacemaker pattern emerges (Grace and Bunney, 1984a, b).

The use of *in vitro* midbrain slices containing the SN and VTA has extended the data gleaned from *in vivo* intracellular studies. Work by Johnson and North (1992) has shown that VTA DA neurons have an input resistance that is substantially higher than that measured in nigral cells *in vivo* (~192 vs. 45 MΩ). Additionally, they report an AHP that is longer (100-200 ms) and of greater amplitude (27 mV) than *in vivo* figures mentioned above (Johnson and North, 1992). It has been argued that in slices, the absence of incremental depolarization by afferents is responsible for these differences (Grace, 1987).

Mechanisms of Spontaneous Firing

In the absence of afferent excitatory drive, slice preparations cannot be used to study bursting phenomena the way they can *in vivo*, but it is the lack of these afferents that lends slice work its transparency and the ability to isolate intrinsic physiologic properties of the cell. One of these properties is the regular, pacemaker-like single spike firing found in DA cells.

In a classic paper using slice preparations containing SNpc and VTA, Grace and Onn confirmed much of the previously reported data from *in vivo* intracellular studies
(Grace and Onn, 1989). For example, present were the typical long action potential (>2 ms) preceded by a slow depolarization and followed by a pronounced AHP. Beyond this, however, the paper also provided the first model of the native physiological properties of the DA neuron and the mechanisms that underlie its rhythmic firing pattern.

The slow depolarization (~22 mV) which eventually ramps up to threshold for action potential generation was proposed to originate from the initial segment (IS) and to be a voltage-dependent phenomenon mediated by sodium conductance, since it could be elicited by current injection and blocked by tetrodotoxin (TTX) but not the calcium channel blocker cobalt. While cobalt was able to block spikes obtained by weak depolarizing current pulses, stronger currents were found to produce fast, TTX-sensitive spiking from a higher threshold (-30 mV, Figure 15). These spikes were different than those observed under drug-free control conditions, in that they were of much shorter duration (~1 ms), and displayed no AHP. With no appreciable AHP, the ISI of these spike trains was very short. Together, these features were taken to mean that these rapid, TTX-sensitive spikes were from the IS, that they had an intimate role in action potential generation, and that the AHP may be the rate limiting feature in cell pacemaker firing.

![Figure 15. Ionic contributions to action potential generation in dopamine neuron.](image)

Intracellular recording shows A, slow rise to spike threshold and subsequent undershoot after spiking. B, Presence of calcium channel blocker cobalt in perfusion media prevents depolarizing current from inducing spikes. C, stronger currents, however, can produce short duration, high threshold spiking. This effect is blocked by D, presence of cobalt and tetrodotoxin (TTX), suggesting a sodium dependent component of the high threshold spikes seen in C. Adapted from Grace and Onn (1989).

Interestingly, depolarizing pulses were not the only electrical stimulus that was capable of eliciting this kind of spike. Small hyperpolarizing currents, when given to a resting cell, were able to trigger a “rebound” depolarization and accompanying spike that was also shown to be TTX-sensitive. In these rebound events, only the spike proved TTX-sensitive, while the preceding low-threshold depolarization proved to be cobalt-
sensitive, indicating a calcium component. Inasmuch as the TTX-sensitive IS spike is responsible for action potential generation, such rebound events may have a role in maintaining the cycle of depolarization-spike-hyperpolarization that underlies pacemaker firing.

These findings became controversial, however, when Kang and Kitai produced data suggesting that the putatively sodium-dependent slow depolarization must have a calcium component (Kang and Kitai, 1993a). Like Grace and Onn (1989), they found the slow depolarization to be highly voltage-dependent, but further, that depending on the baseline holding potential, small current injections could trigger the slow depolarizations in the presence of TTX and sodium free perfusate, but not in the presence of another divalent cation, cadmium, suggesting some calcium channel involvement (Kang and Kitai, 1993b). These authors suggested that because intracellular injection of calcium chelators prolongs the falling phase of the oscillatory slow depolarization, the role of calcium may ultimately be to activate repolarizing potassium currents via the SK family of calcium-activated potassium channels (Sah, 1996). This notion was confirmed in later experiments on SNpc cells by Ping and Shepard, and more recently by several other laboratories (Ping and Shepard, 1996; Wolfart et al., 2001; Koyama et al., 2005). Wolfart et al. used a powerful combination of traditional patch-clamping with single cell RT-PCR and confocal immunolabeling to determine the presence of SK3 transcripts and functional channels in SNpc and VTA neurons. They determined that while SK3 channels do not mediate excitability or action potential induction in DA neurons, they do regulate the AHP in a calcium-dependent manner. Remarkably, they found that this effect was preferentially exerted in SNpc cells as compared to VTA cells, which had less of the protein. For the moment, there is consensus that the presence of SK channels confers the regular frequency of pacemaker firing of DA neurons. Additionally, it has been proposed elsewhere that this is one of the intrinsic properties that delimits bursting episodes engendered by excitatory afferents (see Overton and Clark, 1997).

The classic work of Grace and Onn (1989) uncovered a third important cobalt-sensitive current. The high-threshold calcium spike (HTS) appears only when the potassium channel blocker tetraethylammonium (TEA) is added to slices already treated with TTX. In the absence of TEA, even very strong depolarizing current injections fail to
elicit the HTS. Because of their large amplitude (75 mV), substantial duration (12 ms),
and long AHP, it seems likely that the HTS is the major contributor to the cell’s
somatodendritic action potential.

A final feature of DA cell physiology that may shape membrane potential and
firing properties is the presence of strong anomalous rectification. Originally described in
vivo (Grace and Bunney, 1984b), Grace and Onn (1989) and Kang and Kitai (1993b),
later reported this phenomenon in vitro as non-linear “sags” in membrane potential
during stepwise hyperpolarizing currents, especially during strongly hyperpolarizing
steps (-100 mV). The absence of this effect in the presence of TEA suggests the
possibility that it is mediated by classic KCNH-type inward rectifiers (Hille, 2001). Grace
(1989) has suggested that in keeping with its activity in other cell types, the function of
this type of current may be to maintain resting potential close to firing threshold.

Pharmacology and Synaptic Regulation of Midbrain Dopamine Neurons

Given the rapid growth of neuroscientific data over the past two decades, much of it
driven by molecular biological approaches, there are a chastening number of cloned and
characterized receptors for an equally dizzying array of neuroactive substances and
canonical synthetic ligands. Not surprisingly, the effect of many of these substances on
VTA DA neuron function has been evaluated experimentally. Rather than provide here a
complete recitation of every substance and its effects on DA cell activity, the sources of
major afferent input and their transmitters will be discussed.

Glutamatergic Inputs

The VTA receives glutamatergic afferents from almost every level of the brain (see
Oades and Halliday, 1987). Anatomical or functional experiments have described fibers
from the pedunculopontine tegmentum (Pan and Hyland, 2005), lateral hypothalamic area
(Shizgal, 1989; You et al., 2001), the subthalamic nucleus (Smith and Grace, 1992), and
the bed nucleus of the stria terminalis (Georges and Aston-Jones, 2002). However, none
of these have received the attention that those from the prefrontal cortex (PFC) have
Experimental data suggest that excitatory afferents from medial PFC may be responsible for driving the burst-firing activity of VTA DA neurons (Tong et al., 1996a, b). These authors found that bursting could be induced by single-pulse stimulation of the mPFC and that this effect could be blocked when glutamate receptor antagonists were iontophoresed next to the neuron under observation (Tong et al., 1998).

These data highlight one of the more robust and interesting findings with regard to glutamate’s influence on VTA DA activity: that burst activity is dependent on NMDA receptor activation, and presumably the attendant calcium current through that receptor (White, 1996; Overton and Clark, 1997). Perhaps unremarkably, application of AMPA, NMDA, and glutamate itself will all incite firing rate increases, while blockade of these receptor subtypes diminishes excitability. Only NMDA, however, appears able to induce bursting and this effect is seen only in vivo. While some in vitro preparations have used co-application of NMDA and the SK channel poison apamin to induce bursting, it has been suggested that the resulting bursts are not analogous to natural bursts, in that their ISI decreases over the course of the burst rather than increasing as it would in vivo (Overton and Clark, 1997). Thus, various reports of drugs or transmitters causing bursting in vitro demand additional evidence to substantiate their likeness to the natural phenomenon.

The presence of NMDA and AMPA receptors on VTA DA cells immediately suggests the potential for activity-dependent plasticity. Indeed, much of the literature on addiction focuses directly or indirectly on the neuroadaptations shown by VTA DA neurons following exposure to drugs of abuse (White and Kalivas, 1998; Vanderschuren and Kalivas, 2000; Nestler, 2001). This large body of literature is beyond the scope of this review, but at least one study has investigated the basal, drug-free neuroplastic properties of VTA DA neurons.

Bonci and Malenka (1999) used midbrain slice preparations containing the VTA to probe both DA and non-DA (GABA) cells for their ability to display long-term potentiation (LTP, Bonci and Malenka, 1999). Adapting a classic LTP protocol (a train of 200, 1 Hz stimulations in a cell held at +10 mV) from hippocampal CA1 neurons to the perforated patch technique, the authors were able induce LTP in VTA DA neurons. This
was apparent as a ~30% increase in synaptic strength as measured by per cent EPSC amplitude over the pre-stimulation baseline. The effect was long-lived, appearing at 35 minutes after the induction procedure. Importantly, the effect was blocked by bath application of the NMDA receptor antagonist APV, suggesting it was indeed a form of classical LTP. Interestingly, the same protocol when applied to adjacent GABA cells was ineffective in eliciting LTP. Taken together, these data confirm earlier behavioral experiments that show NMDA receptor activation mediates sensitized responses to drugs of abuse, and further, that in addition to regulating acute burst-firing, NMDA receptors in VTA DA neurons are also responsible for classic forms of neuroplasticity like LTP.

In addition to its rather straightforward effects at AMPA and NMDA receptors, glutamate released from excitatory projections can also exert more complex actions via metabotropic glutamate receptors (mGluR) located both pre- and post-synaptically. Some postsynaptic Group I mGluRs appear to be excitatory, facilitating both spontaneous (Mercuri et al., 1993) and burst firing, although the latter effect was observed in vitro (Zheng and Johnson, 2003).

The mGluR$_1$ receptor, on the other hand, also appears able to exert paradoxical-seeming inhibitory effects (Fiorillo and Williams, 1998). These phenomena were first observed in intracellular recordings when the GABA$_B$ antagonist CGP 35348 failed to block the late component of a biphasic IPSP in VTA DA cells. The late component (~300 ms to peak) appeared to be apamin- and TTX-sensitive, suggesting both SK channel mediation and sodium-dependence. Its amplitude was, however, reduced by about $\frac{3}{4}$ with the mGluR antagonists (S)-MCPG, (S)-4-CPG, and ~40% with (RS)-AIDA, arguing strongly for the involvement of the mGluR$_1$ receptor. Intriguingly, the effects of this receptor on cell potential can be made to be bidirectional, depending on the kind of precipitating stimulus. For example, brief pressure injections of aspartate induced the mGluR-mediated IPSP, while longer applications induced the IPSP followed by a long-latency (800 ms) EPSP, which was also mGluR-dependent.

In line with the finding that glutamate can cause more than simple excitatory effects, Bellone and Lüscher (2005) have recently produced data showing that mGluR$_1$-mediated long-term depression (LTD) can be evoked in VTA DA neurons (Bellone and Luscher, 2005). Deploying a IPSP-induction procedure developed by Fiorillo and...
Williams (a train of two 5-pulse, 66 Hz stimulations), these investigators recorded a ~33% reduction in EPSP amplitude following the procedure. These reductions could be induced by the mGluR agonist DHPG alone and blocked by an mGluR$_1$ antagonist. The loss of rectification following mGluR-LTD suggested that the underlying mechanism may be an upregulation within AMPA receptors of the GluR2 subunit, thereby impairing calcium conductance and excitability (see Bredt and Nicoll, 2003).

**GABAergic Inputs**

While there is little question that GABAergic projection neurons from various forebrain areas (e.g. NAc, ventral pallidum) affect the activity of VTA DA neurons (Wu et al., 1996), many functional studies assume substantial contributions by local interneurons. The earliest extracellular recordings found iontophoretic application of GABA reduced firing rates in all DA neurons tested, an effect that was blocked by the GABA$_\Lambda$ antagonist picrotoxin (Yim and Mogenson, 1980). In the classic *in vitro* study by Johnson and North (1992), when it was found that both TTX and bicuculline reduced the frequency (but not amplitude) of spontaneously occurring IPSPs in DA cells, it was assumed that these potentials originated from GABAergic interneurons within the slice, and further, that DA cells may receive tonic inhibitory input from neighboring GABA cells. While induced IPSPs could be blocked by GABA$_B$ antagonists, no spontaneous potentials showed such regulation by GABA$_B$. This led the authors to posit that projections to DA neurons that signal through GABA$_B$ receptors originate somewhere outside the midbrain. This pattern of responsiveness, while not as prevalent in recorded GABA neurons, was still present. This raises the possibility of both direct inhibition of DA activity by projection neurons, or their disinhibition by GABA action at interneurons.

The idea that GABA$_B$ activation by forebrain afferents might differentially regulate DA activity has been explored more recently using single-cell RT-PCR and patch clamping (Cruz et al., 2004). These investigators showed that the strength of the association between the G-protein coupled inward rectifier (GIRK, Kir3) with GABA$_B$ differed between VTA DA cells and inhibitory interneurons, with the association being weaker in DA neurons. Because this kind of GIRK is a major source of hyperpolarizing
currents, the authors advance a model where low synaptic concentrations of GABA would be able to preferentially inhibit interneurons, because they would be better able to hyperpolarize, in light of their tight coupling to GIRKs. This would then disinhibit DA neurons and presumably increase DA release in terminal areas like NAc. On the other hand, higher synaptic GABA would be required to reduce DA activity by acting directly on weakly GIRK-coupled GABA_B receptors on DA neurons. Like glutamate’s biphasic effects described above, GABA can also exert effects that are complex, and more subtle than one might assume likely with these two ‘fast-acting’ amino acid (aa) transmitters.

**Autoregulatory Dopamine and Associated D_2 Receptor Pharmacology**

The release of DA within the VTA from dendrites appears to proceed in a manner very much like what occurs in dopaminergic varicosities or terminals in the forebrain. It is impulse- and calcium-dependent, in that it is blocked by TTX and exclusion of calcium from perfusate in microdialysis studies (reviewed in Adell and Artigas, 2004). This locally-released DA appears to act on G_i/o-coupled D_2 receptors expressed by DA neurons to effect hyperpolarizing potassium conductances (Grenhoff and Johnson, 1997). In this regard, D_2 functions as a classic somatodendritic autoreceptor. While there is some anatomical evidence for D_3 receptor expression in both DA and non-DA cells in the VTA (Diaz et al., 2000; Bentivoglio and Morelli, 2005), the extent to which these receptors have any functional/physiological significance is controversial at best (White, 1996; Grenhoff and Johnson, 1997). While D_1/5 receptors seem regulate afferent drive onto VTA DA neurons, particularly in the context of drug-induced neuroadaptations (Vezina, 1996; White and Kalivas, 1998), there is no evidence for direct, postsynaptic effects of these receptors either.

The putative autoregulatory role of D_2 receptors has been explored using several elegant pharmacological manipulations. The exposure of DA neurons to D_2 antagonists (e.g. sulpiride, haloperidol) can induce in these neurons rapid firing to the point of “depolarization block” where additional spikes cannot be generated until the cell becomes repolarized (see Grace et al., 1997, Figure 14D). Repolarization itself can also be achieved by pharmacological means, by applying GABA or the D_1/D_2 agonist
apomorphine. When given under basal conditions, both of these compounds decrease firing rates of DA cells, often rendering them silent. However, in preparations driven into depolarization block by D₂ antagonist pretreatment, inhibitory transmitters such as GABA can induce robust firing in DA cells as they repolarize sufficiently. Indeed, the usual test for depolarization block is the activation of DA cells by ligands that typically inhibit DA cell activity, like GABA or apomorphine.

**What Does In Vivo Electrophysiology Tell Us About Behavior?**

Ultimately, all of the preceding data seeks to comment in some way on how midbrain DA affects behavioral output, and investigators working at each level of analysis generally agree that midbrain DA has something to do with goal-directed, locomotor activation. The exact nature of this relationship is, of course, the subject of intense scrutiny by many laboratories, and open debate in the literature. In this final section certain of the proposed explanations will be presented.

**Studies in Awake, Freely Moving Animals**

Earlier, the relevance of different *in vivo* and *in vitro* preparations to unanaesthetized, ambulatory condition was discussed. As noted, investigators have been concerned from the start that artifacts from these unnatural preparations may obscure our view of native midbrain DA function. It is satisfying then that a number of studies, some of them quite recent, have set themselves the task of validating earlier data by making observations with chronically implanted wire electrodes in awake, freely moving animals.

One of these studies found that most of the classical features of midbrain DA activity hold true for recordings made in freely moving animals (Hyland et al., 2002). For example, they report firing rates ranging from 3.5-4.5 Hz with approximately 70% of cells showing bursting activity, consistent with the study by Gessa’s group (2004). Another paper similarly confirmed, in freely moving animals, the basic physiologic responses of the DA neurons, but added to this the view that there may be inhomogeneity in responses within the VTA (Kiyatkin and Rebec, 1998). Both of these works report
increased bursting (i.e. decreased inter- and intraburst intervals) when animals are given sucrose rewards.

While these and other studies (e.g. Kosobud et al., 1994) have evaluated DA responses to simple reward contingencies in ambulatory rats, the greatest contribution in this area has been made using non-human primates. The “error signal” hypothesis of DA function advanced by Wolfram Schultz is at once intuitive and amenable to various mathematical modeling techniques (see Schultz and Dickinson, 2000; Schultz, 2002, 2006).

Working in macaques and recording from identified DA units in SNpc and VTA, Schultz discovered that 50-70% of neurons would increase their rate at the moment a fruit juice reward was dispensed for correct responses in a simple visual discrimination task. Once the task is learned, however, DA responsiveness diminishes. Additionally, after the task is learned, when the correct response is made and a reward is expected but not given, there is a pause in DA neuron firing. From these data Schultz has suggested that DA is a kind of ‘teaching signal’ about reward contingencies where the intensity of the dopamine response is equal to the difference between the reward received and the reward expected based on prior experience (if any). Thus, when a reward arrives unexpectedly the DA response is maximal, and contrariwise, the omission of an expected reward results in a negative DA response.

As simple as it appears, it is to date the most coherent account of midbrain DA function. It is not, however, without its critics. Jon Horvitz (2000) has consistently argued that data depicting increases in DA efflux during aversive stimuli weakens Schultz’s case for increased DA activity as a “reward” with positive hedonic valence. This must be reconciled with recent experiments (cited above) that fail to find increased VTA DA activity with aversive stimuli (Ungless et al., 2004). Horvitz has also produced data showing that phasic firing increases occur in response to stimuli that are presumably hedonically neutral, but may be novel or salient (e.g. loud clicks, and bright flashes of light). However, in the case of such stimuli, reward-related responses and novelty or orienting responses need not be mutually exclusive. The VTA is intimately connected with its ‘upstairs neighbor’ in the optic tectum (Dommett et al., 2005), and it seems
feasible that DA-dependent orienting responses must be involved in the detection of rewards that precedes the energized phase of reward procurement.
4. WHAT IS THE ROLE OF MESOLIMBIC DOPAMINE IN MOTIVATED BEHAVIOR?

While the doctrinal debate over the role of NAc DA in behavior continues, the last twenty years of work in this area has produced two major theories. The first, best termed reinforcement hypotheses (Wise and Rompre, 1989; Wise, 2004), derive in part from the discarded notion that DA is responsible for subjective pleasure associated with natural rewards. Instead of implicating DA itself in hedonic states, current reinforcement models stress that DA is a requirement for the kind of associative learning that occurs as a previously neutral stimulus takes on rewarding properties. Reinforcement models rely on the retroactive nature of the DA signal in coordinating neural plasticity associated with the ‘burning-in’ of stimulus-response (S-R) associations, but that once established, DA is not a requirement for their behavioral expression. The second theory, falling under the heading of incentive-motivation (Robinson and Berridge, 1993; Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999), stresses the activational, or affectively ‘propulsive’ effects of the transmitter in reward procurement, and indeed, that reward procurement may be dependent on DA activation. These theories are not mutually exclusive, however, and reinforcement models acknowledge the “amplifying” role DA may play in active reward seeking (Wise, 2004). The purpose of this section is to briefly examine these models in the context of the behavioral experiments that gave rise to them, and fit them with the anatomical and functional data addressed above. In doing so, a sharper picture of the role of NAc DA in motivated behavior may be drawn.

Reward (Wise)

Reinforcement theories are based on the initial observation that animals treated with DA receptor antagonists often fail to learn instrumental tasks that are typically reinforced by food or other rewards (Wise et al., 1978; Gerber et al., 1981; Wise and Schwartz, 1981). Presumably, in the absence of the DA signal, these rewards lose their reinforcing value and the behavior is neither established nor maintained. In animals previously trained under drug-free conditions, administration of DA receptor antagonists produces within-
session decreases in responding, such that animals exhibit normal responding initially, but responding rapidly tapers and operant behavior is not maintained (Wise et al., 1978). This too is viewed as evidence in support of DA’s role in reinforcement, and that without it, normally reinforcing rewards become extinct.

These findings have met two criticisms. The first centers on Wise’s initial use of the term “anhedonia” to describe the effects of DA antagonists (Wise et al., 1978; Wise, 1982), which would appear to indicate that under normal circumstances, DA is responsible for pleasure or positive affects (see Salamone et al., 1997). Wise has since ceased to imply direct hedonic effects of DA (Wise, 2004), and has cited evidence from the human psychopharmacology literature showing neuroleptics do not produce anhedonia (Brauer and De Wit, 1997). Another early criticism was that DA antagonists produce performance deficits by impairing locomotor behavior, rather than by devaluing rewards or diminishing the motivation for them. This critique has been answered in a number of studies demonstrating that the low doses of DA antagonists commonly used affect reinforcement strength, but not behavioral responding or locomotor output (Franklin and McCoy, 1979; McFarland and Ettenberg, 1995; but see Salamone et al., 1997).

The experimental focus on the role of DA in the NAc emerged from studies using electrical intracranial self-stimulation (ICSS) of the medial forebrain bundle (MFB) in place of conventional rewards. It had long been known that neuroleptics blunt this form of ICSS (Olds, 1958), and since the MFB contains DA fibers from the mesolimbic and nigral systems, it was reasoned that the NAc could be one possible site of action. These older data took on new life with the work of Mogenson (Mogenson et al., 1980), and it was quickly shown that enhanced DA transmission in these areas was reinforcing (Hoebel et al., 1983). Wise also mapped the midbrain for sites that were maximally sensitive to the ICSS paradigm, and he found that the VTA, with its projections to NAc, but not other areas (e.g. locus coeruleus), possessed the lowest current threshold for ICSS (see Wise and Rompre, 1989). Since that time, a large number of microinjection studies have confirmed the sensitivity of the VTA-NAc pathway to dopaminergic manipulations, and shown that these manipulations may reinforce behavior. The overwhelming consensus remains that any drug that increases DA activity appears to be strongly
reinforcing (McBride et al., 1999; Wise, 2002; Ikemoto and Wise, 2004). This hypothesis immediately implied a neural substrate for drug addiction (Wise and Bozarth, 1987), and that notion has engendered a massive literature on the neurobiology of substance abuse, principally concerned with DA, the NAc, and the VTA (surveyed in Koob and Le Moal, 2006). While developments in in vivo neurochemistry enabled experimenters to show that drugs of abuse (Di Chiara and Imperato, 1988; Di Chiara, 1995) and ICSS enhance NAc DA transmission (Phillips et al., 1989; Phillips et al., 1992; You et al., 2001), it was also demonstrated that natural rewards like food (Hernandez and Hoebel, 1988a, b; Ahn and Phillips, 1999), and sex also had this effect (Pfaus et al., 1990b; Damsma et al., 1992; Wenkstern et al., 1993; but for review of DA in microdialysis see Joseph et al., 2003).

Even with its relatively long sampling time (minutes vs. milliseconds with electrophysiology), microdialysis studies of DA in the NAc made it clear that enhanced DA release often occurred prior to the consumption of rewards, and indeed could occur with conditioned cues signaling their impending availability. This observation prompted a number of researchers, especially those with a background in microdialysis, to explore the activational effects of DA on behavior, and led to the articulation of incentive motivation theories (Robinson et al., 1988; Robinson and Berridge, 1993).

**Incentive-Motivation (Robinson and Berridge)**

The first tenet of incentive motivation theory is that DA possesses behaviorally activating effects that operate within both perceptual and motivational domains. Incentive-motivation theorists have argued that increases in DA seen prior to the consumption of rewards (e.g. Schultz and Dickinson, 2000; Schultz, 2002) indicate that the transmitter has a role not only in activating goal-seeking behavior, but also vectoring animals towards goals by affecting the incentive or perceptual properties of rewards in the environment (Berridge and Robinson, 1998). This process is termed “incentive-salience attribution,” and DA is believed to be a key mediator in enhancing the salience, or desirability of previously neutral stimuli (readers interested in computational modeling of the theory are directed to McClure et al., 2003; Montague et al., 2004).
Robinson’s view is derived in part from the literature on behavioral sensitization, where a progressive increase in drug seeking and NAc DA release is observed following repeated administration of DA agonists (see Kalivas and Stewart, 1991; Lorrain et al., 2000; Vanderschuren and Kalivas, 2000). Such data are taken as evidence of the enhanced salience of drug-associated cues, though not necessarily their hedonic impact. Presumably, if increased DA itself were rewarding, or had a subjective hedonic charge, it should be highest during the actual consumption of rewards (not their predictors), and hedonic responses would remain relatively stable. This is often not the case, as when human drug users report decrements in the pleasurable effects of drugs over time, and when that drugs that are not frankly euphorogenic (e.g. nicotine), but do enhance DA transmission, are potently addictive (see Robinson and Berdige, 1993). Human studies have also shown that drugs like morphine, which have potent euphoric effects, may enhance responding in a fixed ratio schedule, even when users are being given doses that cause no subjective effects at all (Lamb et al., 1991). These data underscore the notion that drugs that increase DA transmission may affect incentive properties of environmental stimuli (i.e. the active, morphine-lever) via processing that occurs outside of conscious awareness and with little effect on subjective pleasure (Robinson and Berridge, 2003).

Such observations lead to the second tenet of incentive-motivation theories: that “wanting” and “liking” are separate and dissociable neural processes, with DA being critical to the former and not the latter. This dichotomy may be viewed as the descendant of ideas about the appetitive versus consummatory phases of adaptive behavior that date at least to Sherrington (Sherrington, 1906). Berridge also turns to the historical literature for tools that may dissociate “wanting” and “liking.” Using an ethologically grounded taste reactivity paradigm that owes a debt to Darwin’s The Expression of the Emotions in Man and Animals (Darwin, 1872/1998), Berridge has argued that he is able to tap the distinct processes of “wanting” and “liking.”

In a typical experiment (Wyvell and Berridge, 2000), animals were trained to lever press for a sucrose reward associated with a light cue (CS). Subsequent tests in which the CS was presented without the sucrose reward it predicted showed that intra-NAc amphetamine increased responding on the active lever. This is taken as an indication
of the enhanced salience of the CS. In a parallel experiment, intra-NAc DA was found to actually decrease hedonic facial responses and increase aversive ones. Together, these and other studies have allowed Robinson and Berridge to argue that elevations of DA seen to accompany the presentation of primary or secondary reinforcers mediate “wanting” of those reinforcers, and that there is little evidence for DA as a hedonic reinforcer.

**Cellular Substrates of Dopamine Action in Adaptive Behavior**

Both incentive-motivation and reinforcement theories of NAc DA are systems-level models concerned with the action of a single transmitter in a single brain structure. The network for expressing different modes of adaptive behavior is undoubtedly more complex (Everitt and Wolf, 2002; Kalivas and Volkow, 2005; Kelley et al., 2005), but there is notable support for both theories at the cellular and molecular level.

As described in a preceding section, activation of D1 receptors in MSNs of the NAc brings those neurons closer to their “up state,” (-60 mV) while activation of D2 receptors has opposite, hyperpolarizing effects (O'Donnell et al., 1999). Schultz and others have argued that this arrangement may allow DA to enhance the signal to noise ratio when multiple cortical and allocortical inputs bearing information about environmental stimuli converge on MSNs (Schultz, 2002). Under normal circumstances, tonic DA maintains the “down state” (-80 mV) in MSNs via D2 activation. The presence of rewarding stimuli would induce burst firing in DA neurons, phasically increasing local DA concentrations enough to activate D1 receptors in their low affinity state (Richfield et al., 1989; Schultz, 2002). These incrementally depolarized MSNs would then be ready to fire, given the proper – reward relevant – combination of cortical and allocortical inputs (Goto and O'Donnell, 2002; Casassus et al., 2005). This work has been extended recently by \textit{in vitro} experiments that show the presence of DA in NAc slice preparations to preferentially inhibit stimulation-evoked IPSCs and that this effect is frequency dependent (Hjelmstad, 2004). In these studies, inhibition of GABAergic IPSCs far outlasts a similar effect on glutamatergic EPSCs, and the effect occurs at higher stimulation frequencies. This not only suggests a net excitatory effect, but another means
by which DA may enhance the signal to noise ratio of convergent, perhaps reward relevant (e.g. high frequency), input from cortical structures (Casassus et al., 2005).

This type of excitatory cortical input would be summed with the effects of phasic DA in “up state” MSNs, driving them to the threshold for action potential. Together, these data provide a model whereby the hodologic and electrical properties of the NAc MSNs function as a band-pass filter, allowing only certain types of information to induce MSN firing, and thus ultimately disinhibiting thalamocortical afferents to areas involved in movement and motor planning. This is consistent with some of the models discussed above on the role of the basal ganglia in motoric control (Redgrave et al., 1999). The NAc, however, receiving heavy input from structures involved in reward valuation and mnemonic processes (e.g. basolateral amygdala, hippocampus, see Pennartz et al., 1994; Cardinal et al., 2002; Phillips et al., 2003), may be viewed as a part of the striatum that co-evolved with those allocortical organs to translate their affectively-tinged output into goal-directed behavior. This is in essence Mogenson’s original formulation (Mogenson et al., 1980), but it is compatible with more recent incentive-motivation models that stress the acute actions of NAc DA in priming or driving goal-directed behavior. “Incentive-salience attribution” may simply be the behaviorist’s term for the process electrophysiologists observe when DA appears to enhance the signal to noise ratio amongst convergent inputs to the NAc.

While incentive-motivation models are supported by cellular analyses of the immediate consequences of DA release in NAc, reinforcement models have benefited from demonstrations of DA-dependent neuroplasticity in MSNs. Borrowing heavily from the literature on hippocampal LTP and learning (see Malenka and Bear, 2004), it has become clear that similar processes may be at play in motivation and reinforcement. In the NAc, learning about rewarding cues or “burning in” of S-R relationships is thought to be a phenomenon of heterosynaptic plasticity that is particularly dependant on the D_1 receptor activation that occurs with phasic DA release (discussed above). Most important to this phenomenon is the synaptic arrangement of excitatory glutamatergic and modulatory dopaminergic inputs on the spines of MSNs. This “triadic junction” allows for DA to effect plasticity at the level of single synapses that presumably encode reward-relevant information from cortical or allocortical structures. Associative learning about
reward contingencies may occur with concomitant activation of NMDA and D\textsubscript{1} receptors (see Wickens and Kotter, 1995; Berke and Hyman, 2000; Hyman and Malenka, 2001; Nestler, 2001; Jay, 2003; Kelley, 2004). When activated during reward learning, the two receptors may act in a synergistic fashion to enhance synaptic efficacy via their respective intracellular signaling cascades (Dong et al., 2004; Mangiavacchi and Wolf, 2004; Boudreau and Wolf, 2005; Dong et al., 2006). An in-depth treatment of these intracellular signaling pathways is beyond the scope of this discussion, but at this time, ample evidence exists to suggest that DA-glutamate interactions in the NAc engender profound changes in gene expression that are apparent not just at the molecular level, but at the level of cell ultrastructure (e.g. Robinson and Kolb, 2004), and behavior (e.g. Carlezon et al., 1998). Together, this catalog of neuroadaptations is thought to underlie the reinforcing effects of DA in behavior.
5. THE LATERAL HYPOTHALAMIC AREA AND MOTIVATED BEHAVIOR

As the rather general term “area” would indicate, the lateral hypothalamic area (LHA) has always been something of a terra incognita, both anatomically and functionally. It has few tightly grouped nuclei, its boundaries are ill-defined, and these features along with the presence there of multiple fiber bundles thwart discrete electrical stimulation or injection of anatomical tracers. Even still, such technical demands haven’t prevented a large number of investigations into this piece of diencephalic tissue.

Prior to the NAc’s ascendance as the “final common output path” for motivated behavior, the LHA enjoyed this title. While numerous early investigations reported the LHA to regulate a number of vegetative functions (reviewed in Clark et al., 1938; Morgane, 1979), it was the work of Hess that pushed the LHA into the fore as a possible substrate for volitional motoric output (Hess, 1954). Electrically stimulating near the fornix of awake, freely moving cats, Hess cataloged a remarkable diversity of behaviors, from sleep and atonia, to locomotion and defensive (aggressive) responses. Around the same time, other investigators found that lesions of LHA produced profound aphagia and adipsia (reviewed in Teitelbaum and Epstein, 1962), while more medially located damage had the opposite effects (Anand and Brobeck, 1951). Other investigators found LHA electrical stimulation to evoke other motivated behaviors, like copulation (Vaughan and Fisher, 1962; Caggiula and Hoebel, 1966), and this work was extended by Elliot Valenstein and Bart Hoebel throughout the 1960s and 70s (Valenstein et al., 1970; Valenstein, 1973; Hoebel, 1976).

While the “dual-center” theory of feeding and other hypothalamic “centers” for motivated behavior fell out of favor during the 1970s and 80s (see Stricker and Zigmond, 1976; Stricker et al., 1978), it has been rehabilitated in modified form with the discovery of numerous orexigenic and anorectic peptides there (Sawchenko, 1998; Kalra et al., 1999; van den Pol, 2003; Zigman and Elmquist, 2003; Leibowitz and Wortley, 2004). As the study of adaptive behavior enters the 21st Century, the role of the LHA in motivation is undergoing a reappraisal, particularly in light of its anatomical connections to fore- and midbrain structures (e.g. NAc, VTA) that have dominated the field for the past 30 years. Below is given a brief review of functional and anatomical data on the LHA, with special
attention to its interaction with other nodes in the “motive circuit.” Thereafter, the hypocretin (orexin) peptides will be introduced and offered as a neurochemical link between homeostatically regulated hypothalamic circuitries and those structures of the mid- and forebrain implicated in motivation and behavioral output.

**Gross Anatomy and Cytology**

The boundaries of the LHA have always been somewhat arbitrary, with new parcellation schemes arising periodically (Swanson, 2004; Swanson et al., 2005). Work by Maxwell Cowan’s group, however, provides a well accepted account based on patterns of efferent connections from three major subregions of the LHA (Figure 16, Saper et al., 1979). The anterior portion of the LHA (aLHA) begins just caudal to the lateral preoptic area (IPOA) and runs ventromedial to the substantia innominata and internal capsule to the level of the ventromedial nucleus (VMH). The portion of the LHA

![Figure 16. Lateral hypothalamic area and environs.](image)

Rendered in a horizontal schematic. Abbreviations for major hypothalamic, perihypothalamic, and mesencephalic landmarks: AHN, anterior hypothalamic nucleus; ADP, anterodorsal preoptic nucleus; AVP, anteroventral preoptic nucleus; AVPV, anteroventral periventricular nucleus; DMH, dorsomedial hypothalamic nucleus; EW, Edinger-Westphal nucleus; ICN, interstitial nucleus of Cajal; IPN, interpeduncular nucleus; LPO, lateral preoptic area; MEPO, median preoptic nucleus; MM, medial mammillary nucleus; MPN, medial preoptic nucleus; MPN, midbrain reticular nucleus; ND, nucleus of Darkschewitsch; PAG, periaqueductal gray; PD, posterodorsal preoptic nucleus; PVH, paraventricular hypothalamic nucleus; PVpo, preoptic periventricular nucleus; PVR, periventricular region; RCH, retrochiasmatic area of lateral hypothalamus; RR, midbrain reticular nucleus (retrorubral area); RT, reticular nucleus of thalamus; SBPV, subparaventricular zone; SCH, suprachiasmatic nucleus; SFO, subfornical organ; SO, supraoptic nucleus; STN, subthalamic nucleus; SUM, supramammillary nucleus; VMH, ventromedial hypothalamic nucleus; VTA, ventral tegmental area. Adapted from Swanson (2004).
that is then coextensive with the VMH is commonly called the “tuberal LHA, (tLHA)”,
and this designation gives way to the “posterior” division (pLHA) with the appearance of
the premammillary nuclei. The caudal termination of the pLHA is then near the rostral
portion of the lateral supramammillary nucleus and the beginnings of the VTA. This
close anatomical association with the VTA is, of course, notable where motivated
behavior is concerned, and the LHA was actually once considered as a rostral extension
of this structure (see Morgane, 1979). Along the medial-lateral dimension, the LHA has
been considered to be that hypothalamic tissue lateral to the fornices, though this
designation is, again, somewhat arbitrary and has been the subject of criticism (Bernardis
and Bellinger, 1993).

The LHA is estimated to contain 250,000 neurons (both hemispheres in 200 g
rat), and these cells, along with glia and processes, account for almost 45% of the total
volume of the hypothalamus itself (Palkovits and Van Cuc, 1980). The neurons there are the largest
in the hypothalamus (Palkovits and Van Cuc, 1980), and their dendritic processes can easily
stretch a millimeter or more from the fornix to the
pial surface of the brain (Figure 17, Millhouse,
1969). Most of these neurons are fusiform
(McMullen and Almli, 1981), and their moderately spiny dendrites receive an estimated
16,000 synapses per neuron (Palkovits and Van Cuc, 1980). Compared to the striatal
MSNs addressed in a previous section (~11,000 synapses/neuron Wilson et al., 1983;
Wilson, 2004), it is clear that neurons of the LHA are the site of substantial convergence,
and may fulfill a similar integrative function.

**Hodology and Integrative Function**

The LHA was long viewed as the “bed nucleus” of the MFB (Morgane, 1979;
Bernardis and Bellinger, 1993), and the difficulty of anatomically parsing connections of
loosely packed cells that interdigitate and contribute their own processes to the complex
MFB has been commented on by no less an anatomist than Larry Swanson (Swanson, 1987):

“No aspect of hypothalamic circuitry is less clear at the present time than the origin and topographic distribution of neural inputs to neurons in the lateral zone. The major problem is that the medial forebrain bundle passes through this region and contains fibers that establish bidirectional connections between the telencephalon, the medial zone of the hypothalamus, and the brainstem. In fact, it appears safe to conclude on the basis of current anatomical evidence that no part of the central nervous system gives rise to fibers that end in the lateral zone without also passing to more medial parts of the hypothalamus, or to the telencephalon or brainstem.”

Indeed, until fiber-sparing lesions were deployed in the 1970s, it was presumed that many of the behavioral effects of LHA electrical stimulation, or conversely, lesions of this area, were artifacts originating from fibers of the MFB. This is now known not to be the case (Stricker et al., 1978). Shortly after Swanson’s review, the first modern papers were issued in which as many as a dozen discrete ‘sets’ or cellular groups across the extent of the LHA are cytoarchitectonically distinguished (Geeraedts et al., 1990a; Geeraedts et al., 1990b). The efferents of these neurons have been studied, and there is a marked heterogeneity in projection patterns based on the rostrocaudal domain into which anterograde tracer is applied. Most fibers from all regions join the MFB, traveling in both directions, with aLHA projecting rostrally to the lPOA, medial septum and diagonal band, as well as the thalamic paraventricular nucleus and lateral habenula (Saper et al., 1979). These authors report that the anterior region also projects caudally to most of the medial hypothalamic nuclei (e.g. anterior VMH), to the medial and central amygdala via the stria terminalis, and then as caudal as the supramamillary region and the surrounding VTA. Certain of these projection patterns are seen with the tLHA, but this portion of the LHA appears to send fewer telencephalic projections and to do so in a more restricted fashion (e.g. only to the lPOA), and to preferentially innervate the midline hypothalamic nuclei (e.g. DMH, VMH) and thalamus, and finally, to send most of its efferents caudally to supramamillary nuclei and VTA, as well as the central gray. The most caudal extreme of LHA (pLHA), not surprisingly, continues the pattern of caudal projections not only to the mammillary complex, the VTA and the red nucleus dorsal to it, but also the
compact part of the substantia nigra, the dorsal raphe, locus coeruleus, and as far as the parabrachial nucleus. These findings have been replicated and expanded by other investigators, and it has become clear that the LHA projects as far rostral as the neo- and allocortices, and as far caudal as the spinal cord (Berk and Finkelstein, 1982; Swanson, 1987; Veening et al., 1987).

Of particular interest, however, is the rich innervation the LHA seems to provide to midbrain and brainstem aminergic nuclei. This has prompted many investigators to view the LHA and certain of its inhibitory sleep-active inputs (e.g. ventrolateral POA, Alam and Mallick, 1990; Steininger et al., 2001) as a complex for regulating monoaminergic transmission in the context of arousal and “behavioral state” (Saper et al., 2001; Saper et al., 2005a). It has long been known that the LHA contained wake-active neurons (Ono et al., 1986; Steininger et al., 1999; Alam et al., 2002), and these are now believed to regulate arousal by their descending efferents to wake-active aminergic nuclei like the raphe and locus coeruleus (Aston-Jones et al., 1991; Jacobs and Fornal, 1997; Berridge and Waterhouse, 2003). Perhaps more interestingly, it is believed that neurons of the LHA perform this arousal-related function after integrating information from the midline hypothalamic nuclei. These nuclei, particularly the suprachiasmatic nucleus with its relay in the DMH (Saper et al., 2005b), and the arcuate situated on the third ventricle and expressing receptors for adiposity signals like leptin (Schwartz et al., 2000), inform the LHA arousal-behavioral activation system with circadian cues and information regarding energy balance from the internal milieu (Saper et al., 2002). Thus, arousal and behavioral activation effected by the LHA is coordinated with appropriate environmental and homeostatic stimuli. The recognition of the LHA’s integrative function in this regard has largely been the product of new parcellation schemes based on various neurochemical markers (Abrahamson and Moore, 2001), many of them intimately involved in energy homeostasis and sleep (Sawchenko, 1998).

What is the Lateral Hypothalamic Area?

While the striatum and NAc receive highly processed sensory input and possess neurons that are responsive to rewarding stimuli, there are no reports of neurons there
that show acute electrical responses to glucose or gonadal steroids (Orsini, 1981; Orsini, 1982; Oomura, 1988), that are wake active (Steininger et al., 1999), or inhibited as an animal reaches satiety (Ono et al., 1986; see Rolls, 1999; Rolls, 2005). All of these, in addition to reward-responsive neurons, are present in the LHA, and this is by way of noting the structure’s intimate connections (via periventricular/midline nuclei) with the internal, humoral milieu. The circuitry of the basal ganglia may be important for finding behavioral solutions to potentially reinforcing contingencies, but it has no innate means of deducing when pursuit of rewards like food or sex are appropriate given certain neurohumoral cues and homeostatic parameters.

The hypothalamus may be viewed as a homunculus, with the LHA as the most rudimentary mechanism by which goals (e.g. food, mates) are detected and goal-directed motor programs are enacted – an “extra-extrapyramidal” system as Hess might have had it (Hess, 1954). Indeed, the hypothalamus is the most caudal level of the neuraxis, which, when left intact during decerebrate procedures, allows for spontaneous behavioral expression (Grill and Norgren, 1978a, b). Clearly, the cortices and basal ganglia vastly expand the flexibility and plasticity of adaptive behavior, but the hypothalamus, and LHA in particular, may be conceptualized as the original, ‘primitive,’ substrates of adaptive behavior that were relieved of this duty over evolution in classical Jacksonian fashion (see Swanson, 2000). One link between this primitive motivation system and the products of cephalization in the forebrain may be the hypocretin (orexin, hcr/orx) neurons. The reasons for their importance in this role is treated below.
6. THE HYPOCRETIN (OREXIN) PEPTIDES

Though they constitute only ~1% of the neurons of the LHA (Kilduff and Peyron, 2000), the hcrt/orx cells have emerged recently as an important mode of signaling in the hypothalamus. These neurons are now thought to be involved in the expression of almost all of the effects historically associated with the LHA and addressed above only in brief. It would not be too grandiose to refer to the identification of the hcrt/orx peptides as a revolution where modern molecular biologic techniques have named genes and gene products as the neurochemical phlogistion that had previously seemed to power the remarkable array of behaviors that emerged from the LHA: Autonomic control, feeding and energy balance, arousal, and goal-directed behavior have now all been examined as likely products of hypocretinergic output from the hypothalamus. No fewer than three articles about hcrt/orx have appeared in Annual Reviews since their discovery eight years ago (Willie et al., 2001; Taheri et al., 2002; Siegel, 2004). This trend is not limited to publications with double-digit impact factors, and at this writing, 202 of the 1224 (~17%) published reports listed in PubMed under the terms “hypocretin or orexin” are reviews. Clearly, scientific anticipation over the hcrt/orx system is high. The following section will explain the history, neuropharmacology, anatomy, and behavioral neuroscience of these peptides, and attempt to justify the enthusiasm now felt for them in various fields of neuroscientific study.

Discovery and Nomenclature of Hypocretin (Orexin)

The discovery of the hcrt/orx peptides was an episode of scientific coincidence where two research groups, working independently on opposite sides of the globe converged on the same peptides at the same time. In January of 1998 Luis De Lecea and Greg Sutcliffe’s group published their discovery of a 130 aa gene product expressed solely in neurons of the hypothalamus which they named “hypocretin” (de Lecea et al., 1998). Within a month, Takeshi Sakurai and Masashi Yanagisawa’s group published evidence for the existence of a gene product of identical weight, sequence, and pattern of anatomical expression, which they termed “orexin” (Sakurai et al., 1998). The peptides
were immediately recognized as identical. As evidenced by the “hcr/orx” construction used throughout this dissertation and most of the professional literature on the subject, neither group has conceded to a uniform nomenclature. The reasons for this will be addressed below.

It is scientifically pleasing that both groups arrived at their discoveries by different means. De Lecea’s group used a molecular genetic strategy of subtractive cloning that they had perfected in the early 1990s (Usui et al., 1994). More formally called “directional tag PCR subtractive hybridization,” the method follows older subtractive hybridization techniques where mRNA species selectively expressed in one tissue or brain area are isolated by several rounds of hybridization, or “subtraction,” between two cDNA libraries from different tissues. Message that is common to both tissues will hybridize, leaving unique, single-stranded species to be separated and cloned. These clones can then be used to probe libraries made from target tissue, eventually allowing identification and sequencing of genes expressed only in that tissue. Sutcliffe’s method was, of course, able to capitalize on the amplification power of PCR, but by including PCR tag sequences in a directional cDNA library, and by using mRNAs themselves (instead of cDNAs) for the subtraction, this greatly enhanced the specificity, simplicity, and ease of separating target sequences after subtraction and amplification.

Using their modified subtractive cloning procedure to search for hypothalamic feeding peptides, Sutcliffe and his colleagues identified 43 mRNAs that were selectively expressed in hypothalamus following two rounds of subtraction using hippocampal and cerebellar cDNA libraries (brain structures thought to have little relevance to feeding or energy balance, Gautvik et al., 1996). One of these was hcr/orx, though not yet by that name. In that 1996 paper, hcr/orx appeared in southern blots and in in situ hybridization data as “clone 35.” The work of characterizing “clone 35” would take another year, but publishing priority for the discovery of hypocretin must go to Gautvik, DeLecea, Kilduff, and Sutcliffe. In 1998, with a more complete picture of hcr/orx’s DNA sequence (GenBank: AF019565 and AF019566), anatomical expression, and electrophysiological effects on in vitro slice preparations, De Lecea et al. published their discovery of hypocretin (de Lecea et al., 1998). Eschewing a nomenclature based on presumed functional effects that are so commonly found to become obsolete (cf. oxytocin,
vasopressin, cholecytokinin - CCK), De Lecea’s group named their peptides for their pattern of anatomical expression in the hypothalamus, and their sequence homology to secretin and other members of the incretin gene family. Thus, with the discovery of a “hypothalamic secretin” homolog, the usual sequence of gut-brain peptide discovery is inverted: Unlike CCK, NPY, galanin, etc. which were discovered first in the periphery (see Moran and Ladenheim, 1998; Strand, 1999), then found to be expressed in CNS, hcrt/orx was found in brain, and only recently reported in the enteric nervous system (see Kirchgessner, 2002). It is in part De Lecea’s classification of hcrt/orx as an incretin homolog that precipitated the ongoing debate over hcrt/orx nomenclature. Citing sequence homology between the receptors for hcrt/orx and NPY (26% homology with Y2R), Sakurai has disputed claims that hcrt/orx’s are, in fact, relatives of the incretins (Mieda and Sakurai, 2006).

Sakurai and Yanagisawa’s group arrived at the discovery of orexin through a brawnier high throughput, reverse-pharmacology strategy in which they created 50 lines of HEK293 cells stably transfected with a single “orphan” G-protein coupled receptor (GPCR). Also hunting for hypothalamic feeding peptides, Yanagisawa’s group extracted and fractionated rat hypothalamic tissue by HPLC, then treated each transfected cell line with a different fraction, measuring increases in intracellular Ca++ by the Fura-2 AM fluorophore (Sakurai et al., 1998). Extracts that produced intracellular Ca++ transients were then subjected to several further rounds of reverse-phase HPLC, and peptide content of extracts was identified by mass spectrometry and Edman degradation sequencing. These procedures identified two purified peptide products, orexin A, and orexin B, of distinct mass, and able to markedly induce Ca++ transients, presumably via the transfected orphan GPCR. During protein chemistry experiments, it also became clear that these peptides were derived from a single gene product of higher molecular weight – the preprohormone prepro-orexin. The cDNA coding for prepro-orexin was then quickly found with primers based on deduced sequences of the prohormone’s cleavage products.

With the ligands for these orphan receptors identified, it was then simply a matter of naming them and reporting their sequences. Yanagisawa reported the discovery of two GPCRs that bound orexin A and orexin B, and named these receptors OX1 and OX2, though the genes themselves are listed in GenBank under the hypocretin appellation.
Aside from basic neuropharmacology (i.e. probable signaling via a $G_q$-coupled receptor), and neuroanatomy (localized expression of hcrt/orx mRNAs to the perifornical hypothalamus), Yanagisawa et al., also performed the first behavioral studies with the peptides and reported the findings in their initial paper of 1998. It was observed that both orexin A and orexin B, given intracerebroventricularly to rats, would dose-dependently increase food consumption in a free-feeding paradigm. This prompted the naming of their discovery “orexin,” after the Greek $ορεξίς$, or “appetite.” This immediately sparked contention with the discoverers of hypocretin, who have forcefully argued that the hcrt/orx peptides are not orexigens proper, and have a varied array of non-feeding related behavioral effects (see below, “Role of Hypocretin/Orexin in Arousal”).

Ironically, “orexin” may turn out to be a satisfactory descriptor as more is learned about the peptide’s role in motivated behavior (Harris and Aston-Jones, 2006). This view comes from a more literal translation from the Greek. As when paired with a privative (i.e. anorexic), the association of the Greek root in clinical medicine and pharmacology has always been with feeding and food intake. However, a more faithful transliteration of $ορεξίς$ is simply to mean “desire,” or “longing,” broadly - not necessarily for food. There are dozens of more specific expressions for the subjective state of hunger, but the most familiar cognate to English speakers must be $βοϊλεμα$, from which the modern psychiatric diagnosis of bulimia nervosa is derived. As evidence accumulates to support a broader role for hcrt/orx in goal-directed seeking behavior of all kinds (Mileykovskiy et al., 2005), “orexin” may gain new currency as a specialist term if students of the peptide consult their Greek lexica.

**Biochemistry, Molecular Biology, and Neuropharmacology of Hypocretin (Orexin)**

As indicated above, hypocretin-1/orexin A and hypocretin-2/orexin B are cleaved from the 130 aa prepro-orexin, most likely by prohormone convertase 1 (Nilaweera et al., 2003). The human $Pporx$ gene (GenBank: AF118885) contains a 1432 bp structural gene consisting of two exons (143 bp and 473 bp) that correspond to the neuroactive hcrt-1/orx A and hcrt-2/orx B products. These coding regions are separated by an 816 bp intron, and the structural gene itself is within a 3149 bp 5’-flanking region containing several likely
initiation (TATA) sites between bases 5-10 and 33-35, and a predicted promoter region at position -291 (Sakurai et al., 1999). The structural gene and its open reading frame are followed by a 364 bp 3'-flanking region. Because the hcrt/orx peptides show such a spatially constrained pattern of anatomical expression, separate studies in mice have sought to further characterize the regulatory elements in the 5'-flanking region that are responsible (Moriguchi et al., 2002). These studies have revealed the presence of two Orexin regulatory Elements (OE1, OE2) in this region. A core, conserved region of OE1 (-258 to -201 bp) is required for normal expression of Pporx in LHA. Deletion of this sequence is found to cause ectopic expression of reporter genes (LacZ) driven by the Pporx promoter. Expression is found to shift from the LHA medially to the DMH, and particularly the arcuate nucleus. While OE1 appears to confer spatial specificity of Pporx expression, it appears to work cooperatively with OE2, so that deletions of both abolish all hypothalamic Pporx promoter-driven expression.

The evolutionary origins of the Pporx/Hcrt gene have been examined, and hcrt/orx signaling appears to have arisen amongst early vertebrates, as a Pporx/Hcrt gene is not found in the urochordate Ciona intestinalis (the common sea squirt, Alvarez and Sutcliffe, 2002). This finding, together with the sequence homology observed between divergent phyla (60% from pufferfish Takifugu rubripes to frog Xenopus laevis, and 58% from frog to human) suggests the Pporx/Hcrt gene arose near the time that urochordates and vertebrates diverged 650 million years ago. These authors suggest that the Pporx/Hcrt gene arose as a circular permutation of a parent gene in the incretin family. By this scheme, a tandem duplication of the parent gene was followed by a fusion and subsequent mutations which left roughly half of the secretin 2A (Scrt2-A) gene coding for its C-terminus, fused to a quarter of the N-terminal coding region of Scrt2-B. Thus, hcrt/orx is, in part, a “reverse”-secretin homolog. Evidence for this head-to-tail arrangement comes from the N-terminus amino acid sequences of secretin that are found in the C-terminus of hcrt/orx.

The 569 bp (rat) cDNA sequence originally reported for Pporx/Hcrt predicts the 130 aa product noted above (de Lecea et al., 1998; Sakurai et al., 1998). The prepro-hormone is believed to be processed in the following manner: After removal of the secretory signal at the N-terminus, the peptide is cleaved at two sites containing pairs of
basic amino acids. This gives the 3.5 kDa, 33 aa (residues 28-66) hcrt-1/orx A, and the 2.9 kDa, 28 aa (residues 69-97) hcrt-2/orx B (Sutcliffe and de Lecea, 2002; Sakurai, 2006). While the former is linear, the latter takes on tertiary structure as two intrachain disulfide bonds are formed between the four cysteines of hcrt-1/orx A (Cys⁶-Cys¹², Cys⁷-Cys¹⁴). Additionally, hcrt-1/orx A has secondary structure in the form of two helical regions from Asp⁵ to Gly⁹ and Leu¹⁶ to Gly²² (Miskolzie and Kotovych, 2003). The disulfide bonds hold these helical regions in a bent conformation, with the bend coming at Lys¹⁰ to Ser¹³. Sequence homology between the two cleavage products is about 46% across species, and the conserved N-terminal sequence GNHAAGILT is believed to confer receptor binding ability, though deletion of C-terminal residues can also potently disrupt receptor affinity (Lang et al., 2004; Lang et al., 2006).

The differences between hcrt-1/orx A and hcrt-2/orx B in sequence identity and structural features confers receptor specificity. Hcrt-1/orx A binds with low nM affinity to the OX₁ receptor, while hcrt-2/orx B binds to this receptor with affinity that is several orders of magnitude lower (Sakurai et al., 1998). The OX₂ receptor, however, binds both peptides with similar affinity (IC₅₀ of 20 nM in CHO cells), suggesting it is the nonselective member of this pair of receptors (for review see Smart and Jerman, 2002).

The distribution of OX receptors in rat brain has been mapped by in situ hybridization (Hervieu et al., 2001; Marcus et al., 2001; Cluderay et al., 2002), and while there is often overlap in the expression patterns of the proteins, there are some notable differences (for review see Marcus and Elmquist, 2006). Most notable is the presence of OX₂ mRNA in parvocellular paraventricular nucleus (PVN) of hypothalamus, medial preoptic area (mPOA), and tuberomammillary nuclei (TMN), where OX₁ signal is totally absent or substantially weaker.

It has been known since the first published reports of OX receptor pharmacology that the receptors are able to increase intracellular Ca²⁺ concentrations and are neuroexcitatory (Sakurai et al., 1998). Subsequent work in CHO cells showing phosphatidylinositol hydrolysis that is thapsigargan-sensitive suggests a conventional Gq-PLC mediated mechanism for this effect (Smart et al., 1999). However, in vitro slice experiments have shown that a good portion of the increased Ca²⁺ in hcrt/orx-stimulated neurons is actually extracellular in origin (Lund et al., 2000), and that substitution of
EGTA or cadmium for Ca\textsuperscript{++} in buffer solutions abolishes the effect (van den Pol et al., 1998). These authors also demonstrated that inhibitors of protein kinase C (PKC) block hcrt/orx-mediated Ca\textsuperscript{++} transients, suggesting OX receptors may influence Ca\textsuperscript{++} conductances by enzymatic phosphorylation of ion channels. Several different research groups have found that in other midbrain and brainstem structures, including the VTA, depletion of intracellular Ca\textsuperscript{++} stores has no effect on hcrt/orx-mediated Ca\textsuperscript{++} increases, but that blockade of L-type Ca\textsuperscript{++} channels with nifedipine is effective (Uramura et al., 2001; Kohlmeier et al., 2004). Together, it seems reasonable to conclude that OX receptor activation increases intracellular Ca\textsuperscript{++} by enhancing currents through L-type channels after activation of PKC.

While most research on the neuroexcitatory character of hcrt/orx and OX receptors has focused on the Ca\textsuperscript{++} signaling cascades addressed above, there is some evidence from CHO cells to suggest activation by hcrt-1/orx A of adenylyl cyclase, perhaps via a novel isoform of PKC – PKC\textgreek{d} (Holmqvist et al., 2005).

Though the consensus is that the hcrt/orx peptides are excitatory, recent evidence suggests that hcrt/orx signaling may exert more subtle, inhibitory effects in some systems. In serotonergic neurons of the dorsal raphe, hcrt-2/orx B can actually decrease glutamatergic EPSCs (Haj-Dahmane and Shen, 2005). Intriguingly, reductions in glutamatergic drive by hcrt-2/orx B appear to be due to release of endocannabinoids following activation of postsynaptic OX\textsubscript{2} receptors. While the signaling pathway from OX\textsubscript{2} to \textit{de novo} endocannabinoid synthesis and release is unclear, it probably centers on PLC, which has been offered as a member of the 2-arachadonoyl glycerol synthetic pathway (see Freund et al., 2003). Endocannabinoids released following OX receptor activation bind to presynaptic CB\textsubscript{1} receptors in glutamatergic terminals to dampen transmitter release in a manner consistent with endocannabinoid effects described in other brain areas (Wilson and Nicoll, 2001; Freund et al., 2003). Finally, when considering alternative modes of hcrt/orx signaling, it should not be overlooked that, on a systems level, hcrt/orx may participate in classic feed-forward inhibition by exciting GABAergic interneurons. Such effects have also been demonstrated in the dorsal raphe (Liu et al., 2002).
Ontogeny and Anatomy of the Hypocretin (Orexin) System

In the rat, most neurons double-immunolabeled for bromodeoxyuridine (BrdU) and hcrt/orx appear to arise on embryonic day 12 (E12, Amiot et al., 2005), and to show a marked medial to lateral gradient, with slightly earlier born cells (E10/E11) found in the LHA, and later born cells (E15/E16) appearing more medially, near the ventricle. These data are somewhat at odds with another study that used *in situ* hybridization and immunohistochemistry for hcrt/orx, but not BrdU (Steininger et al., 2004), and reported evidence of hcrt/orx only at E18. The reasons for this discrepancy can only be speculated on, but it may be due to the vagaries inherent in qualitative histological procedures (fixation, antibody avidity, etc.).

As far as their anatomical distribution in hypothalamus, it is not uncommon to read that the hcrt/orx neurons reside in the LHA, and indeed most of them do. However, this designation is one of convenience, because the mediolateral domain of the hcrt/orx neuronal field extends from the lateralmost reaches of the LHA, where hcrt/orx neurons may be encountered above the optic tract, then ranging medially to within a few hundred microns of the third ventricle (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999; Swanson et al., 2005). It has now become common to refer to three somewhat arbitrarily defined subpopulations – a medial population of cells found within and adjacent to the DMH; a dense, perifornical population that enshrouds the fornices and extends dorsally, for the most part, to the zona incerta; and a lateral population that extends as described above throughout the LHA proper (Figure 18).

The rostrocaudal extent of hcrt/orx distribution is such that most of the population of ~4092 neurons (rat) is coextensive with the course of the DMH (Kilduff and Peyron, 2000; Swanson et al., 2005). Isolated hcrt/orx neurons...
immunopositive neurons can be found beginning at the level of the PVN, and these neurons will often be encountered at the border between that structure and the zona incerta, anterior hypothalamic nucleus, and subparaventricular region. The bulk of the hcrt/orx neurons appears more caudally in the perifornical region at the level where DMH, VMH, and arcuate are all equitably sized. This mass of cells then tapers caudally with those nuclei. In the posterior hypothalamus, where the third ventricle has divided into its hypothalamic and mammillary recesses, isolated hcrt/orx neurons can still be found. These disappear at the appearance of the supramammillary nucleus. The entire rostrocaudal extent of the population is about 1 mm.

Hcrt/orx neurons are medium in size (25-30 \( \mu \text{m} \)), with 2-3 branched, primary dendrites occasionally bearing spines. Immunolabeling with light microscopy reveals the presence of both prepro-orx/hcrt and its cleavage products throughout the cytosol, and electron microscopy refines this picture, showing hcrt/orx immunoreactivity in dense granules at the Golgi apparatus (Peyron et al., 1998).

The anatomy of the hcrt/orx system – its pattern of afferent and efferent connections is certainly a major factor in the enthusiasm for the peptides. The hcrt/orx neurons are centrally located and project from the hypothalamus as far rostral as the olfactory bulb (Peyron et al., 1998; Caillol et al., 2003) and as far caudal as thoracic levels of the spinal cord (van den Pol, 1999; Date et al., 2000; Llewellyn-Smith et al., 2003).

Within the forebrain, hcrt/orx innervates layers V-VI of the cortex and the central, medial and paraventricular nuclei of the thalamus, where it is believed to enhance thalamocortical processing related to attention and executive function (Bayer et al., 2002; Lambe and Aghajanian, 2003; Bayer et al., 2004; Lambe et al., 2005; Huang et al., 2006). Dense hcrt/orx innervation of the PVN, BNST, septum, locus coreuleus, and central amygdala are thought to involve the transmitter in adaptive responses to stress (Horvath et al., 1999b; Baldo et al., 2003; Sakamoto et al., 2004). Hcrt/orx fibers found in the NAc shell and VTA are being investigated for their role in motivated behavior and reward (addressed below, Martin et al., 2002; Baldo et al., 2004; Harris and Aston-Jones, 2006). The hcrt/orx system innervates most of the hypothalamus, except for the suprachiasmatic and supraoptic nuclei, suggesting a role for the peptides in energy homeostasis, endocrine
function, and hypothalamic control of sleep (Saper et al., 2002; Saper et al., 2005b; Taheri, 2006). Finally, hcr/orx neurons send robust projections to cholinergic and monoaminergic nuclei in the midbrain and brainstem, and in this way regulate arousal and wakefulness (addressed below).

One notable aspect of hcr/orx hodology is that many of the structures that receive hypocretinergic input send return projections to the hcr/orx system. This is particularly true for GABAergic neurons of the POA, cholinergic neurons in the medial septum and basal nucleus of Meynert, serotonergic neurons of the raphe, as well as many sources within the hypothalamus and LHA itself (interneurons, Sakurai et al., 2005). Sakurai’s anatomical study is notable in that it used a relatively novel molecular anatomic approach of employing the Pporx promoter to drive expression of a retrograde tracer/reporter molecule (tetanus toxin C/green fluorescent protein – TTC::GFP – fusion protein) in hcr/orx neurons. The authors provide evidence to suggest trans-neuronal uptake and transport of the secreted TTC::GFP into axon terminals synapsing on hcr/orx neurons; however, failure of this process may account for discrepancies between these findings and those from studies using more conventional neuroanatomical methods (Yoshida et al., 2006). These authors, for example, used injections of biotinylated dextran into various brain structures to examine synaptic appositions of anterogradely-labeled fibers at hcr/orx neurons. While these data largely mirror those from the Sakurai et al. study, Yoshida’s group reports innervation (~26% neurons in one counting plane) of hcr/orx neurons by cells in the VTA. This pattern of projections was not detected by Sakurai’s group. Subsequent experiments have shown that projections from many of these sites of afferent input (medial preoptic nucleus and substantia innominata) make use of GABA, glutamate, and acetylcholine (Henny and Jones, 2006).

As mentioned in a previous section, the reason more tracing work of this nature hasn’t been performed is due to the diffuse nature of the hcr/orx neuronal population, precluding discrete injections into areas having high densities of cells of that phenotype. What is known about the connectional relationships between the hcr/orx system and other brain structures will be integrated into a theoretical model for the role of this transmitter in behavior at the conclusion of this dissertation.
The Role of Hypocretin (Orexin) in Arousal

The posterior portion of the LHA containing hcrt/orx neurons has long been implicated in arousal maintenance (for historical review of hypothalamic role in sleep see Sterman and Shouse, 1985; Saper et al., 2001). It is now accepted that the wake-promoting effects of this tissue are owed to the hcrt/orx neurons. The neurons seem to promote arousal by sustaining the activity of “wake-active” aminergic and cholinergic systems known to drive wakefulness and cortical desynchronization (Steriade and McCarley, 2005). Electrophysiological studies have shown hcrt/orx to potently excite serotonergic neurons of the raphe (Brown et al., 2001; Liu et al., 2002; Kohlmeier et al., 2004; Takahashi et al., 2005; Tao et al., 2006), histaminergic neurons of the tuberomammillary nucleus (Eriksson et al., 2001; Huang et al., 2001; Ishizuka et al., 2002), noraderenergic cells of the locus coeruleus (Hagan et al., 1999; Bourgin et al., 2000; Ivanov and Aston-Jones, 2000), and cholinergic neurons of the basal forebrain (Eggermann et al., 2001). In many of these studies, this hcrt/orx-mediated activation was correlated with behavioral and electroencephalographic measures of arousal. As might be expected, when exogenous hcrt/orx peptides are infused into the ventricles, animals spend more time awake (Espana et al., 2002), and microdialysis studies in cats have shown that hcrt/orx levels peak in LHA and locus coeruleus during waking, particularly active waking that is accompanied by locomotor output (Kiyashchenko et al., 2001).

A number of Fos studies have shown that hcrt/orx neurons are most active during waking and that psychomotor stimulants that promote wakefulness induce fos expression in hcrt/orx neurons (Scammell et al., 2000; Estabrooke et al., 2001; Espana et al., 2003). More recently, studies using juxtacellular neurobiotin labeling have been able to identify and record from single hcrt/orx neurons in the LHA (Lee

Figure 19. Hypocretin (orexin) neuron in vivo. A, Waveform from identified hypocretin (orexin) neuron in wake, freely moving animal. B, Correlation of hypocretin (orexin) unit activity with novel food presentation and consumption, and various electromyo- and encephalographic measures of motor activity and arousal. Adapted from Milejkovskiy et al. (2005).
et al., 2005; Mileykovskiy et al., 2005). These experiments show that hcrt/orx firing is highest during waking, especially when animals are behaviorally active or have postural muscle tone. Hcrt/orx decrease their activity during quiet waking or automatisms (e.g. grooming), and fall silent during REM sleep episodes that are accompanied by muscular atonia (Figure 19). These data support conclusive evidence that implicates the loss of hcrt/orx signaling in narcolepsy, a neurological disorder characterized by intrusion of REM sleep (“sleep attacks”) and its associated loss of postural muscle tone (cataplexy) into normal waking.

After discovering hcrt/orx, Yanagisawa’s group pursued its suggested role in feeding by creating transgenic (knockout) mice lacking a functional gene for hcrt/orx. While effects of the genetic manipulation on feeding were modest, in the course of their observations they noticed that \( \text{Pporx}^-/- \) mice showed what appeared to be cataplectic attacks (Chemelli et al., 1999). Electroencephalographic recordings verified that this was the case and that \( \text{Pporx}^-/- \) mice showed frequent REM episodes not preceded by non-REM sleep, a classic feature of narcolepsy. In another remarkable coincidence, Emmanuel Mignot’s group published similar findings a month later (Lin et al., 1999). These researchers had been working for some time with narcoleptic Doberman pinschers in hope of identifying the sequence of a fully penetrant, autosomal recessive allele, \textit{canarc-1}, that is responsible for the disorder in these animals. Following a positional cloning strategy, Mignot and colleagues discovered that \textit{canarc-1} was, in fact, a loss-of-function mutation in the gene for the hcrt-2/orx B receptor. These data were then complemented by mouse knockout studies by Yanagisawa’s group where \( \text{HcrtR2}^-/- \) animals show a narcoleptic phenotype (Willie et al., 2003).

Together, these preclinical data argued strongly in favor of a role for hcrt/orx in human narcolepsy. In the year following the publication of the animal data, it was shown, again, by several independent research groups that human narcoleptics have massive losses (~93% of the total neuronal population) of hcrt/orx immunolabeling in hypothalamus (Peyron et al., 2000; Thannickal et al., 2000). That year it was also demonstrated that human narcoleptics have extremely low levels of hcrt/orx in their CSF (Nishino et al., 2000). It has subsequently been shown that the absence of hcrt/orx neurons and peptide is due to immune-mediated destruction of these cells during early
life (Blouin et al., 2005; Crocker et al., 2005; Fromherz and Mignot, 2006). Though the pathways to narcolepsy appear to differ between canine and human forms of the disease (genetic vs. autoimmune, receptor mutation vs. peptide insufficiency), it is now clear that the endpoint is the same: impaired hcrt/orx signaling.

In a way, it is hcrt/orx’s role in narcolepsy that has afforded the clearest view of what function the peptides fulfill in non-pathological states. Using Yanagisawa’s hcrt/orx−/− mice, Thomas Scammell’s group has further scrutinized the sleep architecture in these animals (Mochizuki et al., 2004). Remarkably, they find that hcrt/orx knock out mice, compared to wild-type controls, show only slight increases in the cumulative time spent asleep across a 24 hour day, and these occur only in the first hour or two during the nocturnal period. Thereafter, differences cannot be distinguished. What Scammell does report, however, is a marked fragmentation of wakefulness, with hcrt/orx knock outs showing many more transitions between all behavioral states, be it from waking to non-REM sleep, non-REM sleep to waking, waking to REM sleep, etc. This is as might be expected given the clinical presentation of human narcolepsy, but it should also be noted that while the most salient features of the disease are sleep attacks with cataplexy, narcoleptics are often poor sleepers, who have as much difficulty staying asleep as they do staying awake. In light of these findings, Scammell proposes that the duty of hcrt/orx is not to drive wakefulness, as if it were some endogenous stimulant drug – animals and humans without hcrt/orx would thus be in a permanent state of somnolence. Rather, hcrt/orx peptides stabilize behavioral state by facilitating activity in wake promoting aminergic cell groups of the mid- and hindbrain. With Cliff Saper, Scammell has formalized this thinking in a simple model for regulation of arousal by hcrt/orx known as

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Figure 20. The “flip-flop” switch

A. During waking, hypocretin (orexin) neurons stabilize activity of midbrain aminergic nuclei that coordinate arousal by their projections to forebrain. These projections also inhibit sleep active GABA neurons in the preoptic area.

B. During sleep, hypocretin (orexin) neurons and wake-active aminergic nuclei are inhibited by projections from preoptic area. LC, locus coeruleus; ORX, orexin; TMN, tuberomammillary nucleus; VLPO, ventrolateral preoptic area. Adapted from Saper et al. (2005).
the “flip flop switch” (Saper et al., 2001; Saper et al., 2005a). The model describes two, mutually inhibitory loci: sleep-active, sleep-promoting GABAergic neurons of the vlPOA, and wake-active, wakefulness-promoting aminergic neurons of the midbrain and brainstem (Figure 20). During normal waking, hcart/orx cements the activity of aminergic nuclei, as described above, while these neurons, in turn, are found to inhibit the activity of the sleep-promoting neurons of the vlPOA. During sleep, however, increased activity of the vlPOA is able to inhibit both aminergic cell groups as well as hcart/orx neurons, until it is time to wake. In the absence of hcart/orx, clinical symptoms of narcolepsy represent a dysregulated toggling between states, as the two major elements of the flip-flop switch alternately inhibit one another.

The Role of Hypocretin (Orexin) in Feeding

As above with arousal, the LHA has long been associated with feeding and ingestive behavior (exhaustively reviewed in Bernardis and Bellinger, 1993, 1996). As mentioned in a previous section, interest in this structure’s role in feeding behavior had dissipated in the 1980s and early 1990s, but was renewed not just by the discovery of hcart/orx there, but also by the discovery of a host of hypothalamic peptides or receptors (e.g. leptin, ghrelin) that are responsive to homeostatic signals related to energy balance (Zigman and Elmquist, 2003). The first studies of hcart/orx reported that the peptides have orexigenic properties (Sakurai et al., 1998), and that small molecule antagonists at hcart/orx receptors are anorectic (Haynes et al., 1999; Haynes et al., 2000; Rodgers et al., 2001). This line of research continues.

Neurons in the LHA were reported to alter their firing rate in response to energy molecules as simple as glucose in the 1960s (Oomura et al., 1969; Oomura, 1988). Only recently have these glucose-responsive neurons been discovered to contain hcart/orx (Yamanaka et al., 2003; Burdakov et al., 2005). The relationship between glucose and hcart/orx neuronal activity appears to be negatively correlated, such that increased concentrations of glucose, as would be found in recently fed animals, inhibit activity of hcart/orx neurons. Burdakov et al., have explored the mechanism for this effect and report that glucose inhibits hcart/orx neurons by inducing outward currents through tandem pore
potassium channels (K_{2P}, TASK family), and further that this mechanism endows hcrt/orx neurons with exquisite sensitivity to glucose dynamics well within the physiological range (Burdakov et al., 2006). Consistent with glucose’s inhibitory influence on hcrt/orx neurons, studies have also shown that glucoprivic challenges (fasting, insulin, or 2-deoxyglucose treatment) that induce hypoglycemia increase expression of Pporx/Hcrt mRNA, release of the peptide, and induction of Fos in hcrt/orx neurons (Cai et al., 1999; Griffond et al., 1999; Moriguchi et al., 1999; Briski and Sylvester, 2001; Cai et al., 2001).

Larger molecules than glucose also appear to relay information about energy balance to the hcrt/orx system. The adiposity peptide leptin, secreted from white adipose tissue during times of energy abundance, acts on two populations of neuron in the arcuate which, in turn, project to hcrt/orx neurons in the LHA. Intravenous injections of leptin appear to inhibit neurons containing the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), as these neurons fail to show Fos induction but do show increased transcription of the suppressor of cytokine signaling-3 (SOCS-3) gene known to be induced by leptin receptor (OB-Rb) activation (Elias et al., 1999). Conversely, the same authors report that leptin increases both SOCS-3 and Fos expression in neurons containing the anorectic peptides proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), suggesting that these neurons are activated. Together with other functional and anatomical data mapping the peptidergic projections to the LHA, and the presence of receptors for these peptides on hcrt/orx neurons, a simple model has been proposed whereby the hcrt/orx system functions as a second order effector system for feeding that acts on energy balance information that is integrated and relayed by neurons in the arcuate (Broberger et al., 1998; Elias et al., 1998; Horvath et al., 1999a; van den Top et al., 2004). By this model, rising leptin under conditions of positive energy balance inhibits NPY/AgRP projections to hcrt/orx neurons that would normally induce feeding. On the other hand, high leptin is thought to increase activity of POMC inputs to hcrt/orx neurons, inhibiting their output and thus feeding behavior (Schwartz et al., 2000). Acting in opposition to leptin, a second peripheral (GI tract, stomach) factor, ghrelin, is believed to signal negative energy balance and the necessity to feed (Tschop et al., 2000; Cummings et al., 2001). Ghrelin’s orexigenic effects appear
to be mediated by the hcrt/orx neurons (Olszewski et al., 2003; Toshinai et al., 2003). Thus, under circumstances of rising ghrelin and falling leptin, a state of negative energy balance would be detected in the arcuate and relayed to hcrt/orx neurons in the LHA to coordinate appropriate behaviors and arousal states (feeding and food seeking).

The notion that hcrt/orx regulates arousal in relation to energy balance has been investigated, and thus, the stories of hcrt/orx’s involvement in arousal and feeding have been integrated (Yamanaka et al., 2003). These authors report that hcrt/orx knockout mice fail to show fasting-induced increases in locomotor exploration when compared to wild-type controls. This difference was not found to be due to locomotor impairments – most importantly cataplectic attacks – when these were taken into account. Presumably, increased arousal and behavioral activation is an evolutionarily adaptive response to satisfy homeostatic parameters by first locating and consuming food. These findings have been extended by studies in which hcrt/orx knockout mice fail to show food anticipatory activity that is typically entrained by a restricted feeding schedule (Mieda et al., 2004).

**The Role of Hypocretin (Orexin) in Goal-Directed Behavioral Activation**

In addition to prolonging wakefulness and inducing feeding, exogenous hcrt/orx, when given centrally, increases locomotor exploratory behavior, much like psychomotor stimulants like amphetamine and cocaine (Kotz et al., 2002; reviewed in Kotz, 2006). Because this effect can be markedly attenuated by pretreatment with DA receptor antagonists (Nakamura et al., 2000), it is possible that one effector system for hcrt/orx-induced behavior is the mesolimbic DA pathway. Like other aminergic systems that the hcrt/orx system is known to affect in the context of arousal, anatomical data demonstrate that there is rich innervation of the VTA by hcrt/orx neurons (Peyron et al., 1998; Fadel and Deutch, 2002), and that neurons there express receptors for hcrt/orx (Marcus et al., 2001). In light of these anatomical findings, several groups engaged in research on addiction and motivated behavior have evaluated the extent to which hcrt/orx can affect the function of the mesolimbic DA circuit.

One of the first reports used *in vitro* electrophysiology and single-cell RT-PCR to identify and record from DA cells in the VTA (Korotkova et al., 2003). These
experiments show that in cells that express TH and OX₁ receptors, bath application of hcrt-1/orx A increases firing frequency and induces burst firing. This latter finding is somewhat questionable in that burst firing as not commonly regarded as a property of DA neurons *in vitro* (Grace, 1987). Nonetheless, increases in rate activity of VTA neurons is predicted based on studies of hcrt/orx effects in other systems (see above section on arousal) and the intracellular signaling pathways (PLC, PKA) known to be engaged in by hcrt/orx receptors in VTA DA neurons (Uramura et al., 2001).

A more recent study also performed on *in vitro* slices containing VTA suggests that hcrt/orx not only exerts acute effects on DA cells there, but may affect NMDA receptor-mediated plasticity (Borgland et al., 2006). In VTA DA neurons voltage clamped at +40 mV, bath application of hcrt-1/orx A dose-dependently enhanced NMDAR-mediated EPSCs by ~50%. This effect was blocked by pretreatment with the OX₁ antagonist, SB 334867. As predicted by the earlier work of Uramura et al. the effect of hcrt/orx on NMDAR-mediated EPSCs uses a PLC and PKC-dependent mechanism, as inhibitors of these enzymes block the effect. In a separate experiment in this paper, it was shown that a 5 minute application of hcrt-1/orx A increased the AMPAR/NMDAR ratio computed from miniature EPSC amplitudes at 3-4 hours after application. This suggests that hcrt/orx is able to engage AMPAR trafficking mechanisms to durably enhance the excitability of VTA DA neurons. This effect has ramifications for drug addiction, where this kind of plasticity is known to be induced by drugs of abuse during of the behavioral sensitization paradigm (Ungless et al., 2001; Borgland et al., 2004). With this in mind, Borgland et al. were able to show that compared to cocaine-sensitized rats, animals pretreated with SB 334867 prior to cocaine showed neither behavioral sensitization nor significant increases in AMPAR/NMDAR ratios.

*In vivo* neurochemical data from microdialysis studies shows that infusion of hcrt-1/orx A into VTA causes increased DA release in NAc (Narita et al., 2006). These authors also show that in hcrt/orx knockout mice, this effect is attenuated, and that place preference for morphine is attenuated compared to controls. Other studies have used the conditioned place preference paradigm together with Fos-labeling in hcrt/orx neurons to show that when animals are conditioned to expect a food or drug reward, activity in hcrt/orx neurons is highest (Harris et al., 2005). These authors’ work, because of its close
relevance to topics treated in the experimental chapters in this dissertation will be addressed more thoroughly in that section. Other operant behavioral studies have shown that in fixed and progressive ratio schedules of reinforcement, hcr-t-1/orx A appears to enhance the incentive value of sweet pellets (Thorpe et al., 2005). When similar operant procedures are used for food or cocaine rewards, centrally administered hcr-t-1/orx A reinstates lever-pressing after it has become extinguished by several sessions of non-delivery of reward (Boutrel et al., 2005). It is notable, however, that when MFB stimulation is used as the reinforcer, hcr-t-1/orx A increases the current threshold for ICSS, suggesting a negatively valenced hedonic state the authors have linked to stress responses.

Work on the role of hcr-t/orx in reward, addiction, and motivated behavior is still a nascent state, with most of the few high profile papers published within the last year. The new scientific content in this dissertation is meant both to add to the literature on hcr-t/orx and motivated behavior, as well as echo that already done on hcr-t/orx and feeding. After all, male reproductive behavior, like feeding is a motivated behavior dependent largely on chemical factors from the periphery (gonadal steroids), and which can be seen to engage the mesolimbic DA circuitry. A precis of the literature on these two themes is given below.
7. MALE SEXUAL BEHAVIOR AS A MODEL MOTIVATED BEHAVIOR INVOLVING HYPOTHALAMIC AND MESOLIMBIC CIRCUITS

Assessing Sexual Motivation

Since the early days of behavioral neuroendocrinology, male sexual behavior has been dichotomized into motivational or consummatory components (Beach, 1956). Beach hypothesized that these behavioral features must correspond to distinct neural processes. The appetitive mechanism was assumed to be responsible for sexual arousal and pursuit of the female, as well as the initiation of mounting. Thereafter, a more reflexive “intromission and ejaculation” mechanism would be engaged to coordinate the sequence of stereotyped behaviors seen during actual copulation. As a heuristic, the motivation-performance dichotomy has been substantiated by more recent scholarship using factor analytic techniques (Sachs, 1978; Pfaus et al., 1990a), and it continues to color our view of the behavior as a whole.

One criticism of the motivation-performance concept has been that there have been few “pure” measures of sexual motivation that are not to some degree confounded by or conflated with those of sexual performance (Hull et al., 2006). Motivation is often operationalized as the latencies to mount or intromit, but as these authors point out, such measures may be confounded when experimental manipulations (e.g. drugs, hormones, lesions) affect motor function or sensory processing that may be motivation’s necessary antecedent.

Over the years, a number of investigators have attempted to fashion experimental paradigms that would tap motivational processes in a relatively undiluted manner. Some of these attempts have been more successful than others. Simple runway or T-maze procedures that use run time as their dependent measure are, of course, handicapped for the reasons discussed above, but more meaningful data on motivational parameters have been produced using an X-maze apparatus. In these studies, a receptive female is placed into the goal box at one arm of the maze, and a male, a non-receptive female, or food may be placed in others. In this design, motivation is separated from motor effects as it is measured by the number of successive trials where the male chooses the arm containing
the estrous female. Motor effects of pharmacological manipulations or lesions may then be accounted for by observing latencies and runway speeds (Hull et al., 1991; Warner et al., 1991).

Another strategy to assess motivation is based on level changing behavior in a bilevel chamber in males that had previously copulated in the apparatus (Mendelson and Pfau, 1989). These authors argue for the ethological validity of level-changing as an anticipatory searching response for sexual reward, and have demonstrated by factor analysis that level changing loads onto other behaviors that are viewed as anticipatory (Pfaus et al., 1990a). Other investigators have shown that level changing decreases in animals that had ejaculated immediately prior to introduction to the apparatus, suggesting that low motivation during the post-ejaculatory interval (PEI) is reflected in the test. However, as above, the level changing measure is dependent on locomotor output and sensory processing – particularly olfaction (Van Furth and Van Ree, 1996) – and is thus confounded as a pure measure of sexual appetite.

Though instrumental procedures suffer somewhat from the usual confounds, they offer some transparency because they are completely dissociated from the consummatory aspect of sexual behavior. Rather than level-changing or runway time, lever pressing is used as the dependent measure. Barry Everett has provided the most recent account of operant responding for access to estrous females (Everitt et al., 1987). In this paper, males quickly began to discriminate between lever pressing for a conditioned stimulus (CS+) that had previously been paired with the presentation of a receptive female, and a CS- that had not. Using a fixed ratio (FR) schedule, males would work for about 15 minutes for female access. When the schedule of CS+ presentation was then switched to a fixed interval of 15 minutes (FI-15 min), responding took on the classic scallop shape common to FI schedules indicating that responding had come under the control of the CS+. In a separate experiment, Everitt tested responding in animals during the sexually refractory PEI when motivation for female contact should be at its lowest. Compared to baseline rates, responding during the PEI was greatly diminished. Similar results were had by substituting a non-estrous female as the reward. Over repeated exposures to a non-receptive female, baseline responding fell to what was normally elicited during the PEI. Because these experiments did not employ potentially impairing pharmacological or
surgical manipulations, the data can be assumed to reflect a relatively pure measure of a motivational process related to female-associated cues.

In a subsequent paper, Everitt did examine the effects of POA lesions and castration on instrumental responding (Everitt and Stacey, 1987). While animals given excitotoxic POA lesions showed marked decrements in sexual performance (i.e. mounts, intromissions, and ejaculations) compared to that of sham-treated controls, operant responding for estrous females was only modestly reduced on the last of four testing days. This finding is interpreted by the authors as evidence for structures other than POA contributing to sexual motivation, though this has been disputed (Warner et al., 1991). In the same study, castration was found to produce the expected decrements in sexual performance, but it also substantially reduced instrumental responding for females. Both of these effects were rapidly reversed by testosterone replacement.

The importance of parsing appetitive versus consummatory facets of male sexual behavior takes on extra significance when considering the possible contributions of the mesolimbic system to one or the other of these aspects of the behavior. Below this topic is given lengthier treatment.

**Mesolimbic Dopamine and Male Sexual Behavior**

**Pharmacological and Neurochemical Evidence**

Dopaminergic transmission has been implicated in the motivational features of male sexual behavior at least since the time that L-DOPA was found to induce hypersexuality in Parkinsonian patients (Bowers and van Woert, 1972; Hornykiewicz, 1974; Brown et al., 1978). DA antagonists, on the other hand, are noted to be anaphrodisiacal in humans (Petrie, 1985). These effects have been reproduced in animal models using systemic delivery of any number of selective and non-selective ligands for DA receptors or transporters (Hull et al., 2007). Because it is one of the largest populations of DA-containing neurons in the brain, many preclinical studies have focused on the mesolimbic system in an attempt to arrive at a mechanistic understanding of the drug effects described above. Because of its putative role in other motivated behaviors,
DA action in the mesolimbic system has also been argued to operate within this domain
in the context of sex behavior, perhaps to encode the reinforcing properties of receptive
females (see Everitt, 1990). It should be noted, however, that DA neurons in the
hypothalamus (A14, periventricular system) are believed to make major contributions to
the control of both appetitive and consummatory aspects of masculine sexual behavior
via their action on cells of the mPOA (see Dominguez and Hull, 2005). Given the
experimental focus in this dissertation on descending hcrt/orx projections, attention will
be paid mainly to the mesolimbic DA system.

A thorough pharmacological analysis that was able to contrast effects of
dopaminergic manipulations in either mPOA or NAc is provided by Pfaus and Phillips.
These authors were able to show that infusion of haloperidol into the mPOA of sexually
experienced males produced marked impairments in measures of both performance and
motivation, while intra-NAc injections disrupted measures only of the latter (Pfaus and
Phillips, 1991). Although the data here from mPOA injection experiments confirms
several other reports (Pehek et al., 1988; Warner et al., 1991), the results from the NAc
injection studies are somewhat at odds with other findings that suggest that
pharmacological impairment of mesolimbic DA transmission preferentially affects
motoric features of copulatory behavior (Hull et al., 1991; Moses et al., 1995). The
disparity in results may be due to the vagaries inherent in pharmacological manipulations
(i.e. dosing and receptor selectivity, etc.), because electrical stimulation of the VTA
enhances both appetitive and consummatory features of copulation (Markowski and Hull,
1995), while lesions there increase the postejaculatory interval (Brackett et al., 1986).
Furthermore, radiofrequency and DA-depleting (6-OH-DA) lesions of NAc can be seen
to impair various motivational indices like non-contact erections and intromission latency
(Liu et al., 1998). Together, these data would seem to make the mesolimbic DA system a
strong candidate for the control of male sexual behavior, but with the details of the nature
of this control (e.g. appetitive vs. motoric) needing further study.

One alternative approach has emerged from Roy Wise’s theory of DA and
reinforcement. With regard to sexual behavior, this view predicts that prompt release of
DA in the mesolimbic tract may not be an absolute necessity for the commission of
copulatory behavior, but rather may be critical over time to ‘stamp in’ an association
between copulatory cues and their positive hedonic properties. As with food reward (Wise and Schwartz, 1981), DA blockade in naïve animals during their initial exposures to estrous females should cause decrements in subsequent tests of sexual motivation under drug-free conditions. This phenomenon was demonstrated by López and Ettenberg, who gave naïve males ten daily sexual experience sessions that were preceded by treatment with several systemic doses of haloperidol or vehicle (Lopez and Ettenberg, 2000). After completing the course of experience sessions, runway times to an estrous female were tested and found to be increased in animals that had previously received the 0.1 mg/kg dose of haloperidol before their experience sessions. Thus, it can be argued that DA blockade during the acquisition phase impaired the reinforcing properties of, and the motivation for estrous females.

While reinforcement theories of DA in sexual motivation appear to be ably supported by the above study, there is substantial evidence for DA’s participation in incentive sensitization processes along the lines of those proposed by Terry Robinson. Many of these data come from microdialysis or voltammetry studies in which probes installed in the NAc recovered enhanced DA release prior to the consummatory phase of copulation (Pfaus et al., 1990b; Louilot et al., 1991; Damsma et al., 1992; Wenkstern et al., 1993; Mas et al., 1995). In a typical experiment, progressive increases in NAc DA follow increasingly proximate copulatory cues. For example, DA levels will rise from baseline following exposure to soiled female bedding, rise further with gated exposure to an estrous female, and reach their peak when animals are allowed to copulate. These responses are muted or not present when non-estrous females are used or when probes are placed in the dorsal striatum. Further, the behavioral specificity of female-induced NAc DA is suggested by failure to achieve similar increases during wheel running or novel object stimuli. That NAc DA release may in some way correlate with the internal motive state of the animal is supported by experiments showing that DA increases mirror sexual behavior during the famous “Coolidge effect” (Fiorino et al., 1997). In this experiment, animals who had become sated with one female, and whose NAc DA levels had returned to baseline after repeated bouts with her, were quickly introduced to a novel female. As expected, both copulation and NAc DA release were renewed.
The release of DA prior to actual copulation and its sensitivity to the incentive properties of receptive females are consistent with Robinson’s suggestion that DA mediates “wanting” of salient rewards, rather than the subjective hedonic experience of their consumption. This argument is strengthened by the demonstration of cross-sensitization between DA agonists, sexual performance, and NAc DA release (Fiorino and Phillips, 1999a, b). These authors show that, in sexually naïve animals, prior exposure to amphetamine facilitates copulation during drug free tests several weeks later. Compared to vehicle-treated controls, amphetamine-exposed animals showed decreased mount and intromission latencies, and generally became more proficient copulators ahead of their vehicle-treated counterparts. These effects were also bolstered by dialysis data that showed animals that had experienced amphetamine, showed an augmented response to estrous females. Together these data suggest that the same circuits (i.e. NAc and mesolimbic tract) that undergo drug-dependent neuroplasticity during classical sensitization (Kalivas and Stewart, 1991; Robinson and Berridge, 2003), overlap with or are identical to those responsible for the motivational facet of male sexual behavior.

**Neuroanatomical Evidence**

As the proto-oncogene c-fos became recognized as a marker of neuronal activation, it superceded existing 2-deoxyglucose methods and allowed for functional mapping with cellular resolution (Plum et al., 1976; Sheng and Greenberg, 1990; Morgan and Curran, 1991). Immunolabeling for Fos protein allowed several early investigators to show increased activation of neurons of the NAc in animals that had recently mated (Robertson et al., 1991). This effect has been replicated using various behavioral paradigms that used olfactory stimuli or other copulatory cues to suggest that Fos induction in NAc was related to the anticipatory component of sexual behavior (e.g. Lopez and Ettenberg, 2002; Kippin et al., 2003). Subsequent studies have also demonstrated significant Fos induction with mating in the origins of the mesolimbic tract in the VTA (Balfour et al., 2004). Together, these results can be seen to complement neurochemical findings discussed above, in which increased dopaminergic activity in these structures accompanies sexual behavior and estrous female cues.
Hormonal Control of Male Rat Sexual Behavior with Reference to Endocrine Influences on Mesolimbic Dopamine Function

Since the emergence of modern behavioral neuroendocrinology with the work of Frank Beach, Robert Goy, Julian Davidson, and many others, the knowledge base on andrology has become too vast to offer more than a brief survey here. However, as this dissertation contains experiments having a neuroendocrine focus, it will be important to outline the basic tenets regarding hormonal control of copulatory behavior.

Much of our understanding of the endocrine regulation of sexual behavior flows from elegant experiments using the castration-replacement paradigm. Sexually experienced rats when castrated show a strong, albeit gradual, diminution in sexual behavior, beginning at about two weeks to a month post-castration. The latency to behavioral deficits is variable and shows individual differences amongst test subjects, with a few individuals occasionally retaining full sexual competence several months post-castration (Davidson, 1966). Aside from the retention of behavior long after the point when residual gonadal steroids are cleared (~24 hr. Krey and McGinnis, 1990), another notable feature of the post-castration syndrome is the consistent order of behavioral losses that follow removal of the testes. Latencies for all measures are dilated in the weeks following castration, with ejaculation being the first feature to disappear completely, this is followed by intromission, and later mounts. Mounts eventually disappear, although this feature seems more durable than the others, with Davidson reporting increases in mount frequency at about a month post-castration, which later tailed off. The entire sequence of deficits is remarkable, as it suggests differential sensitivity to hormones of the central and peripheral substrates for each component of behavior. The durability of mounting behavior could be interpreted along a number of lines, one of them being that the motivational substrates for sexual behavior overlap with the generic motivational circuitry and that these remain potent despite the loss of steroids. Thus, mounting, and presumably motivation for sex, declines after multiple attempts at copulation that are unsuccessful due to failure of central and peripheral systems required for erection. In keeping with Wise’s theory and microdialysis data, these attempts would
result in DA release that is insufficient for robust reinforcement. Since it is only weakly rewarding, over time the behavior is extinguished and further attempts at copulation are abandoned.

The pattern of post-castration behavioral losses is also notable because with hormone replacement they are restored in reverse order. As might be expected, exogenous testosterone (T) renews the full behavioral profile in relatively short order – typically more promptly than the time the individual behaviors took to disappear, though this depends on the interval allowed after castration before T is given (Davidson and Bloch, 1969; Larsson, 1979; Hull et al., 2006).

Replacement studies of this type also revealed an unexpected level of biochemical complexity in the hormonal control of male sex behavior, when it was found that other androgens, even those with higher affinity for their receptor (Wilbert et al., 1983), like dihydrotestosterone (DHT), were ineffective in restoring normal behavior (Whalen and Luttge, 1971). Moreover, it was known that estradiol \((E_2)\) was effective in this capacity (Davidson, 1969). Soon it became clear that in rodents, T functions as a prohormone, and that while androgenic hormones are not without effects on behavior (Feder et al., 1974), it is T’s aromatized metabolite E_2 that is responsible for much of the behavioral repertoire (see Balthazart and Foidart, 1993). Confirmation of the ‘aromatization hypothesis’ came in the form of studies where inhibitors of E_2 synthesis were found to impair copulation in castrates maintained on T, but not when given exogenous E_2 (Christensen and Clemens, 1975; Beyer et al., 1976).

The central loci on which the steroid hormones act to facilitate the expression reproductive behavior have been mapped by autoradiography, immunohistochemistry or \textit{in situ} hybridization for the androgen receptor (AR) and two isoforms of estrogen receptor (ER-alpha and ERß, e.g. McEwen et al., 1979; Simerly et al., 1990). Neuroanatomical mapping of the gonadal hormone receptors have revealed a constellation of structures across the brain that appear to form a circuit for integrating chemo- and somatosensory information regarding proximate sexual cues, and in turn for orchestrating voluntary and reflexive locomotor outputs associated with the expression of sexual behavior (reviewed in Hull et al., 2006). Forebrain structures showing high steroid hormone receptor expression are often those that have been classically associated with
sex behavior by early lesion studies and that show increased Fos expression following mating (Baum and Everitt, 1992; Coolen et al., 1997). Among the most important are the mPOA, medial amygdala, and BNST. It should be noted here, and will be expanded on further later, that each of these structures enjoys substantial connections with the LHA and the hcrt/orx neurons there. The putative role of each structure in elaborating reproductive behavior by these connections will be discussed in the conclusion section.

As a putative substrate for the appetitive features of sexual arousal, it would seem to make sense for VTA or NAc to be endowed with steroid hormone receptors. Disappointingly, many of the first anatomical surveys (above) of steroid hormone receptor expression depicted little, if any, AR or ER in either structure. More recent analyses have detected scant, but distinct populations of TH-positive neurons in VTA that contain AR or ER (Kritzer, 1997). This evidence is encouraging, but for the time being it may be assumed that any endocrine influence on the function of the mesolimbic system is indirect or via non-genomic mechanisms akin to those described in mPOA or striata of female rats (Pfaff and Pfaffmann, 1969; Mermelstein et al., 1996). In functional neurochemical terms, this influence appears to be potent, with castration producing reductions in DA content of NAc that were reversible with T or E$_2$ (Mitchell and Stewart, 1989). This finding is in accord with other experiments showing that intra-NAc T will condition a place preference, and that this effect is dependent on DA transmission, since it is blocked with pretreatment with a DA antagonist (Packard et al., 1997; Packard et al., 1998; but see Wood, 2004).
8. EXPERIMENTS

Experimental Hypothesis

In the preceding pages are described a set of heterogeneous but interconnected structures from whose mutual influence may emerge highly organized goal-directed behaviors like copulation. One of these structures is the LHA and its hcrt/orx neurons. Recent work has shown these neurons to be important for general arousal and wakefulness. Other investigators have suggested a role for these neurons in feeding and reward. The broad hypothesis of this dissertation is that hcrt/orx neurons may be important not only for wakefulness, but determining which behaviors are enacted during arousal. The experimental work that is detailed below aims to outline how neurohumoral information relevant to a specific motivated behavior, in this case copulation, may be integrated by the hcrt/orx system, which may, in turn, act on structures known to effect the expression of that motivated behavior.

This hypothesis allows several predictions that are evaluated in the experiments below: the hcrt/orx system should show activation during sexual behavior, as indexed by increased expression of immediate early genes. The hcrt/orx neurons should also be influenced in some fashion by gonadal steroids known to be critical for the expression of sexual behavior. The character of this influence should become apparent with classic castration and replacement studies and by the presence in hcrt/orx neurons of nuclear steroid hormone receptors. That the hcrt/orx system is acutely involved in sexual behavior should also be apparent by pharmacological manipulations that block hcrt/orx signaling, thus impairing the behavior. Positive findings in each of these experiments presented below has confirmed that a role for the hcrt/orx system in male reproductive behavior is plausible.

The manner in which the hcrt/orx system effects this behavior is the subject of additional experiments. Given the substantial projections of hcrt/orx neurons to the VTA, it is reasonable to assume that the peptides exert marked neuroexcitatory effects on dopaminergic neurons there, and these effects will be apparent in unit recordings of DA neurons in the VTA. The effect of hcrt/orx on VTA DA neuronal activity, in turn, has
ramifications for reinforcement and behavioral output. Finally, an anatomical picture of hcrt/orx-VTA connections should be drawn, where hypocretinergic innervation of that structure accompanies increased immediate early gene activity during the behavior in question.

While many more experiments will be necessary to fully characterize the proposed hcrt/orx-DA motive circuit, the experiments below provide an essential outline of how these structures may interact in the context of goal-directed behavior. The basic schematic installs the LHA-hcrt/orx system as a central “selector” node that integrates and facilitates the activity of inputs from forebrain structures (“detectors”) responsible for apprehending sensory information that is relevant to a specific mode of goal-directed behavioral expression. These inputs, once integrated by the LHA, are passed for action by the hcrt/orx system to behavioral “effectors” such as the VTA and its associated circuitry in the basal ganglia. This model will be discussed further in light of the data presented below.

**General Methods**

**Animals and Surgery**

Adult (300-350 g) male Long-Evans/Blue Spruce rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually in plastic tubs in a climate-controlled colony with lights off at 11 A.M. and on at 9 P.M. Food and water were available ad libitum. Animals in the colony were handled daily for general health examination and weighing. Ovariectomized, hormone-replaced (10 µg estradiol benzoate at 48 hr before and 500 µg progesterone at 4 hr before testing) females used in sexual behavior tests were housed in a separate room under the same conditions. Surgeries were performed in aseptic conditions under ketamine HCl/xylazine HCl (80/10 mg/kg, i.p.), and animals were given postoperative analgesia (buprenorphine HCl, 0.3 mg/kg, i.p.) and local anesthesia at wound margins (5% lidocaine HCl). For terminal procedures, animals were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg).

**Behavioral Testing**
Standard measures of male sexual behavior were recorded. These are: *mount* (and latency to first mount), identified by the male’s forebody over female’s hindquarters, flank clasping, and a shallow thrust; *intromission* (and latency to intromit), identified by an intra-vaginal thrust with a springing, retropulsive decoupling after the intromission; and frequency and latency to *ejaculate*, where after multiple intromissions a deeper thrust is followed by a slower dismount and physical inactivity -- the **post-ejaculatory interval (PEI)**. Sexual motivation will be operationally defined as mount latency, but other measures (e.g. intromission frequency preceding ejaculation, PEI) will also be considered. Theoretical issues associated with assessment and differentiation of sexual motivation and performance are discussed in Hull et al., 2006.

**Perfusion, Fixation, and Tissue Sectioning**

Sodium pentobarbital-anesthetized animals (as above) were perfused transcardially via the ascending aorta with 200 mL cold 0.1 M phosphate buffered saline (PBS, pH 7.4, 0.9% NaCl). After exsanguination was complete, animals received 200 mL 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Thereafter, brains were removed and submerged in 15 mL of 4% paraformaldehyde for 4 hr. Brains were then submerged in two changes of 30% sucrose solution in PBS until they lost buoyancy (~36 hr). Before sectioning, brains were blocked in two by a single sagittal razor cut near the pons, and pieces flash frozen (10 sec) in 2-methylbutane cooled by dry ice. While still frozen, brains were mounted on stages and cut into 40 μm sagittal sections on a cryostat at -16° C. Resulting sections were then stored in ethylene glycol cryoprotectant solution (see Watson et al., 1986; Hoffman and Le, 2004) at -20° C until immunolabeling.

**Immunohistochemistry**

Free floating, paraformaldehyde fixed 40 μm sections were rinsed in 0.1 M PBS, and endogenous peroxidase activity blocked in 0.3% H₂O₂ for 20 min. Sections were then given 2 x 10 min PBS rinses and incubated 2 h in a 5% solution of normal goat serum (NGS) in PBS + 0.3% Triton X-100 (PBST). Brain sections were incubated for 12 (cytosolic proteins) or 48 hr (nuclear proteins) in rabbit primary antibodies diluted in this NGS-PBST blocking solution. After incubation in primaries, sections were given 6 x 10
min rinses in PBS, and incubated in biotinylated goat anti-rabbit IgG in PBST (1:200, Vector Labs, Burlingame, CA), and subsequently rinsed for 3 x 10 min in PBS. Tissue was then incubated for 1 hr in avidin biotin horseradish peroxidase detection reagent (1:100, Vectastain Elite Kit, Vector) and rinsed for 3 x 10 min in PBS. Peroxidase chromogen was visualized in a solution of 3′3-diaminobenzidine (200 μg/mL) with H2O2 substrate with (nuclear proteins) or without NiSO4 intensification (cytosolic proteins). Labeled sections were mounted on SuperFrost slides (Fisher Scientific, Hampton, NH), dehydrated in graded ethanols, cleared with Hemo-De (Fisher) and coverslipped with distyrine DPX mountant (Electron Microscopy Sciences, Hatfield, PA).

**Western Immunoblotting**

Fresh brains were quickly removed from sodium pentobarbital-anesthetized animals killed by decapitation. Brains were then flash frozen as described above and kept at -80° C until extracted. Frozen hypothalami were then blocked according to anatomical landmarks (Glowinski and Iversen, 1966) and homogenized by sonication. Protein was extracted into modified radioimmunoprecipitation (RIPA) buffer (pH 8.0) with protease inhibitors (Roche Complete Mini protease cocktail; Roche, Indianapolis, IN) and extracts aliquoted after centrifugation. Frozen aliquots were kept at -80°C until further processing. After spectrophotometrically (580 nm) determining total protein concentration for extracts from each subject (BioRad Protein Assay kit, Hercules, CA), 40 μg protein from each animal was loaded into separate wells of a 15% SDS-PAGE gel. Electrophoretic separation, transfer to polyvinylidene difluoride membranes were performed at 80 and 15 V for ~5 hr and over night, respectively (BioRad, Hercules, CA). Immunolabeling of PVDF membranes was performed according to the method of Laemmli (Cleveland et al., 1977), using 1:1,000 rabbit anti-prepro-hcrt/orx antibody, and later after stripping, membranes were reprobed with 1:2,000 anti-β-actin (Sigma) as a loading control. Both primaries were then bound with 1:5,000 goat anti-rabbit IgG secondary (Santa Cruz Biotech, Santa Cruz, CA). All antibodies were dissolved in a 5% powdered milk blocking solution in Tris-buffered saline (50 mM, 0.9% NaCl). Protein bands were visualized by enhanced chemiluminescence kit (ECL, Amersham/GE Healthcare, Piscataway, NJ) and Kodak BioMax films (Kodak, Rochester, NY). Films were exposed for 40 sec, digitally
Experiment One

Aim

The LHA has long been implicated in motivated behaviors like feeding (Valenstein, 1973) and sexual behavior (Caggiula and Hoebel, 1966). The recent discovery of the hcrt/orx neurons there (de Lecea et al., 1998; Sakurai et al., 1998), and their projections to other midbrain areas implicated in motivation (e.g. VTA, Fadel and Deutch, 2002), suggests these neurons may be one output path by which information about rewards integrated in the hypothalamus affects other parts of the brain’s “motive circuit.” Older studies have shown that copulation and estrous female odors increase cellular metabolism in LHA (Orsini et al., 1985), and more recent experiments find that hcrt/orx neurons show increased fos expression when animals expect food or drug rewards (Harris et al., 2005). The purpose of this experiment is to show that Fos induction in hcrt/orx neurons occurs during male sexual behavior. In this experiment, increased fos expression is presumed to be a marker for recent neuronal activation (Sheng and Greenberg, 1990; Morgan and Curran, 1991) and to implicate increased hcrt/orx transmission in the expression of male reproductive behavior.

Methods

Subjects and Design

Experiment 1a. Adult (~325 g), sexually-experienced male Long-Evans rats (Harlan, Indianapolis, IN, USA) were kept and used in accordance with the National Institutes of Health Guidelines for the Use of Animals, and all procedures were approved by the University’s Institutional Animal Care and Use Committee. In the week prior to testing, all animals were allowed to have 4 daily 1-hr sexual experience sessions with an ovariectomized female brought to estrus by estradiol benzoate (10 µg, s.c.) and
progesterone 48 hr later (400 µg, s.c., Sigma-RBI, St. Louis, MO, USA, given 4 hr before testing). Behavioral testing in all experiments was performed 2 hr into the animals’ usual nocturnal period in their home cage under light from a single 40 watt red incandescent bulb. For copulation experiments, one group (n=6) of males was allowed to copulate in their home cage with an estrous female to a single ejaculation, after which the female was removed. All animals mounted almost immediately and intromitted in less than 4 min. The mean ejaculation latency for this group was 632.6 sec ± 169.64 (SEM, n = 6). A control group (n=6) was used to control for activity level. These animals were visually monitored to verify that they were awake and active for a 15 min period, which corresponded to the duration of the experimental group’s copulation testing period. Cage lids were opened and closed at the beginning and end of this 15 min period, but otherwise animals were left undisturbed. Animals that were behaviorally quiescent (flat body posture, resting, non-ambulatory) for longer than 1 min during the observation period were excluded from the experiment. Sixty min after the start of copulation sessions or control observations, animals were deeply anesthetized, sacrificed, perfused, and their brains removed and prepared for immunohistochemistry as described above.

Experiment 1b. Ten sexually experienced male rats (n=5) as described above were used in this experiment. In addition to these experimental animals, estrous females and adult males as described above were also used as stimulus animals. As above, all experiments were performed 2 hr into the animal’s usual nocturnal phase while the animal remained in its home cage. Experimental animals were exposed to either an estrous female or a male, suspended in a mesh cage immediately above (on top of) the experimental animal’s home cage for 15 min. No physical contact with stimulus animals was allowed. Sixty minutes after stimulus onset, experimental animals were anesthetized with sodium pentobarbital and perfused for immunohistochemical identification of Fos and prepro-hcrt/orx as described above. Antibodies, immunohistochemistry protocols, microscopy and cell counts were also performed as in Experiment 1a.
Primary Antibodies

For detection of Fos, rabbit anti-human Fos polyclonal antibodies were used at a dilution of 1:10,000 (Ab-5, Oncogene, San Diego, CA). Detection of hcrt/orx precursor was performed with rabbit anti-mouse prepro-hcrt/orx polyclonal antibodies diluted to 1:100 (Chemicon, Temecula, CA).

Immunohistochemistry

A detailed description of the immunohistochemistry protocol is given above under General Methods. In this experiment, labeling for Fos was performed first with Ni+++ intensification, giving black reaction product in the nucleus. This was followed by heavy blocking with NGS and labeling for prepro-hcrt/orx using DAB to give a brown reaction product in the cytosolic compartment. The resulting sections thus presented three cell types for quantification: black nuclei labeled for Fos, brown somata and processes labeled for prepro-hcrt/orx, and double-labeled neuronal somata containing both elements (black Fos-labeled nuclei within brown somata labeled for prepro-hcrt/orx). In no case was artifact detected in the form of black labeled somata or brown labeled nuclei.

Cell Counts and Data Analysis

Cell counts were performed under high-magnification using an Olympus microscope and camera on a single section at a consistent rostrocaudal level (~2.45 mm posterior to bregma, Swanson, 2004). In each hemisphere, cells that fell within two 470 x 630 µm counting fields were tagged using digital image analysis software (Image-Pro Plus; MediaCybernetics, Silver Spring, MD) by an examiner blind to experimental conditions. The numbers of Fos-only, hcrt/orx-only, and double-labeled cells with both hcrt/orx and Fos immunoreactivity (ir) were recorded and subjected to a independent samples t-test.

Results

Experiment 1a. The percentage of hcrt/orx-ir cells that showed Fos-ir differed significantly between groups, with copulating animals showing increased Fos-ir in
hcrt/orx cells (Figure 21, Table 1, \(t_{(10)} = 7.71, p<0.01\)). Mean numbers of double-labeled hcrt/orx also differed significantly between groups \((t_{(10)}=6.03, p<0.001\)). The mean number of hcrt/orx-ir neurons in each section did not differ significantly between treatment groups \((269.3 \pm 25.5 \text{ control vs. } 284.8 \pm 20.2 \text{ copulation, } \pm \text{ SEM})\). The total mean number of Fos-ir nuclei (Fos-only + double-labeled hcrt/orx neurons) was significantly greater in copulating animals compared to non-copulating controls \((t_{(10)}=16.97, p<0.001\)). Although copulating animals also showed higher numbers of Fos-only nuclei (those not in hcrt/orx cells), this trend did not achieve statistical significance. Together this suggests that a considerable portion of behaviorally relevant Fos-ir expressed in the LHA is within neurons of the hcrt/orx phenotype.

**Experiment 1b.** The mean number of hcrt/orx-ir neurons in this experiment was 247.60 ±12.47 (Table 1, \(n = 10\)) and did not differ significantly between treatment groups; \(t_{(8)}=1.34, \text{n.s.}\) Mean Fos-ir was higher in animals exposed to estrous females, as compared to the control group exposed to males, though this failed to reach statistical significance. Again, while the mean number of Fos-only cells in female-exposed animals was higher than the male-exposed group, this number failed to achieve significance; \(t_{(8)}=1.01, \text{n.s.}\) Similar to data from the copulation experiment above, mean percent of hcrt/orx+Fos cells in the experimental group was also significantly greater than that in the control group; \(t_{(8)}=3.23, p<0.05\). Therefore, the stimulus properties (e.g. olfactory, visual) of estrous females increased Fos-ir in hcrt/orx neurons of sexually experienced rats above that which is found with exposure to males.

**Discussion**

**Experiment 1a.** The increased Fos-ir in hcrt/orx neurons in the LHA following copulation suggests that activation of these cells accompanies male reproductive behavior (Morgan and Curran, 1991). These data are consistent with earlier 2-deoxyglucose studies reporting increased metabolic activity in LHA following exposure to estrous female odors (Orsini et al., 1985). This effect may reflect enhanced hcrt/orx transmission in hcrt/orx neuron terminal areas like the medial preoptic area (mPOA), where hcrt/orx has been shown to facilitate male copulatory behavior (Gulia et al., 2003). Sex-related
Fos induction in hcr/t/orx neurons is consistent with the notion that the hcr/t/orx neurons are sensitive to natural reinforcers. A recent study demonstrated that Fos-ir increased in hcr/t/orx neurons of rats conditioned to expect a food reward and that this increase in Fos-ir correlated with the animals’ preference score in the conditioned place preference paradigm (Harris et al., 2005). The activation of hcr/t/orx neurons may be a reward-related phenomenon, because the above study showed a lack of robust increases in Fos-ir hcr/t/orx cells in animals exposed to a novel object stimulus. These authors also report percentages of hcr/t/orx neurons expressing basal (15%), novelty-induced (18%), and food-conditioned (50%) Fos-ir that are compatible with those we report here (12% basal vs. 40% copulation-induced). These observations suggest that hcr/t/orx neurons are activated by natural rewards such as food and sex.

*Experiment 1b.* These data show a robust non-specific arousal effect of both male and female stimuli on Fos expression in hcr/t/orx neurons. Beyond this, however, there appears to be a preferential effect of the female stimulus on Fos expression. One explanation for this finding is that it represents a conditioned reward effect that is consistent with those previously demonstrated in the conditioned place paradigm with food and drug rewards (Harris et al., 2005). Animals used in this experiment were sexually experienced, and chemosensory cues emitted by estrous females would presumably signal the impending reward of copulation (Kippin et al., 2003). Together, these data endorse a role for hcr/t/orx neurons in processing reward-relevant information.
Table 1. Cell counts of Fos and hcrt (orx)-ir following copulation or female cues.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hcrt/orx-only</th>
<th>Hcrt-orx+Fos (double-label)</th>
<th>Total hcrt/orx</th>
<th>Fos only</th>
<th>Total Fos</th>
<th>% Double-labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-copulation</td>
<td>237.5±25.5</td>
<td>31.8±5.8</td>
<td>269.3±25.5</td>
<td>22.5±5.9</td>
<td>54.3±11.3</td>
<td>12.0±2.1</td>
</tr>
<tr>
<td>Copulation</td>
<td>170.5±13.7</td>
<td>114.3±12.4</td>
<td>284.8±20.2</td>
<td>32.2±10.3</td>
<td>146.5±6.9***</td>
<td>40.0±2.9**</td>
</tr>
<tr>
<td>Male stimulus</td>
<td>161.2±12.6</td>
<td>105.8±11.8</td>
<td>267.0±7.9</td>
<td>48.0±10.9</td>
<td>153.8±20.4</td>
<td>40.1±3.8</td>
</tr>
<tr>
<td>Female stimulus</td>
<td>105.0±7.5</td>
<td>126.6±17.4</td>
<td>231.6±22.5</td>
<td>70.4±19.1</td>
<td>197.0±25.1</td>
<td>54.6±2.6*</td>
</tr>
</tbody>
</table>

Mean number of hcrt/orx neurons not showing Fos-ir, neurons double-labeled for Fos and hcrt/orx, total hcrt/orx both with and without Fos-ir, individual nuclei showing Fos-ir but not hcrt/orx in surrounding cytoplasm, total neurons showing Fos-ir, and mean percent of total hcrt/orx sample also showing Fos-ir (double labeled).

Mean (±SEM) *p<0.05, **p<0.01, ***p<0.001. Asterisks indicate a significant difference between an experimental group and the control reported immediately above.
Figure 21. Increased Fos immunoreactivity in hcrt/orx neurons during copulation. 
A, hcr/orx neuron (on right) showing Fos-ir, scale bar = 25 μm. B, representative micrograph from right counting field in left hemisphere of non-copulating control male, scale bar = 200 μm. C, micrograph from copulating male, scale bar = 200 μm. Arrows indicate hcr/orx neurons showing Fos-ir, asterisks indicate Fos-ir nuclei in non-hcrt/orx cells, fx = fornix.
Experiment Two

Aim

The activation of hcrt/orx neurons during sexual behavior raises the question of whether hcrt/orx neurons may be one locus at which gonadal steroids act to facilitate the expression of that behavior. While older studies have shown short-latency responses of neurons in the LHA to systemic androgens (Orsini, 1981; Orsini, 1982, 1985), no reports of their genomic effects in hcrt/orx neurons have been presented. The purpose of this experiment is to determine whether gonadal hormones may regulate expression of hcrt/orx in the hypothalamus. Given its prosexual effects hcrt/orx expression should be driven by gonadal steroids.

Methods

Subjects, surgery, and immunohistochemistry, and protein quantification

Experiment 2a. Adult male (~325 g at the beginning of experiments) Long-Evans rats were anesthetized (75 mg/kg ketamine HCl, 10 mg/kg xylazine HCl, i.p.) and castrated. Animals were allowed either 7, 14, or 28 days survival time after surgery before they were anesthetized, perfused and their brains immunostained for prepro-hcrt/orx as above (all groups n=5). These animals were compared to a sham-surgery group that received a scrotal incision under identical anesthesia (n=5). Shams were also sacrificed at 28 days. Cell counts were performed as above at the same coronal plane under lower magnification.

Experiment 2b. Animals identical to those described above were castrated (n=5) or given sham surgeries (n=5) and sacrificed 28 d after orchidectomy. Brains were then blocked and processed for western immunoblot measurement of hypothalamic prepro-hcrt/orx levels as described above.

Experiment 2c. Male rats as described above were given sham surgeries (n=3), or castrated and injected every second day for 28 days with dihydrotestosterone (500 μg,
s.c., n=4), estradiol benzoate (20 μg, s.c., n=3), or oil vehicle (0.1 μL, n=3). Both hormones were purchased from Sigma. Hormone doses were chosen for their ability in previous studies to maintain copulation in castrates (Putnam et al., 2005). On the 28th day after surgeries, animals were sacrificed and their hypothalami blocked for measurement of prepro-hcrt/orx content by western immunoblot as described above.

**Results**

*Experiment 2a.* When compared to sham-treated controls, counts of hcrt/orx-ir neurons showed a significant decrease in cell number 28 days following castration (Figure 22A, $F_{(3,16)}=7.60$, $p<0.005$). This represents a 31.8% decrease in the number of cells in the observed population.

*Experiment 2b.* Subsequent western immunoblot analysis of hypothalamic prepro-hcrt/orx found a significant decrease in mean optical density of hcrt/orx bands from 28 day castrates when compared to sham treated controls (Figure 22B, $t_{(8)}=3.21$, $p<0.05$). Analysis of β-actin loading controls revealed no change in this protein between groups.

*Experiment 2c.* At 28 days following castration or sham-surgery, prepro-hcrt/orx levels in oil-treated animals were significantly lower than those for sham- and E$_2$-treated groups ($F_{(3,9)}=8.47$, $p<0.005$, Fig. 22C). One-way ANOVA with post hoc (Tukey) tests found that prepro-hcrt/orx levels measured in sham- and E$_2$-treated animals did not differ from each other, and that the E$_2$ group did not differ from DHT-treated animals.
Figure 22. Castration decreases hcrt/orx-ir in male rat hypothalamus. A, representative micrographs of hcrt/orx-labeled neurons in one hemisphere show significant decreases in cell number by 28 days after castration. Inset values are mean cell counts for both hemispheres ± SEM, **p<0.005. Scale bar=200 μm, fx=fornix. B, Western blots show significant decreases in hypothalamic prepro-hcrt/orx of 28 day castrates. Each band represents the signal from one animal in either group. Values are mean (± SEM) optical density units for hcrt/orx relative to β-actin, *p<0.05. C, Immunoblots for prepro-hcrt/orx in 28 day castrates show E2 to maintain hypothalamic hcrt/orx content equivalent to that of shams when compared to oil-treated controls. Groups with same lower case letter are not significantly different, p<0.05.
Discussion

The estrogenic regulation of hcr/orx described here provides further evidence for possible hypocretinergic control of male sexual behavior. It is notable that the time-course of hcr/orx loss reported here is compatible with classic behavioral data showing that male sexual behavior takes weeks to decline following castration, and further that it is E$_2$ rather than DHT that is required for reinstatement of behavior (see Hull et al., 2006). Without further experiment, the mechanisms underlying hcr/orx’s continued presence in the absence of E$_2$ can only be speculated. The simplest explanation may be that the lag time to decreased hcr/orx levels mirrors the latency to a readily quantifiable depletion in vesicular stores of the transmitter. As discussed below, the absence of ERs in hcr/orx neurons suggests regulation of hcr/orx expression by inputs from neurons containing those receptors. Inasmuch as neuropeptide synthesis (Enyeart et al., 1987), motility (Shakiryanova et al., 2005), and release are activity dependent phenomena (Fulop et al., 2005), any post-castration decreases in hcr/orx neuronal excitability (e.g. Smith et al., 2002) may negatively affect these processes, slowing the kinetics of peptide release such that reduced levels of synthesis might not be apparent for some time. By whatever mechanism, it seems likely that action of the steroid is the first element of a complex cascade responsible for maintaining basal levels of the peptide.

Just as hcr/orx neurons seem to regulate food intake in response to humoral factors related to energy balance (Olszewski et al., 2003; Burdakov et al., 2006), hcr/orx neurons also appear to be sensitive to the hormonal milieu and may facilitate reproductive behavior in a similar manner. Data presented here suggest that basal hcr/orx expression is maintained by E$_2$. In gonadally intact animals expressing the full complement of hcr/orx, this transmitter would presumably facilitate processing in structures important to male sexual behavior and reward. The hcr/orx neurons enjoy substantial reciprocal connections with areas like the mPOA, bed nucleus of the stria terminalis (BNST), and VTA (Peyron et al., 1998; Sakurai et al., 2005) that are known to be important for expression of male sexual behavior (reviewed in Hull et al., 2006). Decreases in hcr/orx following castration would be expected to diminish an important source of excitatory input to these structures, thereby impairing behavior.
Experiment Three

Aim

Nuclear steroid hormone receptors are ligand-activated transcription factors that regulate the expression of a wide array of neuropeptides (Tsai and O'Malley, 1994; Burbach, 2002). In light of results described in Experiment 2, where the presence of gonadal steroids like E2 appear to maintain basal levels of hcr/orx peptide in hypothalamus, these experiments are intended to show an elegant mechanism for this effect. Presumably, the most efficient way gonadal steroids could affect hcr/orx expression is via nuclear ERs located in hcr/orx-expressing neurons themselves. In these experiments, tissue from the LHA was double-labeled for AR or ER and hcr/orx and melanin concentrating hormone (MCH) which shows a similar pattern of anatomical expression as hcr/orx, and which has similar functional effects.

Methods

Subjects, Antibodies, and Design

Experiment 3a. Brain sections from the LHA were immunolabeled for AR (1:750, Santa Cruz, \( n = 6 \)) or ER\( \alpha \) (1:2500, Santa Cruz, \( n = 9 \)) using nickel-intensified 3’,3’-diaminobenzidine. After labeling for AR, tissue was then labeled for prepro-hcr/orx as described above.

Experiment 3b. Brain sections from the LHA were immunolabeled for ER\( \alpha \) (1:2500, Santa Cruz, \( n = 9 \)) using nickel-intensified 3’,3’-diaminobenzidine. Sections were then double-labeled for prepro-hcr/orx as above.

Experiment 3c. Brain sections from the LHA were immunolabeled for ER\( \alpha \) (1:2500, Santa Cruz, \( n = 4 \)) using nickel-intensified 3’,3’-diaminobenzidine. Sections were then double-labeled for MCH (1:1000, Phoenix Pharmaceuticals, Belmont, CA).
Mapping Cell Populations

After mounting, sections that closely matched one of four coronal layers of the LHA in the atlas of Swanson (2004) were hand-drawn under the microscope using a camera lucida attachment and double- and single-labeled cells in each section tagged. Drawings were digitally scanned and superimposed on atlas levels using Adobe Illustrator, and each population of cells was mapped onto atlas illustrations (Swanson, 2004). In the case of ERα+hcrt/orx labeled sections, numbers of double- and single-labeled hcrt/orx neurons were counted.

Results

Experiment 3a. Nuclear AR was ventrally removed from the main hcrt/orx neuron population, spreading mediolaterally from the arcuate nucleus to the optic tract. In no cases were nuclear ARs and hcrt/orx found in the same neuron.

Experiment 3b, c. While ERα showed a similar pattern of distribution in the ventral extent of the hypothalamus, ERα labeling was more extensive in the ventromedial nucleus (Figure 23). More dorsally, ERα labeling was found in tapered bands of cells that extended medially from the internal capsule beneath the zona incerta to the dorsomedial nucleus (DMH). These ERα-labeled cells were commonly found adjacent to neurons labeled for hcrt/orx or MCH. Label for ERα was seldom found in hcrt/orx neurons (<1% of the population surveyed). When ERα was seen to colocalize with hcrt/orx it was typically in neurons within or just rostral to the DMH. Thus, while very few hcrt/orx neurons in the hypothalamus express ERα, a high proportion (~65%) of those located in or near the DMH do. In no cases was ERα found in neurons expressing MCH; however, as with the hcrt/orx cells, MCH neurons are also intermingled with ERα labeled neurons in the LHA.
Figure 23. ERα is coextensive with hcrt/orx and MCH. ERα nuclei are represented by closed circles, hcrt/orx neurons by open circles, double-labeled ERα+hcrt/orx cells by stars, and MCH by open squares. Numbers are mm caudal to bregma.
Discussion

The manner in which ER activation maintains basal hcrt/orx expression awaits further study; however, it is likely to be driven by afferents from ERα-expressing brain areas that project to LHA (Simerly et al., 1990; Yoshida et al., 2006), particularly those structures found to have some excitatory projections (e.g. BNST, mPOA, Georges and Aston-Jones, 2002; Henny and Jones, 2006). We report no colocalization of AR with hcrt/orx, and, although a few hcrt/orx neurons were ERα-immunopositive, these cells are not numerous enough to explain the marked effects of castration, nor are they in register with those seen to decrease their hcrt/orx content after castration (Figs. 22A, 23). We also report that ERα-ir nuclei and hcrt/orx neurons are often juxtaposed, raising the possibility of local regulation of hcrt/orx neuronal and gene expression activity by neighboring ERα-containing cells. The importance of excitatory local circuit activity of this type has been described in the hcrt/orx system (Li et al., 2002). However, until the requisite anatomical experiments show excitatory synapses made by ERα-containing cells onto hcrt/orx neurons, hormone-dependent, afferent-driven expression of hcrt/orx can not be assumed. Hcrt expression fluctuates diurnally (Taheri et al., 2000), during pregnancy (Kanenishi et al., 2004), and in response to various dietary manipulations (Cai et al., 1999; Griffond et al., 1999). The molecular mechanisms that regulate the dynamics of hcrt expression have not been characterized, for example, functional estrogen response elements or AP-1 sequences have not been reported for the Hcrt promoter. In this way, tracing a path from nucleus to membrane, and naming candidate signaling molecules that may affect hcrt/orx expression is difficult at this time.

Experiment Four

Aim

In light of the above data that suggest hcrt/orx neurons to be activated as part of male reproductive behavior, as well as other studies showing infusions of hcrt/orx into mPOA to have prosexual effects (Gulia et al., 2003), it is important to show that pharmacological impairments in hcrt/orx signaling impair male sexual behavior. Fortunately, several small molecule antagonists at OX receptors exist, and these have been shown to impair the expression of other motivated behaviors like feeding (Haynes et
al., 1999; Haynes et al., 2000). The purpose of this experiment is to evaluate the effects of systemic OX$_1$ blockade on male sexual behavior.

**Methods**

*Subjects, drug, experimental procedure*

Adult male (~350 g at the start of experiments) Long-Evans rats ($n=9$) were given four 1 hr sexual experience sessions with a sexually receptive female during the week prior to behavioral testing. Animals that failed to ejaculate at least once during the first 30 min of the final test were excluded from the experiment. Experience sessions and behavioral testing were performed under 40 watt red incandescent light 2 hr into the animal’s nocturnal period. Tests of copulatory behavior were performed in the male’s home cage and were 30 min in duration. Distinct features of male copulatory behavior (i.e. mounts, intromissions, and ejaculations) were scored by an observer blind to experimental treatments using custom computer software that recorded frequency and latency data for each behavioral event. Thirty min prior to behavioral testing, animals were injected with either the OX$_1$ antagonist SB 334867, $N$-(2-methyl-6-benzoxazolyl)-$N''$-1,5-naphthyridin-4-yl urea (20 mg/kg, i.p.), or DMSO vehicle (0.5 mL/kg). The experiment followed a simple counterbalanced within-subjects design such that 5 animals received drug injections on the first day’s testing and the remaining 4 received vehicle. After a 48 hour drug washout period, animals treated with drug on day one were given vehicle and vice-versa.

**Results**

Compared to vehicle, pretreatment with the OX$_1$ antagonist SB 334867 increased mean latencies to intromit and decreased mean ejaculation frequency (Table 2). Paired samples $t$-tests revealed a significant effect of SB 334867 on intromission latencies ($t_{(8)}=3.31$, $p<0.05$) and ejaculation frequency ($t_{(8)}=2.40$, $p<0.05$). Drug treatment also appeared to produce non-significant increases in mean latencies to mount and ejaculate and decreases in mean number of intromissions.
Table 2. OX₁ antagonist SB 334867 impairs male copulatory behavior.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mount Latency (sec)</th>
<th>Intromission Latency (sec)</th>
<th>Ejaculation Latency (sec)</th>
<th>Mount Frequency</th>
<th>Intromission Frequency</th>
<th>Ejaculation Frequency</th>
<th>PEI (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>22.4±7.7</td>
<td>39.0±9.2</td>
<td>585.7±80.7</td>
<td>13.7±1.5</td>
<td>16.9±1.5</td>
<td>2.1±0.2</td>
<td>485.0±37.3</td>
</tr>
<tr>
<td>SB 334867</td>
<td>48.2±8.8</td>
<td>107.7±20.4**</td>
<td>804.5±103.9</td>
<td>13.7±1.7</td>
<td>12.4±1.7</td>
<td>1.3±0.2*</td>
<td>548.0±44.5</td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM. *p<0.05, **p<0.01

Discussion

The notion that hcrt/orx signaling is involved in reinforcing behaviors like sex is also supported by data showing impairments in sexual behavior following treatment with the OX₁ antagonist SB 334867. At a dose similar to those used to block stress-induced reinstatement of cocaine self-administration (Boutrel et al., 2005), SB 334867 significantly increased intromission latency and decreased numbers of ejaculations, suggesting that blockade of hcrt/orx transmission may affect the incentive properties of estrous females.

It is also notable that SB 334867’s inhibitory effects on sexual behavior are intimated by increased mean mount and ejaculation latencies, and by a longer PEI in drug treated animals. Mean mount latency in animals treated with SB 334867 is more than double that of vehicle-treated controls. Though these means fell just short of achieving statistical significance, their trend is consistent with other measures showing impairments. Interestingly, mean mount frequency was unaltered by SB 334867, and was in fact, identical in both groups. This is reassuring inasmuch it can be used to argue against any drug-induced locomotor impairments that may be confounding: With both treatments, test subjects were able to pursue and mount females with equal frequency.

Experiment Five

Aim

The mesolimbic DA tract, with its origins in VTA and termini in NAc and PFC, has long been viewed as a substrate for motivated behaviors, including sex (Pfaus et al., 1990b). The hcrt/orx neurons innervate DA neurons of the VTA (Fadel and Deutch,
2002) and have been shown to have robust excitatory effects there in vitro (Korotkova et al., 2003). In light of data presented above that shows the hcrt/orx system to be steroid-sensitive and to be activated during sexual behavior, the mesolimbic pathway is one possible downstream effector by which hcrt/orx projections exert their prosexual effects. At this time there are no in vivo reports on the effects of hcrt/orx peptides on VTA DA neuronal activity. To confirm that hcrt/orx does exert neuroexcitatory effects on the VTA DA neurons of live animals the following experiment was performed.

Method

Subjects and Electrophysiology.

Adult male Sprague-Dawley rats (~350 g) were anesthetized with chloral hydrate (400 mg/kg, i.p. for induction, 100 mg/kg/h thereafter for maintenance) and mounted on a stereotaxic instrument. The skull and dura over the VTA were removed. Each animal first received an infusion of aCSF (0.5 μL/5 min; from lambda, AP: +2.6 mm or +3.4, ML ± 0.8, DV -7.0, flat skull according to a stereotaxic atlas (Paxinos and Watson, 1998)), with a 32 ga Hamilton syringe (0.5 μL/5 min) on one side of the VTA. Then 10 min later the cells-per-track sampling procedure was performed on the ipsilateral side of the VTA. Animals next received an infusion of hcrt-1/orx-A (American Peptide, Sunnyvale, CA; 0.014, 1.4, or 140 nmol, dissolved in aCSF) into the contralateral VTA before the cells-per-track procedure was repeated on that side. Injections were counterbalanced by hemisphere and by anterior or posterior injection site. Extracellular single-unit recordings were carried out with single-barrel glass micropipettes (1.5 mm o.d. before pulling; World Precision Instruments Inc., Sarasota, Florida) filled with 2 M NaCl. Electrode impedance ranged from 2 to 4 MΩ at 135 Hz. DA neurons were identified by their positive-negative extracellular action potentials that often have a prominent initial segment/somatodendritic (IS/SD) break, wide action potential duration, slow firing rate, and irregular single spike or burst firing pattern (Figure 24 A, B, Grace and Bunney, 1983). To perform the cells-per-track experiment, the recording electrode was passed through a stereotaxically defined block in the VTA (2.8 - 3.4 anterior to lambda; 0.6-1.0 lateral to midline; 6.5 - 8.5 mm below the brain surface) systematically 6
times. Each identified DA neuron was recorded for 2 to 5 min on-line using the Chart data acquisition system (AD Instruments, Mountain View, CA). The average number of spontaneously active DA neurons encountered per electrode track from each animal (cells-per-track) was the index for VTA DA neuron population activity. Mean firing rate of DA neurons was determined from all DA neurons sampled from all animals within each group.

To test for depolarization inactivation in the 140 nmol hcrt/orx group, apomorphine HCl (20 μg/kg, i.p., Sigma) was administered immediately after the completion of post-hcrt-1/orx-A sampling. Ten min after apomorphine injection, 6 additional electrode tracks were sampled in the side of VTA that previously received hcrt-1/orx-A.

Results
The lowest dose of hcrt-1/orx-A (0.014 nmol, n=4) increased mean VTA DA neuron firing rate compared to vehicle treated controls (Figure 24C, pairwise comparison, $F_{(1,18)}=13.83, p<0.01$, following a mixed 2x3 ANOVA, $F_{(1,10)}=13.67, p<0.005$), but no other dose of hcrt-1/orx-A had this effect. This dose appears responsible for a significant main effect of hcrt/orx on firing rate detected in the 2x3 ANOVA. A post hoc (Tukey) test revealed no significant difference in rate between aCSF-treated control groups across dose (Figure 24D). The 1.4 nmol dose of hcrt-1/orx-A (n=5) significantly increased VTA DA neuron population activity (number of spontaneously active neurons detected in each electrode track, pairwise comparison, $F_{(1,24)}=6.42, p<0.05$, following a significant interaction in a 2x3 mixed ANOVA, $F_{(2,10)}=16.71, p<.001$). The highest dose of hcrt-1/orx-A tested (140 nmol, n=6) significantly decreased the population activity of DA neurons (cells-per-track, one-way repeated measures ANOVA on 140 nmol dose $F_{(2,6)}=12.20, p<0.01$). This decrease was reversed by systemic apomorphine (n=4) injection (Tukey’s post hoc test).
Figure 24. Hcrt/orx regulates VTA DA neuronal activity in vivo.

A, waveform of a typical VTA DA neuron showing characteristic wide action potential and an IS/SD break. B, (upper panel), firing rate and pattern of same vehicle-treated neuron showing typical bursting activity; (lower panel), a fast firing neuron observed following local infusion of 0.014 nmol hcrt 1/orx A (n=4). C & D, dose relationship of locally infused hcrt 1/orx A on the firing rate and population activity of VTA DA neurons. Hcrt 1/orx A (1.4 nmol, n=5) increases population activity. The decreased population activity after 140 nmol hcrt 1/orx A (n=4) was reversed by systemic apomorphine (20 μg/kg). Number inside each bar shows number of neurons recorded. Shown are means ± SEM. *p<0.05, **p<0.01.

Discussion

DA is an important neurotransmitter for reward, incentive motivation, and adaptive behavior (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Wise, 2004). We observed a potent dose-dependent excitatory effect of hcrt/orx on VTA DA neuron activity. This finding supports the role of hcrt/orx in reward or incentive motivation and suggests that the mesolimbic DA system is a locus outside the mPOA where hcrt/orx projections may act to enhance male sexual behavior. At the lowest dose tested (0.014 nmol), hcrt/orx increased firing rate without affecting population activity (cells/track). At the intermediate dose (1.4 nmol), the population activity of DA neurons was increased, indicating that previously quiescent, hyperpolarized neurons were activated. At the highest dose (140 nmol), the population activity of VTA DA neurons was decreased. Interestingly, the hcrt/orx-induced reduction in VTA DA neuron population activity was reversed by acute administration of the DA
agonist apomorphine. In normal animals, apomorphine hyperpolarizes DA neurons by activating autoreceptors and reduces their firing rate and population activity. However, after chronic antipsychotic treatment or repeated treatment with drugs of abuse, apomorphine can reverse drug-induced decreases in population activity (Grace et al., 1997; Shen and Choong, 2006). Apomorphine is thought to reverse depolarization inactivation by repolarizing overexcited cells enough to resume firing. We observe that lower doses of hcrt/orx (0.014 to 1.4 nmol) can increase DA neuron firing rate and population activity and the highest dose of hcrt/orx produces decreased population activity that could be caused by depolarization inactivation. Together, these data suggest that hcrt/orx exerts a dose-dependent excitatory effect on VTA DA neurons. At the present time, it is not known if depolarization inactivation can be achieved by endogenous hcrt/orx.

The excitatory effect demonstrated *in vivo* in the present study is consistent with that observed in a previous *in vitro* study (Korotkova et al., 2003). However, because hcrt/orx is also known to exert presynaptic effects (Haj-Dahmane and Shen, 2005; van den Pol and Acuna-Goycolea, 2006), it is possible that the excitatory effect of hcrt/orx observed in the present study is mediated by altered glutamatergic input as well as a direct membrane depolarization.

The importance of descending pathways from the LHA to VTA was explored in a number of experiments (Bielajew and Shizgal, 1986; Shizgal, 1989; You et al., 2001). These data suggest that increases in NAc DA during hypothalamically-mediated motivated behaviors like copulation (Pfaus et al., 1990b; Wenkstern et al., 1993) or feeding (Hernandez and Hoebel, 1988a; Rada et al., 2005) may rely on these descending projections. That such projections contain hcrt/orx is supported by experiments in which NAc DA efflux is seen to increase following intra-VTA injection of hcrt/orx (Narita et al., 2006). Within the hypothalamus, serotonin (5-hydroxytryptamine, 5-HT) can potently hyperpolarize hcrt/orx neurons in the LHA (Li et al., 2002). Selective serotonin reuptake inhibitors or 5-HT itself, reverse-dialyzed into the LHA near the main population of hcrt/orx expressing cells, reduces basal and female-elicited NAc DA release and impairs copulation (Lorrain et al., 1997; Lorrain et al., 1999). In light of data presented above showing activation of hcrt/orx neurons during copulation, and of VTA DA neurons by
hcrt/orx, we argue that descending hcrt/orx projections to the VTA could mediate sex-related NAc DA release. Further, inhibition of these projections by intra-LHA 5-HT may explain 5-HT’s inhibitory effect on NAc DA release and sexual behavior.

**Experiment Six**

**Aim**

In light of data presented in Experiment Five showing that hcrt/orx may excite VTA DA neurons *in vivo*, it becomes important to link this finding to the motivated behavior in question – copulation. This experiment is designed to provide functional anatomic data showing activation of hcrt/orx-innervated VTA DA neurons during copulation.

**Method**

Adult (~300 g) male Long Evans rats were given four 1 hr sexual experience sessions with a receptive female as above. Prior (1 hr) to anesthesia, perfusion, and preparation of tissue for immunolabeling, animals (*n* = 6) were allowed to copulate to a single ejaculation. As above, animals mounted and intromitted almost immediately, and mean ejaculation latency for this group was 588.50 ± 85.03 sec (SEM). The experimental group was compared to sexually experienced controls that were not given access to estrous females for copulation (*n* = 6). As above, testing occurred 2 hr into the animal’s usual nocturnal period under red incandescent light. After sacrifice, fixation, and cold microtome sectioning (as above), four sections from each animal representing four rostrocaudal levels of VTA were selected for immunohistochemistry. Number of subjects (*n*) used for cell counts at each level is given in Table X. Sections were labeled for Fos (1:7,500, Santa Cruz) with DAB as above. Sections were then labeled for prepro-hcrt/orx (1:500) and tyrosine hydroxylase (TH, 1:2,000, Chemicon) with cyanine-conjugated fluorescent secondaries (1:200, Cy3 and Cy2, respectively, Jackson Immunoresearch, West Grove, PA). Cell counts were performed under high (40x) magnification on a Leica DM 4000B microscope using Stereo Investigator software (MBF Bioscience, Williston, VT) by an experimenter blind to treatment conditions. Stereo Investigator software was used to define a counting field that included all DA cells within each level of VTA. At a
single focal length in each of the four rostrocaudal levels of VTA, five cell types were
tagged: TH-positive neurons, TH-positive neurons showing Fos-ir, TH-positive neurons
showing direct (onto somatic plasmalemma) appositions by hcrt/orx fibers, TH-positive
neurons showing both Fos-ir and hcrt/orx appositions, and finally, solo Fos-positive
nuclei not in TH neurons.

Results

Two-way analysis of variance on mean number of Fos-ir nuclei in non-TH
positive neurons showed significant effects of treatment \( (F_{(1,38)}=38.88, p<0.001) \) and
anatomical level \( (F_{(7,38)}=12.59, p<0.001) \), as well as a significant interaction between
these two factors \( (F_{(7,38)}=7.45, p<0.001) \) suggesting Fos induced during copulation
appears preferentially in the anterior counting levels. This was confirmed by subsequent
one-way ANOVA and post hoc (Tukey) tests that reveal the two anterior most levels
(“Rostral” and “Middle 1”) to have significantly greater numbers of Fos-positive nuclei
\( (F_{(3,24)}=9.48, p<0.001) \). The same was not true for non-copulating controls where basal
Fos-ir did not differ by level (Table 3). The percentage of TH-labeled neurons with
hcrt/orx appositions did not differ by experimental treatment; however, this measure did
show a marked rostrocaudal gradient, with the most rostral level having a significantly
higher percentage of these neurons in both groups (Table 4, \( F_{(3,38)}=133.57, p<0.001) \).
This finding is consistent with the higher density of hcrt/orx fibers we observed in the
anterior VTA. The percentage of TH-labeled neurons showing Fos-ir (but not having
hcrt/orx appositions) differed neither by treatment or by rostrocaudal level. The
percentage of TH neurons showing both Fos and hcrt/orx appositions showed a
significant effect of treatment \( F_{(1,38)}=8.62, p<0.01 \), level \( F_{(3,38)}=4.53, p<0.01 \), and their
interaction \( F_{(3,38)}=4.53, p<0.01 \). One-way ANOVA on means from the experimental
group suggests that copulation-induced Fos-ir in TH neurons with hcrt/orx appositions
occurs most prominently within cells located in the rostral VTA \( F_{(3,24)}=4.85, p<0.05 \).
Table 3. Number Fos-ir nuclei in non-TH positive cells of VTA at four rostrocaudal levels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rostral</th>
<th>Middle 1</th>
<th>Middle 2</th>
<th>Caudal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-copulation</td>
<td>2.8 ± 1.3a</td>
<td>0.4 ± 0.2a</td>
<td>2.6 ± 1.3a</td>
<td>0.6 ± 0.4a</td>
</tr>
<tr>
<td>Copulation</td>
<td>21.2 ± 2.5b</td>
<td>18.5 ± 4.0b</td>
<td>6.3 ± 3.5c</td>
<td>2.0 ± 0.8c</td>
</tr>
</tbody>
</table>

Means (± SEM) with the same lowercase letter do not differ significantly (p<0.05).

Table 4. Percent TH neurons showing hcrt/orx appositions, Fos, or both.

<table>
<thead>
<tr>
<th>Level of VTA</th>
<th>Treatment</th>
<th>TH-hcrt/orx</th>
<th>TH-Fos (non-apposed)</th>
<th>TH-Fos-hcrt/orx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rostral</td>
<td>Non-copulation (n=6)</td>
<td>47.6 ± 6.8a</td>
<td>1.3 ± 0.8a</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td>Copulation (n=6)</td>
<td>53.7 ± 8.7a</td>
<td>0.9 ± 0.9a</td>
<td>9.5 ± 3.9b</td>
</tr>
<tr>
<td>Middle 1</td>
<td>Non-copulation (n=5)</td>
<td>12.5 ± 2.1b</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td>Copulation (n=6)</td>
<td>10.3 ± 1.2b</td>
<td>0.8 ± 0.3a</td>
<td>2.2 ± 0.7b,c</td>
</tr>
<tr>
<td>Middle 2</td>
<td>Non-copulation (n=6)</td>
<td>6.2 ± 0.9b</td>
<td>0a</td>
<td>0a,c</td>
</tr>
<tr>
<td></td>
<td>Copulation (n=6)</td>
<td>6.0 ± 0.9b</td>
<td>0.8 ± 0.4a</td>
<td>0.5 ± 0.3a,c</td>
</tr>
<tr>
<td>Caudal</td>
<td>Non-copulation (n=5)</td>
<td>5.2 ± 0.6b</td>
<td>0a</td>
<td>0a,c</td>
</tr>
<tr>
<td></td>
<td>Copulation (n=6)</td>
<td>3.5 ± 0.4b</td>
<td>0.1 ± 0.1a</td>
<td>0a,c</td>
</tr>
</tbody>
</table>

In each counting column, means (± SEM) with same lower case letter do not differ significantly (p<0.05).

**Discussion**

Consistent with previous findings from other laboratories, copulation produced a marked Fos-induction effect in VTA when compared to non-copulating controls (Balfour et al., 2004). As in this previous study, Fos-ir was found in both DA-containing and non-dopaminergic cells (Table 3, 4). Also consistent with previous experiments was a marked rostrocaudal gradient in Fos-ir in both cell types, with the anterior most counting fields showing the greatest effect. This suggests that the locus of copulation-induced increases in DA transmission lies in the rostral most levels of VTA. It is perhaps unsurprising that this should be the case, as this area of VTA shows the densest plexus of hcrt/orx fibers, as well as the highest percentage of TH-positive neurons in receipt of hcrt/orx appositions (Table 4). Finally, the rostral VTA also shows the highest percentage of hcrt/orx-apposed TH neurons with Fos-ir. In fact, neurons of this type were never observed in non-
copulating animals. Together, these data argue for a hypocretinergic contribution to midbrain DA transmission in the context of motivated behaviors like copulation.

Figure 25. Copulation induces Fos-ir in hcrt/orx-apposed TH neurons. A, Micrographs showing TH-, hcrt/orx-, and Fos-labeling in VTA. Arrows denote Fos-positive TH neurons with hcrt/orx appositions, asterisks mark double-labeled neurons without appositions. Scale bar = 45 μm. B, Detail of Fos-positive TH neurons with arrows indicating sites of hcrt/orx boutons in apposition. C, Coronal sections showing rostrocaudal levels of VTA used in counting (dark-shaded). Numbers at upper left of each section are in mm from bregma. Numbers at upper right are mean (±SEM) estimates of cell density at that level. Box indicates area of micrographs in A.
9. GENERAL DISCUSSION

A Role for Hypocretin (Orexin) in Male Reproductive Behavior

Male rat sexual behavior is an energized goal-directed behavior that emerges from coordinated activity across the neuraxis. Prospective mates must be identified and their reproductive status assessed from afar by olfactory and visual cues. Receptive females must then be pursued and mounted until resolution. Each of these features can be parsed further, and most require action of gonadal hormones on sites both central and peripheral (Larsson, 1979; Hull et al., 2006). The action of these hormones is often said to be permissive or to facilitate activity in each node of the complex circuit responsible for the expression of mating behavior. For example, the presence of E$_2$ in the medial amygdala seems to be necessary for the processing and conveyance of integrated chemosensory information transduced from the olfactory bulb (Wood and Coolen, 1997). The outputs of the medial amygdala, in turn, are required for full expression of male reproductive behavior. Similarly, presence of gonadal steroids in the mPOA is a requirement for competent sexual performance (Johnson and Davidson, 1972; Christensen and Clemens, 1974).

With rare exceptions, the LHA has not been considered to be part of the copulatory circuit. This is understandable, given that there are fewer steroid hormone receptors there, compared to the medial amygdala, mPOA, or BNST (Simerly et al., 1990). However, as data presented in this dissertation make clear, they are present, and early studies have also documented short latency, non-classical effects of steroids on this tissue (Orsini, 1981; Orsini, 1982, 1985). The same investigators also found increased cellular metabolism in the LHA when males were exposed to estrous female odors (Orsini et al., 1985). This is consistent with the pattern of Fos activation recorded in LHA hcr/tx neurons in mated animals. Because the LHA, and indeed the hcr/tx neurons there receive input from medial amygdala (Swanson, 1987; Sakurai et al., 2005), and even direct projections from the olfactory bulb (Price et al., 1991), it is possible to view this modality as a major contributor to the activation seen during sexual behavior. Confirmation of this hypothesis, though, will require reductions in Fos activation to
follow bulbectomy or chemical lesions along the pathways to LHA. Given that the LHA is the site of substantial convergence of projections from several other areas known to be critical for male sexual behavior (e.g. mPOA, Yoshida et al., 2006), it is likely that these structures also convey behaviorally-relevant information that affects hcrt/orx neuronal activity.

As noted previously, the same structures that innervate the hcrt/orx neurons receive return projections from hcrt/orx neurons themselves (Peyron et al., 1998). The mPOA is one of these structures. Since it has been shown that exogenous hcrt-1/orx A injections into mPOA potently facilitate male sexual behavior (Gulia et al., 2003), any activation of hcrt/orx neurons during copulation can be presumed to result in higher peptide release in terminal areas, like the mPOA, thus facilitating copulation. That hcrt/orx tone generally is a requirement for unimpaired expression of sexual behavior is supported by data from Experiment 4, where systemic administration of an OX₁ antagonist produced behavioral impairments. The presence of reciprocal connections between hcrt/orx neurons and other forebrain structures suggests the possibility of modulatory or positive feedback loops. How this pattern of connectivity may be important to goal-directed behavioral activation will be addressed below.

**Endocrine Regulation of Hypocretin (Orexin) and Hypocretinergic Outputs to Midbrain**

Studies on the peptidergic control of sex behavior represent a special circumstance where the relationship between gonadal steroids, peptide neuromodulators, and behavior takes on extra transparency. Neuropeptides are gene products whose expression is often regulated by hormone receptors that are in turn activated by their ligands, the gonadal steroids (Tsai and O'Malley, 1994). This regulation may be direct, as when a neuropeptide gene contains regulatory elements to which an activated nuclear hormone receptor binds (Claessens et al., 2001; Klinge, 2001). It may also be indirect in cases where steroid-sensitive neurons endowed with hormone receptors synapse on peptidergic cells and affect their gene expression activity by signals transduced from membrane-bound neurotransmitter receptors (Polston and Simerly, 2003). By either
route, these kinds of interactions, and their relative contributions to behavior, can be evaluated by elegant experiments pairing classical behavioral endocrine paradigms with straightforward anatomical techniques (e.g. castration and replacement with in situ hybridization or immunolabeling).

Joint control of sex behavior by peptides and gonadal hormones is also unique with regard to the properties of the signaling molecules involved. Unlike small molecule neurotransmitters whose action appears to be more spatially and temporally constrained, peptides are released outside of the synapse, travel widely in the extracellular milieu to act on their receptors at very low concentrations (low pM) many minutes after their release (see Ludwig and Leng, 2006). For an example of the nature of peptide signaling, one need look no further than the adenohypophyseal system itself. By contrast, the typical amino acid (e.g. glutamate) usually acts within microns of the synapse, at much higher concentrations (low to mid-μM), and for a much shorter duration before uptake and inactivation (see Seal and Amara, 1999; Danbolt, 2001). In this way, peptides possess a neurohormonal character not unlike that of the gonadal steroids that seem to regulate them, and like steroids, their effects on behavior can be long-lived and profound.

Bearing these features of peptidergic signaling in mind, the approach taken in these experiments has been to acknowledge the possible role of neuropeptides as intermediaries in the hormonal control of male reproductive behavior. Because gonadal steroids are a requirement for copulatory behavior, there are at least two simple scenarios that can emerge from this hypothesis: The first is one in which basal expression of a sexually facilitative peptide is maintained by gonadal steroids. Such a model predicts gene expression and copulatory behavior to decline concomitantly following castration and that these changes be reversible by hormone replacement. Additionally, one might anticipate that acute treatment of castrates with exogenous peptide might reverse any behavioral impairments. Obviously, because a neuropeptide gene would be one of many genes up- or downregulated following castration, including some that may participate in elaborating other complex behaviors, it would be remarkable to observe complete restoration of behavior following replacement of a single gene product. Nonetheless, any significant recovery of function may index that peptide’s contribution to the behavior.
The second scenario would be one in which gonadal steroids suppress the expression of a peptide that inhibits sex behavior. In this model, castration would allow one to observe an inverse relation between the peptide’s expression and copulatory behavior: removal of steroids would disinhibit peptide expression, and behavior would become impaired. As above, these changes should reverse with hormone replacement, and exogenous peptide should easily impair the behavior of gonadally intact males. Leaving aside for the moment questions about long-loop endocrine feedback effects, the above model provides the most basic framework from which to begin an analysis of whether hormones influence reproductive behavior via specific gene products.

Data presented above permits the addition of hcrt/orx to the list of gene products whose expression appears to be affected by gonadal steroids and whose exogenous administration affects male reproductive behavior (Table 5). In the case of hcrt/orx, it was found that the peptide is downregulated following castration, and that treating castrates with either DHT or E\textsubscript{2} returns peptide content of hypothalamic tissue to basal levels. In light of data showing exogenous hcrt/orx to enhance motivation and performance (Gulia et al., 2003), and OX\textsubscript{1} receptor antagonists to impair behavior of intact animals, hcrt/orx falls into the first class of steroid-peptide-behavior relationship, one where levels of a prosexual gene product wane following castration. This immediately raises the question of whether exogenous hcrt/orx by itself would be able to restore behavior in castrates. Only further experiment will tell.

Another question raised by the effects of castration and hormone replacement on central hcrt/orx levels involves its time-course. Decrements in hcrt/orx are not seen until the fourth week following castration. As noted previously, this squares nicely with classic behavioral data showing that behavioral impairments appear around this time, but what is the mechanism for hcrt/orx’s longevity in the absence of gonadal steroids? Synaptic hcrt/orx would be degraded by metalloendopeptidases in relatively short order (Carpentier et al., 2003), so the simplest explanation is that the time course of hcrt/orx losses in tissue mirrors that of vesicular hcrt/orx release. Unfortunately, neurochemical studies on the dynamics of hcrt/orx release and metabolism do not exist, so for the time being this notion is speculative. A second, more nuanced explanation builds on this
hypothesis as well as the findings with regard to steroid hormone receptor expression in hcrt/orx neurons.

Because hcrt/orx exerts prosexual effects when given exogenously, and neurons containing the peptide are active during copulation and project to forebrain structures important to sexual behavior, it would be reasonable to expect these neurons to be endowed with steroid hormone receptors. For the most part this is not the case. While data presented here show that AR and ERα are in close proximity to hcrt/orx neurons, only a small number of them actually co-express ERα. Those neurons that do appear in a spatially restricted compartment of DMH and in the transitional tissue just rostral to this nucleus. Whether or not this small population of cells exerts any behaviorally relevant effects requires further study. Interestingly, immunohistochemical labeling for hcrt/orx post-castration found that decreases in the peptide were found broadly across the entire population of hcrt/orx neurons, and not just those in the region found to contain ERα.

In the absence of nuclear steroid hormone receptors, by what mechanism do gonadal steroids potently influence hcrt/orx levels? There at least two possibilities: the more exotic being that hcrt/orx expression is sensitive to signaling by non-classical, membrane bound steroid hormone receptors that have only recently begun to be
characterized (Falkenstein et al., 2000; Heinlein and Chang, 2002); the most straightforward, however, is that hcrt/orx expression is an afferent driven phenomenon. The afferents in question must then come from neurons containing steroid hormone receptors (for a discussion of this arrangement in the periphery see Breedlove, 1994). These neurons could either be local (within the LHA), as both AR and particularly ERα were detected in quantity in the vicinity of hcrt/orx neurons, or they could be from other parts of forebrain known to both express steroid hormone receptors and project to hcrt/orx neurons in the LHA (e.g. mPOA, BNST, medial amygdala, Yoshida et al., 2006). Any excitatory projections from these structures would presumably be able to influence gene expression via classical Ca<sup>2+</sup>-mediated signal transduction pathways (Hardingham et al., 2001; Deisseroth and Tsien, 2002).

This hypothesis suggests a mechanism (alluded to above) for the longevity of hcrt/orx peptides after castration. If hcrt/orx expression is indeed driven by excitatory afferents from steroid-sensitive brain areas, it may be anticipated that afferent drive would decrease following castration. Inasmuch as neuropeptide synthesis (Enyeart et al., 1987; Enyeart et al., 1990), motility (Shakiryanova et al., 2005), and release are activity dependent phenomena (Fulop et al., 2005), any post-castration decreases in hcrt/orx neuronal excitability (e.g. Smith et al., 2002) may negatively affect these processes, slowing the kinetics of peptide release such that reduced levels of synthesis might not be apparent for some time. Along these lines, a delayed course to decreases in neuronal nitric oxide synthase following castration has been reported recently (Sato et al., 2005). By whatever mechanism, it seems likely that action of the steroid is the first element of a complex cascade responsible for maintaining basal levels of the peptide.

A final question about the possible afferent control of the hcrt/orx system by steroid sensitive areas is simply, why did it evolve in such a way – wouldn’t it have been simpler to place hormone receptors in the hcrt/orx neurons themselves? The answer to this question can only be speculative, but it rests on the nature of hcrt/orx’s relation to motivated behavior. This theme will be elaborated on shortly, but in brief, the hcrt/orx system has been viewed in the feeding literature as a second order effector system – an entity once removed from the sites of homeostatic signal transduction in the arcuate nucleus (Schwartz et al., 2000). As other specific behavioral functions are required of the
The hcrt/orx system (e.g. reproduction), removing the transduction apparatus for gonadal hormones or adiposity signals to earlier levels of processing allows the system flexibility and integrative capacity, such that no ‘channel’ of behaviorally-relevant information may exert hegemony over another from the start. For example, were hcrt/orx expression and neuronal activity wholly dependent on gonadal steroids, and tightly regulated by hormone receptors in each neuron, loss of tonic steroid action, as after castration, would result in hcrt/orx being unable to fulfill its other behavioral functions. Castration would thus result in an animal that was narcoleptic and aphagic. Rather, the system should be able to integrate information related to each behaviorally-relevant input and engage behaviors that are appropriate to that information.

A Theoretical Model for the Role of Hypocretin (Orexin) in Goal-Directed Behavior

The hcrt/orx system has been implicated in arousal and wakefulness (Chemelli et al., 1999; Willie et al., 2003; Mochizuki et al., 2004), feeding and food-seeking (Sakurai et al., 1998; Dube et al., 1999; Yamanaka et al., 2003; Mieda et al., 2004), drinking (Kunii et al., 1999), drug and alcohol seeking (Harris et al., 2005; Lawrence et al., 2006), general locomotor activation (Mileykovskiy et al., 2005; Kotz, 2006), and now sexual behavior. This catalog of energized, often-goal directed behaviors has prompted some to suggest that the hcrt/orx system is a general purpose, non-specific behavioral activation system that is closely tethered to the midbrain (DA) circuitry (Siegel, 2004; Harris and
Aston-Jones, 2006). This view likens the hcrt/orx to a behavioral ‘on switch’ where more hcrt/orx transmission results in more behavior – whatever the behavior happens to be. This hearkens back to Eliot Valentstein’s ideas about “stimulus bound behavior” that can be activated by electrical stimulation of cites in the LHA where we now know hcrt/orx neurons to reside (Valenstein et al., 1970). Valentstein found that when this tissue was stimulated, animals would actively engage in whatever behavior was called for given the environment at hand: were food present, the animal would eat; were water present he would drink; were a receptive female present, vigorous copulation would ensue. Returning to that literature on LHA electrical stimulation-evoked behavior, in almost every case there is a contemporary study demonstrating that central hcrt/orx administration will evoke the same behaviors.

Table 6. LHA stimulation and hcrt/orx evoked behavior

<table>
<thead>
<tr>
<th>Behavior</th>
<th>LHA Stimulation Citation</th>
<th>Central Hcrt/Orx Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding</td>
<td>(Hoebel and Teitelbaum, 1962)</td>
<td>(Sakurai et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>(Margules and Olds, 1962)</td>
<td>(Dube et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>(Hoebel, 1969)</td>
<td>(Haynes et al., 1999)</td>
</tr>
<tr>
<td>Drinking</td>
<td>(Mogenson, 1966)</td>
<td>(Kunii et al., 1999)</td>
</tr>
<tr>
<td>Copulation</td>
<td>(Vaughan and Fisher, 1962)</td>
<td>(Gulia et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>(Herberg, 1963)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Caggiula and Hoebel, 1966)</td>
<td></td>
</tr>
<tr>
<td>Locomotion</td>
<td>(Rolls and Kelly, 1972)</td>
<td>(Kotz et al., 2002)</td>
</tr>
<tr>
<td>Gnawing</td>
<td>(Valenstein et al., 1968)</td>
<td>(Espana et al., 2002)</td>
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<td>(Valenstein, 1973)</td>
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From this comparison, and from the anatomical data showing stimulation sites and hcrt/orx neurons to be in the same location, it seems apparent that it was this population of neurons that was being stimulated and that accounts for the behavioral effects arrived at in these early investigations. Further, the same conclusions may be drawn about the “stimulus-bound” nature of central hcrt/orx administration: When feeding is assessed the peptide appears to be orexigenic; when drinking is observed, a dipsogen; copulation, an aphrodisiac. Even gnawing of non-food objects (wood blocks) – what was called
“displacement behavior” – can be elicited by LHA electrical stimulation or by central hcr/orx treatment.

In light of these data it is rather tempting to say that hcr/orx is indeed a general-purpose behavioral activation system. However, before such an assertion is accepted, the artificiality of these experimental manipulations must be acknowledged and taken into account. Electrical stimulation and bolus injections of exogenous peptide can be argued to ‘hotwire’ a brain system that evolved to express individual behaviors in response to specific sets of environmental cues and neurohumoral contexts. How might the system regulate behavior under normal circumstances when not subjected to experimental manipulations? It seems possible that it is the combination of active inputs to the LHA hcr/orx system that determines which behaviors are enacted. With this in mind, a “three layer” model for hcr/orx control of adaptive behavior is proposed. This model holds that individual behaviors emerge from the hcr/orx system as the system integrates behaviorally relevant sensory and neurohumoral input from other forebrain areas and the internal milieu. The products of this integrative processing are then brought to bear on effector systems, principally in the midbrain, which coordinate arousal state and locomotor output in a manner that is appropriate to external/environmental and internal/hormoral cues for a particular behavior. Each layer of this model will be treated in turn below.

The “Detector” Layer

The detector layer consists of those structures and organs tasked with transducing and integrating behaviorally-relevant sensory input from various modalities. In addition to conventional sensory information, this layer also contains elements that detect and respond to humoral factors (i.e. adiposity peptides, hormones). To simplify the diagram, the many subfeatures and sensory organs (e.g. olfactory bulb, retina) that compose the detector layer have been collapsed and referred to by the character of the information they convey. Also, so that the nature of these inputs and the information they provide the hcr/orx system may be highlighted and tied to existing experimental data, each channel
of input will be addressed with respect two classes of behavior that are well understood: feeding and male reproductive behavior.

![Three Layer Model for hypocretinergic control of adaptive behavior](image)

**Fig. 26. Three Layer Model for hypocretinergic control of adaptive behavior**

*Feeding.* Chemosensory information about the olfactory and gustatory qualities of food reach the LHA directly from the olfactory bulb and caudal brainstem (e.g. nucleus of the solitary tract, NTS, Norgren, 1976; Price et al., 1991; Bernardis and Bellinger, 1993; Scott and Giza, 2000). The former class of information is subjected to further processing in the medial amygdala (Swanson, 1987), while the latter emerges from the NTS in a manner that is informed by blood- and gut-borne satiety signals that ultimately control meal size and feeding architecture (Rolls, 1999, 2005; Schwartz, 2006). Together these inputs would signal the close proximity of consumables, and in an unsated animal, engage a behavioral response. This is not to say that visual inputs relaying information about food do not influence LHA neuronal excitability, as evidence suggests they do in
primates (Rolls et al., 1976; Ono et al., 1989); however, in rats olfaction is likely to be dominant.

Humoral information not only influences outputs from brainstem but also structures in the forebrain like the arcuate nucleus (Elmquist et al., 2005). As discussed in the general introduction under hcr/orx and feeding, the standard model of this behavior hinges on leptin or ghrelin-sensitive neurons in the arcuate, which in turn send peptidergic projections to hcr/orx neurons (Zigman and Elmquist, 2003). Under conditions of negative energy balance, the low ratio of leptin to ghrelin is expected to enhance release of orexigenic peptides (e.g. NPY) onto hcr/orx neurons (Elias et al., 1998; Elias et al., 1999). These neurons may then coordinate arousal state and locomotor output in order to seek and procure food (Coppari et al., 2005). In addition to peptidergic energy signals, neurons of the LHA, and indeed hcr/orx neurons, are sensitive to glucose itself (Oomura et al., 1969; Oomura, 1988; Burdakov et al., 2006). These, and other experiments have shown that increased blood glucose concentrations silence LHA hcr/orx neurons in a manner that would be expected to terminate feeding or food seeking locomotor activity. As expected, blockade of OX1 receptors hastens the onset of satiety and reduces food consumption (Haynes et al., 2000; Rodgers et al., 2001).

Feeding behavior and attendant locomotor activation both show strong circadian rhythmicity (Mistlberger, 1994; Strubbe and Woods, 2004). Food itself can entrain a recently described oscillator in the DMH that interfaces directly with hcr/orx neurons (Mieda et al., 2006). Neurons in the DMH are also entrained by more conventional photic information, as it is passed from the retinohypothalamic tract to the suprachiasmatic nucleus, and relayed to hcr/orx neurons via the subparaventricular zone and DMH (Chou et al., 2003; Saper et al., 2005b). By this arrangement, hcr/orx neurons are subject to a redundant system of zeitgebers that permits an animal to become awake, active, and searching for food at times described by both circadian and circannual cues. For example, the hcr/orx system is privy to information, not only about whether it is an appropriate time of day to feed (when food sources are abundant and risks of predation are acceptable), but also whether it is the appropriate time of year (longer nights) to begin hoarding food and consuming extra calories (Kilduff et al., 1989; but for discussion see Saper et al., 2005b).
Sexual behavior. As with food odors, the LHA of male rodents is likely to gather sexually-relevant olfactory information about the immediate presence of receptive females directly from the olfactory bulbs, and indirectly by way of the medial amygdala (Wood and Coolen, 1997). This input would be expected to engender activation among hcrt/orx neurons and heighten arousal during the pursuit and anticipatory stages of sexual behavior. In addition, somatosensory information integrated in the mPOA probably contributes to hcrt/orx activation and arousal during both anticipatory and consummatory phases (Pehek et al., 1988; Warner et al., 1991; Moses et al., 1995).

The activity of these structures is under hormonal control (Putnam et al., 2001; Hull et al., 2006), and these structures are in turn likely responsible for the hormone-dependent production of hcrt/orx peptide in the hypothalamus (Experiment 2, above). Since hcrt/orx transmission appears to enhance sexual motivation (Experiment 4, above), basal levels of the peptide maintained by input from steroid-sensitive brain areas should facilitate sexual behavior.

Remarkably, male sexual behavior displays circadian rhythmicity that is disrupted by lesions of the SCN (Sodersten et al., 1981). Presumably, such behavior arises from the same SCN-DMH-LHA connections that appear to be important for circadian food anticipatory behavior. As with feeding, circadian rhythms in mating behavior would also be subsumed under circannual rhythms that dictate the mating season (longer days) in some species.

The “Selector” Layer

The function of the LHA appears to be integrative in nature. It receives convergent input from across the body and brain about both the external environment (via sensory systems), and the internal milieu (via hormones and energy homeostasis peptides, Swanson, 1987; Swanson, 2000). Given the responsiveness of neurons in the LHA to these diverse inputs, it seems likely that the integrative capacities of this structure may be thought of somewhat like a checklist: In states of negative energy balance (low leptin to ghrelin ratio), when the time is right (photic inputs), and food odors are present
(chemosensory input), the LHA makes this tally, and feeding and food seeking will proceed. Likewise, given hormonal competence during mating season, presence of estrous female odors will arouse sexual appetite. Specificity in behavioral output may arise with the combination of neurohumoral inputs that is unique to each stimulus (e.g. feeding vs. mating). The LHA must weigh each input and engage behavior in accordance with homeostatic needs such that, say, mating doesn’t come at the expense of feeding at times when caloric needs are more pressing. Likewise, the system, when it has reached satiety with regard to one behavior, shouldn’t then be insensitive given the chance to satisfy another. Rats are still easily roused from their post-ejaculatory quiescence when presented with food and they eat normally (Everitt et al., 1987). Thus, the operations of the ‘general-purpose arousal system’ of the LHA don’t necessarily generalize across behavioral categories, and the system may be activated by a new combination of inputs.

Another architectural feature of this layer, one that has emboldened the use of the term “selector”, are the reciprocal connections between the hcrt/orx neurons and their sources of afferent input that are hypothesized to govern the category of behavioral output that is expressed by an animal (Peyron et al., 1998; Nambu et al., 1999; Marcus et al., 2001; Sakurai et al., 2005; Yoshida et al., 2006). This raises the possibility, given the neuroexcitatory character of hcrt/orx peptides, of positive feedback between hcrt/orx neurons and their sources of afferent input. This would permit the hcrt/orx neurons, once excited by, say, inputs from structures related to feeding, to enhance processing in those structures by return projections. In this way the hcrt/orx could ‘cement’ activity in structures across the brain, binding these activities into a single neural expression that is associated with an individual behavior. This process of circuit stabilization is thought to underlie hcrt/orx’s wake-promoting actions in the midbrain, with which the hcrt/orx system enjoys the same reciprocal connections (Saper et al., 2001; Saper et al., 2005a). There seems to be no reason that this model cannot be extrapolated to other forebrain structures that are connected to the hcrt/orx neurons in a similar fashion. However, rather than determining whether any behavior at all will be emitted (sleep vs. waking), this pattern of forebrain connectivity would have more subtle influence, determining which behaviors are enacted during wakefulness. It should be made clear, however, that discrete behavioral programs do not emerge in toto from the tissue of the LHA, but rather that this
structure, after integrative computations, is able to endorse or select a unique behavior by facilitating processing in other brain structures principally tasked with handling neurohumoral input relevant to a particular behavior.

This “selector” view is interesting because it allows for a number of testable hypotheses. If it is the unique combination of active inputs to the hcrt/orx neurons that is responsible for unique behavioral outputs, this can be made apparent by a number of means. Fiber-sparing lesions in the “detector” layer should negatively affect patterns of immediate early gene expression in hcrt/orx neurons of animals experiencing behaviorally-relevant stimuli. For example, one would expect reduced Fos expression in animals subjected to mPOA or medial amygdala lesions and exposed to estrous female odors. This approach has the advantage of revealing the granular nature of Fos responses in hcrt/orx neurons. It has already been suggested that there are “arousal-related” and “reward-related” populations of hcrt/orx neurons (Harris and Aston-Jones, 2006), but a further parsing with regard to subpopulations involved in a specific behavior may be possible. This may seem like an atavistic return to the discarded “center hypotheses” of motivated behavior, but thus far hodological data (above) support the idea of a non-homogenous functional anatomy of hcrt/orx cells. More likely than “centers,” for a particular behavior, gradients of neurons may be resolved where are overlapping several sources of afferent input.

In addition to altered immediate early gene expression patterns, lesions within the “detector” layer will obviously cause behavioral impairments. What is often interesting about these impairments is that they do not abolish behavior completely, but only certain features of it. One example is the “displacement behavior” that follows attempts at copulation in mPOA-lesioned males (Hansen and af Hagelsrum, 1984). It is similar to that had by LHA electrical stimulation (Valenstein, 1973) in that such animals will often show energized drinking and scratching behavior, suggesting that, while arousal and behavioral output are increased, they are misdirected. These data argue for the convergent and integrative nature of LHA processing where the loss of one critical input may change the animal’s ‘vector’ and the specificity of its response. While the remaining inputs are able to engage the system, increasing its activity and thus that of the animal, critical information about which goal should be the behavioral focus is has been degraded by
lesion. Under such circumstances, the next most active set of inputs (e.g. those for drinking) may become dominant in the stream of behavioral expression following haphazard and incomplete integration with those related to a separate behavior (e.g. copulation).

The “Effector” Layer

This layer consists mainly of aminergic midbrain and brainstem structures involved in vigilance, arousal, and locomotor activation and that receive hypocretinergic inputs (see section on hcrt/orx and wakefulness above). Because this dissertation is specifically concerned with motivated behavior, the VTA is viewed as the chief means by which behaviorally relevant information integrated by the LHA and passed on by hcrt/orx neurons is translated into observable behavior. This is to say that once information converging on the LHA is judged to be actionable, the hcrt/orx system functions as an output that recruits DA neurons of the VTA to effect a behavioral solution to the results of processing in the LHA. As shown in Experiment 5, hcrt/orx potently influences DA neurons in the VTA. The exact nature of what this increased DA transmission may mean for behavior is the subject of ongoing debate (see preceding section, What is the role of mesolimbic dopamine in motivated behavior?), but it can be agreed upon that the correlation between hcrt/orx activity, dopaminergic transmission, and activation of goal-directed behavior is a positive one.

Together the Three Layer model demands that discrete pathways may be followed anatomically from the first layer to the last, and that the origin of these pathways in the forebrain may be related to a particular behavior. This work has been done, in parts, by two laboratories. A putative circuit for male sexual behavior should begin in the mPOA and have projections to hcrt/orx neurons in the LHA. Robert Scammell’s group injected anterograde tracer into the mPOA and mapped synaptic appositions with hcrt/orx neurons (Yoshida et al., 2006). They found that almost 50% of hcrt/orx neurons received inputs from their injection site in the mPOA. These neurons appeared in a group just dorsomedial to the fornix that extends over and partially through the DMH to near the top of the third ventricle. Scott Zahm’s group applied retrograde tracer to the medial aspect
of the VTA and observed labeled cell bodies in the LHA (Geisler and Zahm, 2005). While many were distributed more laterally than hcrt/orx neurons found to receive inputs from mPOA, there is a distinct band of neurons that is in register – arcing above the fornix and through the DMH to the top of the third ventricle. This may represent a simple, disynaptic pathway by which information regarding copulatory cues accesses midbrain DA circuitry that participate in elaborating the behavior.
“I hope…to persuade some readers that limbic structures outside the hypothalamus are crucially involved in the expression of sexually motivated behavior, as they are in other motivated responses, and that the challenge is to define the ways that essential, hormone-dependent hypothalamic mechanisms, which are unique to sexual responses, interface with this wider system involved with incentive motivational processes.”

- Barry Everitt (1990)

When this was written, the discovery of hcrtr/orx was slightly less than a decade away. That discovery is currently reshaping our understanding as the peptides fit together the diverse brain areas that make up pieces in the puzzle of motivation. Everitt’s challenge has guided the work of this dissertation. Like any idea in science, the work here and the Three Layer Model will require expansion and revision, but they may provide a more holistic sketch of how the brain processes an incentive stimulus and moves the body to acquire it. Presented was a hormone-dependent system that is also responsive to proximal reproductive cues, whose transmitter facilitates that behavior and affects the seat of incentive motivational processes in the midbrain. Researchers on the field of addiction have provided a detailed account at every level of analysis of how drugs of abuse co-opt the ‘natural reward circuitry.’ Strangely, we know much less about how natural rewards affect the ‘natural reward circuitry.’ This work is one small step in that direction.
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BIOGRAPHICAL SKETCH

John Whitney Muschamp was born in St. Paul, Minnesota in 1974. He attended St. Paul Academy and St. Paul Central High School. He briefly attended the University of Minnesota, taking coursework in film and theatre. Before matriculating at Gettysburg College, he lived in Los Angeles. After completing the doctorate, he will begin a research fellowship in the Department of Psychiatry at Harvard Medical School.

2005-2007 Ph.D., Neuroscience, Florida State University, Tallahassee, FL.
2001-2005 M.A., Behavioral Neuroscience, University at Buffalo, SUNY, Buffalo, NY.
1998-2001 B.A., Magna cum Laude, Psychology, Gettysburg College, Gettysburg, PA.

Funding


Research Reports


**Chapters and Reviews**


**Academic Honors, Memberships, and Awards**

Phi Beta Kappa, National Honor Society (2001)
Psi Chi, National Psychology Honor Society (2000)
Eta Sigma Phi, National Classics Honor Society (2000)
Society for Neuroscience, Student Member (2001)
Society for Behavioral Neuroendocrinology, Student Member (2001)
J. Douglas Shand Award for Faculty-Student Research, Gettysburg College (2001)
Student Research Award, Office of the Provost, Gettysburg College (1999)

**Research Assistantships**

2006 Graduate Research Assistant, Department of Psychology, Princeton University, Princeton, NJ.

2005 Graduate Research Assistant, Research Institute on Addictions, University at Buffalo, SUNY, Buffalo, NY.

2000 Undergraduate Research Assistant, Behavioral Pharmacology Research Unit, Department of Psychiatry, School of Medicine, Johns Hopkins University, Baltimore, MD.