Modulation of Prepulse Inhibition and Ingestive Behavior by Nucleus Accumbens AMY1 Amylin Receptors

By

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ABSTRACT

Amylin is a feeding-modulatory pancreatic peptide that crosses the blood-brain barrier to access receptors localized within specific sites across the neural axis. Among the densest concentrations of amylin receptors (AMY-Rs) is found in the nucleus accumbens shell (AcbSh). The AcbSh represents an interface between the limbic cortex and di/mesencephalic behavior effector systems, and plays a role in both complex motivated behaviors, and more basic information-processing functions (namely, pre-attentional sensorimotor gating). Hence, we investigated the role of AcbSh AMY-Rs in both functional realms.

In the first set of experiments, we measured sensorimotor gating using the prepulse inhibition (PPI) paradigm, in which sub-threshold auditory stimuli ("prepulses") negatively modulate the behavioral responses to subsequent superthreshold stimuli. AcbSh amylin infusions partially reversed PPI deficits created by the psychotomimetic drug amphetamine. These effects were limited to the AcbSh, where we also found high levels of mRNA for RAMP-1 and CT-R, the two genetic components of the high-affinity AMY1 amylin receptor. In addition, blockade of AcbSh AMY-R receptors created PPI deficits that were reversed by the antipsychotic haloperidol. This suggests that there is an endogenous amylinergic 'tone' in the AcbSh that regulates PPI via interactions with dopamine receptors. Because AMY-Rs are almost absent outside of the medial AcbSh, drugs affecting AMY1 receptors could provide a means of selectively targeting the ventral striatum and thereby avoid dorsal striatum-mediated side effects. Amylin is also a satiety factor, so its use as an adjunct antipsychotic could counteract the diabetes, obesity, and other metabolic side effects commonly seen with existing antipsychotic treatments.

In the second set of experiments, we explored AcbSh amylin's role in appetitively motivated behavior, by examining interactions between AcbSh-localized AMY-Rs and mu-opioid receptors (μ-OR). Amylin infusions in the AcbSh, but not the anterodorsal striatum, potently decreased μ-OR stimulation-induced hyperphagia at doses far lower than needed to reduce non-opioid-driven feeding. Conversely, blockade of AcbSh AMY-Rs significantly reversed the ability of pre-feeding to suppress μ-OR stimulation-induced hyperphagia. This effect of AMY-R blockade was present only after eating, when circulating amylin levels are highest. This represents the first demonstration that endogenous AMY-R signaling negatively modulates μ-OR-mediated appetitive responses at the level of the AcbSh.

Taken together, the results from this thesis suggest that AcbSh AMY1 stimulation may have antipsychotic potential, and that an endogenous telencephalic amylinergic 'tone' regulates sensorimotor gating and appetitive behavior.

Chapter I:

Introduction

Overview

This project brings together several converging lines of evidence supporting the general notion that amylin receptors within the nucleus accumbens (Acb) are functionally relevant targets for the development of new pharmacotherapies to modulate core Acb-based processes of relevance to multiple, divergent psychiatric illnesses. First, I will review schizophrenia to highlight the severity of the illness, the role of the Acb and related circuitry, and the need for identification of new treatments. Schizophrenia is characterized by impairments in sensorimotor gating, which is an important aspect of information-processing. Therefore, I will discuss prepulse inhibition (a behavioral paradigm used extensively in this thesis), a functional measure of sensorimotor gating that has high predictive validity for whether a compound has antipsychotic properties. I will next review the neurobiology of amylin and its receptors - which was the neuropharmacological focus of this thesis - with an emphasis on high-affinity amylin receptors in the Acb. The next topic I discuss will be ingestive behavior, a function separate from prepulse inhibition but which also strongly involves the Acb, particularly zones of the Acb with high AMY-R binding. Finally, I will discuss Acb mu-opioid receptors, which play a role in regulating feeding behavior and feeding dysregulation as in binge-type eating disorders, and which may interact with amylin receptors. To summarize, this thesis investigates how amylin might modulate two distinct functional domains via actions in the Acb: prepulse inhibition, and feeding.

Schizophrenia

Schizophrenia is a serious disorder that affects approximately 0.5-1.0% of the population worldwide (Saha et al. 2005). While schizophrenia is highly heterogeneous, hallmark symptoms are categorized into clusters of positive, negative, and cognitive symptoms. Positive symptoms are traits that are present in the patient but not present in those unaffected by the disorder. Hallucinations (aberrant perceptions) and delusions (fixed, false beliefs) are classical positive symptoms of schizophrenia (Kapur 2003; Keller et al. 2011; Tandon et al. 2008). The subject matter of hallucinations and delusions varies between individuals and is influenced by the individual's cultural context (Kapur et al. 2005). When a trait is generally present in the normal population but not in those with schizophrenia, this is termed a 'negative symptom'. Negative symptoms of schizophrenia include anhedonia (an inability to experience pleasure), avolition (diminished motivation), and blunted affect (Keller et al. 2011; Tandon et al. 2008). Schizophrenia is also characterized by cognitive dysfunction, which is sometimes included as a positive symptom (Carpenter 2004; Jablensky 2006; Tandon et al. 2008; Weickert et al. 2000). Schizophrenia is thought to be a neurodevelopmental disorder, with symptoms often manifesting in late adolescence to early adulthood (Tandon et al. 2008). There is also strong evidence indicating that schizophrenia has a genetic basis and that risk for the disorder is partly heritable (Cardno and Gottesman 2000; Williams et al. 2009). The development of schizophrenia also depends on the environment, with adverse life experiences increasing the risk of developing the disorder, and stress contributing to relapse (Agid et al. 1999; Betensky et al. 2008; Corcoran et al. 2001; Huttunen and Niskanen 1978; Nuechterlein et al. 1994; Walker and Diforio 1997).

There is a large body of work suggesting that dopamine plays an important role in the psychopathology of schizophrenia. Imaging studies have shown that patients with schizophrenia, while psychotic, show heightened synthesis of dopamine, a heightened dopamine release in response to a stimulus, and a heightened level of synaptic dopamine (Kapur 2003; Laruelle and Abi-Dargham 1999; Seeman and Kapur 2000; Soares and Innis 1999). Furthermore, psychostimulant agents such as amphetamine, which trigger dopamine release, are associated with *de novo* psychosis, and they can worsen psychotic symptoms in patients with partial remissions (Angrist et al. 1980; Angrist et al. 1974; Angrist and Gershon 1970; Harris and Batki 2000; Kapur 2003). While it is unlikely that abnormalities in the dopaminergic system are the sole factor in the psychopathology of schizophrenia, this evidence shows that it is likely to be a contributing factor.

Numerous treatment options have been developed to ameliorate the symptoms of schizophrenia. First-generation antipsychotics, also called typical antipsychotics, were developed in the 1950's. They are postulated to exert their therapeutic effects primarily through antagonism of D2 dopamine receptors (Kapur and Remington 2001; Miyamoto et al. 2005). However, there is evidence that D3 receptor blockade may also be involved (Meltzer and Massey 2011). While typical antipsychotics are sometimes successful at ameliorating positive symptoms, they are less effective at treating negative symptoms. Unfortunately, typical antipsychotics can also lead to serious and potentially irreversible side effects such as tardive dyskinesia, parkinsonism, dystonia, and akasthisia (Miyamoto et al. 2005). Second-generation antipsychotics, also called 'atypical' antipsychotics, were developed as an attempted improvement over the first-generation antipsychotics. Atypical antipsychotics modulate other monoaminergic systems in addition to the

dopaminergic system. In addition to D2/D3 blockade, atypical antipsychotics may also block 5-HT2A serotonergic receptors, act indirectly or directly as agonists at 5-HT1A receptors, and to a lesser extent, block 5-HT2C, 5-HT6, and 5-HT7 receptors (Meltzer and Massey 2011). Atypical antipsychotics can be more effective than typical antipsychotics at treating negative symptoms of schizophrenia (Miyamoto et al. 2005). In addition, second-generation antipsychotics are less likely than typical antipsychotics to produce tardive dyskinesia and other extrapyramidal side effects (Kapur and Remington 2001; Meltzer and Massey 2011).

However, many patients with schizophrenia remain unresponsive to any existing treatments, and despite the reduced risk of extrapyramidal side effects, atypical antipsychotics have considerable drawbacks. Atypical antipsychotics can produce problematic side effects such as weight gain, hyperlipidemia, and diabetes (Allison et al. 2003; Carpenter 2004; Newcomer 2005). These side effects create a considerable public health problem. Since schizophrenia is such a devastating disorder, patients with schizophrenia have difficulty maintaining healthy diets and obtaining sufficient amounts of exercise. Unhealthy lifestyles in people with schizophrenia increase their risk of weight gain, diabetes, and cardiovascular disease (Newcomer 2005). As a consequence, patients with schizophrenia have a higher mortality rate than those unaffected by the disorder, even discounting suicide rates (McGrath et al. 2008). Cardiovascular disease is one of the leading causes of death in people with schizophrenia (Brown et al. 2010). Thus, the added burden of medication-induced weight gain and metabolic dysfunction drastically reduces quality of life for patients with schizophrenia, increases risk of mortality, and contributes to patient non-compliance, which is a significant obstacle to treating schizophrenia (Allison et al. 2003; Chiang

et al. 2011; Lambert et al. 2004). Therefore, it is paramount to public health that alternative treatments to schizophrenia be developed.

Circuitry of prepulse inhibition

Sensorimotor gating is an involuntary process by which organisms filter out potentially irrelevant stimuli before they reach motor response networks. This mechanism is proposed to serve as a gauge of information-filtering processes that may confer some protection from potential sensory inundation and cognitive deficits (Geyer et al. 1990). With healthy sensorimotor gating, an organism is able to filter out potentially irrelevant sensory information from the external environment, as well as impulses and thoughts from the internal environment. This allows attention to be focused on the most salient stimuli in the environment. Healthy sensorimotor gating is a crucial part of normal cognitive functioning. Impairments in sensorimotor gating lead to sensory inundation and may ultimately result in cognitive fragmentation. Sensorimotor gating has been shown to be impaired in a wide range of disorders including schizophrenia, autism, attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), Tourette's syndrome, and post-traumatic stress disorder (PTSD) (Braff et al. 2008; Grillon et al. 1998; Grillon et al. 1996; Ornitz and Pynoos 1989; Swerdlow et al. 2008). There are numerous functional ways to measure sensorimotor gating processes in a laboratory or clinical setting, including prepulse inhibition (PPI) and P50/N40 suppression (Swerdlow et al. 2006b).

Prepulse inhibition (PPI) is a phenomenon found in many species, including humans and rats, and it can provide a functional measure of sensorimotor gating (Braff et al. 2001; Geyer et

al. 2001; Swerdlow et al. 2001a). PPI occurs when a brief, non-startling stimulus (prepulse) decreases the startle reflex to a subsequent, more intense stimulus (pulse) (see Fig. A) (Hoffman and Ison 1980; Ison and Hoffman 1983). While the pulse and prepulse are often presented as auditory stimuli when studying rodents, PPI can also occur in response to tactile (air puffs aimed at the backs of rodents or the necks of humans), electrical (cutaneous stimulation), or visual stimuli (bright flashes of light) (Geyer and Swerdlow 2001; Swerdlow et al. 2000). Thus, PPI is a multimodal process that does not solely rely on the auditory circuitry. PPI is often calculated as a percentage that compares the prepulse+pulse startle response to the pulse-alone startle response. For each Prepulse + Pulse trial type, % PPI = 100 – {[(startle response for Prepulse + Pulse trial)/(startle response for Pulse-Alone trial)] x 100}. In order to reduce startle amplitude, prepulses are typically presented 30-500 milliseconds before the pulse. This interval between presentations of the prepulse and pulse has been found to be effective for the prepulse to inhibit the startle response (Hoffman and Searle 1965). Since PPI occurs with a time interval too short to be affected by voluntary cognitive control, PPI is thought to be an involuntary and 'preattentional' process (Swerdlow et al. 2000). The intensity of the prepulse can vary, but is typically 3, 6, 9, 12, or 15 dB louder than the background noise,

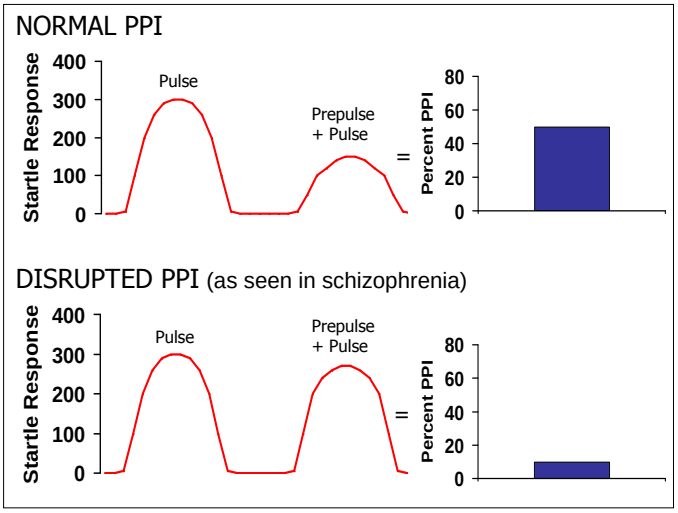

Fig. A: Top panel represents high %PPI due to high inhibition of the startle response by the prepulse in normal circumstances. Bottom panel represents low %PPI due to weak inhibition of the startle response by the prepulse, as observed in schizophrenia.

which is commonly presented as white noise at a continuous intensity of 65 dB (Geyer and Swerdlow 2001). It is worthwhile to employ several different prepulse intensities during a study, since louder prepulses tend to produce stronger PPI. Certain drugs may produce ceiling or floor effects that complicate interpretation of the data, so it is also reasonable to select a range of prepulse intensities that would limit these confounds (Geyer and Swerdlow 2001). PPI has the advantage that it does not need to be learned and it does not diminish with repeated exposure to the testing paradigm (Geyer 2001).

It is worth noting that healthy organisms are able to flexibly shift PPI in order to adapt to different environmental needs; however, consistently low %PPI is indicative of poor sensorimotor gating, and high %PPI generally indicates intact sensorimotor gating processes. For example, a normal organism with intact sensorimotor gating would startle more to the pulse-alone condition than to the pulse preceded by a non-startling prepulse; thus, the normal organism would have a high %PPI. An organism with impaired sensorimotor gating may startle about equally to the pulse preceded by a prepulse as to the pulse when it is presented alone, generating a low %PPI.

Indeed, impairments in PPI have been associated with pathological conditions. As previously mentioned, PPI has been shown to be impaired in a wide variety of disorders including schizophrenia (Braff et al. 2008; Swerdlow et al. 2008). Furthermore, psychotomimetic agents such as amphetamine and PCP have been shown to potently disrupt PPI (Braff et al. 2008; Geyer et al. 2001). Therefore, deficient PPI has been proposed as an endophenotype with which to study the etiology of such illnesses (Braff et al. 2008). Animal models of deficient PPI have been shown to possess face, construct, and predictive validity for gating deficits in

schizophrenia, and have been a valuable tool for identifying antipsychotic treatments (Braff 2010; Braff et al. 2008; Castagne et al. 2009; Ellenbroek 2004; Geyer 2008; Swerdlow et al. 2008; van den Buuse 2010; Weiss and Feldon 2001).

The primary acoustic startle circuit is fairly simple and consists of only a few synapses. Cochlear root neurons in the ear carry auditory information to the caudal pontine reticular nucleus, also known as the pontis caudalis (PnC). Subsequently, the PnC projects to spinal cord motor neurons, which synapse onto muscle cells and generate a whole-body startle response (Davis et al. 1982). It is postulated that the pedunculopontine tegmental nucleus (PPTg) inhibits the startle response through its inhibitory synapse onto the PnC. Structures within the cortico-striatal-pallido-thalamic loop (CSPT) are also thought to converge onto the PPTg and thereby regulate PPI (Fendt et al. 2001; Koch and Schnitzler 1997; Swerdlow et al. 2001a). As suggested by its name, the CSPT includes areas within the cortex, striatum, pallidum, and thalamus that modulate PPI. More specifically, areas implicated in the regulation of PPI include the prefrontal cortex (including the medial prefrontal cortex and orbital cortex), nucleus accumbens (both shell and core), amygdala (medial, central, basolateral divisions), thalamus (particularly the mediodorsal region), and hippocampus (dorsal and ventral) (Bakshi and Geyer

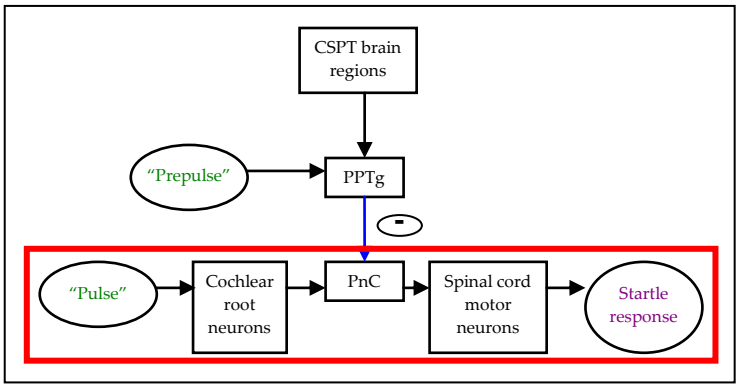

Fig. B: A simplified schematic depicting the circuitry underlying prepulse inhibition of the startle response. The red box depicts the primary acoustic startle circuit. CSPT = cortico-striatal-pallido-thalamic loop. PPTg = pedunculopontine tegmental nucleus. PnC = pontis caudalis. The PPTg converges onto the startle circuit through an inhibitory projection to the PnC.

1999; Cromwell et al. 2005; Kumari et al. 2003; Schwabe and Koch 2004; Swerdlow et al. 2001a; Swerdlow et al. 2001b; Swerdlow et al. 2002; Swerdlow et al. 2008; Vinkers et al. 2010; Weinberger 1987).

Among the most widely studied areas influencing PPI is the Acb, which like other CSPT areas, affects PPI by serial connections that ultimately converge upon the PPTg. Models proposed by (Koch and Schnitzler 1997; Swerdlow et al. 1999) outline these serial connections in greater detail. In these models, the Acb receives inhibitory dopaminergic inputs from the ventral tegmental area, as well as excitatory glutamatergic inputs from the medial prefrontal cortex, hippocampus, and amygdala. The Acb, in turn, sends inhibitory GABAergic projections both directly to the PPTg, and to the ventral pallidum, which also sends inhibitory projections to the PPTg, which interfaces with the primary acoustic startle circuit. In this model, the Acb has the ability to inhibit PPTg activity through direct GABAergic projections, or stimulate the PPTg by inhibiting the ventral pallidum's inhibitory signals to the PPTg. The Acb is an important node in the PPI-regulatory network for this reason.

Pharmacology of prepulse inhibition

The neurotransmitter systems underlying regulation of PPI regulation are quite complex and still not fully understood. Multiple neurotransmitter systems have been shown to regulate PPI, including dopamine, norepinephrine, serotonin (5-HT), and glutamate (Geyer et al. 2001). Dopamine is the most thoroughly studied neurotransmitter in terms of PPI regulation (Swerdlow et al. 2001a). The non-selective dopamine receptor agonist apomorphine, as well as more selective D2 agonists, have been shown to disrupt PPI, an effect that is blocked by D2

antagonists such as typical antipsychotics (Geyer et al. 1990; Mansbach et al. 1988; Swerdlow et al. 2001a). However, there is evidence that other systems also regulate PPI. For example, stimulation of the α1 and β NE receptors has been repeatedly shown to disrupt PPI, and PPI deficits can be reversed by α1 and β NE receptor antagonists (Alsene et al. 2006; Alsene et al. 2010; Alsene et al. 2011; Carasso et al. 1998; Shilling et al. 2004; Swerdlow et al. 2006a; Varty et al. 1999). Stimulation of serotonergic 5-HT2 receptors also causes PPI deficits that can be reversed by 5-HT2 receptor antagonists (Brea et al. 2006; Briody et al. 2010; Farid et al. 2000; Feifel et al. 2003; Kohnomi et al. 2008; Shilling and Feifel 2002; Shilling et al. 2004; Sipes and Geyer 1994; Sipes and Geyer 1995; 1997; Swerdlow et al. 2006b; Varty and Higgins 1995; Wadenberg et al. 2000). Furthermore, 5-HT2 receptors are thought to be particularly important in schizophrenia (Maier et al. 2008). Psychotomimetic drugs such as the non-competitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonists phencyclidine (PCP), ketamine, dizocilpine have also been shown to disrupt PPI (Bakshi and Geyer 1995; 1997; Bakshi et al. 1994; Geyer et al. 2001). Hence, the pharmacology that regulates PPI is quite complex, and there may be many unexplored therapeutic methods for restoring dysfunctional PPI. The ubiquity of dopamine receptors outside of the Acb, such as in other striatal compartments or in the basal ganglia, makes direct dopamine antagonists compromised as antipsychotic agents due to extrapyramidal side effects. Finding a way to selectively create functional dopamine antagonism within the Acb is therefore important.

Amylin and AMY1 receptors

Amylin could potentially play a role in the modulation of the PPI system at the level of the Acb. Amylin is a 37-amino-acid polypeptide hormone that belongs to the calcitonin family of peptides, which also includes calcitonin, two calcitonin gene-related peptides (CGRP1 and CGRP 2), and adrenomedullin (Foord and Marshall 1999; Poyner et al. 2002). Since amylin is colocalized with insulin in β-cell secretory granules of the pancreas, and since it is cosecreted with insulin in response to nutrient stimuli (Butler et al. 1990), amylin is most commonly known for its roles in metabolism and feeding.

Receptors for the calcitonin peptide family consist of two distinct genes for a calcitonin receptor (CT) and a calcitonin receptor-like receptor (CL). The CT and CL are G-protein-coupled receptors (GPCRs) that each consist of seven transmembrane domains (Conner et al. 2004; Foord and Marshall 1999). These receptors are coupled to receptor activity modifying proteins (RAMPs), of which there are three. Each member of the RAMP family is approximately 150 amino acids long, with a short C-terminus domain, a single transmembrane region, and a longer extracellular N-terminus that varies between the three RAMPs (Conner et al. 2004; Young 2005). A complete receptor complex consists of either CT or CL coupled to one of the 3 RAMPs; without the presence of a RAMP, the receptor is not functional (McLatchie et al. 1998). While all three known RAMP proteins are abundant in humans, RAMP-3 is not as highly prevalent in rats (McLatchie 1998). Intracellular co-expression of RAMP-1 and CT-R yields the high affinity AMY-1 subtype whose affinity profile for amylin and related peptides matches the high-affinity binding seen in areas such as the nucleus accumbens (Acb) (Poyner et al. 2002; Young 2005). The presence of CT-R and RAMP-1 in the striatum and Acb has been reported in

whole-brain surveys (Becskei et al. 2004; Lee et al. 2008; Nakamoto et al. 2000; Oliver et al. 2001; Ueda et al. 2001). The medial ventral striatum, including the Acb, also exhibits high-affinity amylin binding (Aiyar et al. 1995; Beaumont et al. 1993; Paxinos et al. 2004; Sexton et al. 1994; van Rossum et al. 1994). Since the Acb contains high-affinity amylin binding and dense expression of the gene components of AMY1-Rs, it is likely that the Acb-localized AMY-R is AMY1.

Amylin has been studied extensively for its peripheral effects, particularly that of glucose metabolism regulation. However, the role of CNS AMY1 receptors is poorly understood. Several studies have found a satiety-like suppression of feeding or a reduction in spontaneous motor activity following infusion of amylin into the lateral or third ventricle, but it is difficult to determine which specific brain regions are responsible for this effect (Bouali et al. 1995; Lutz et al. 1998a; Morley and Flood 1991; Rushing et al. 2000). Current understanding of amylin's feeding-modulatory function is largely limited to studies of amylin's role in ingestive behaviors in the area postrema. Blockade of area postrema amylin receptors and lesions specific to the area postrema both reduce anorectic effects induced by systemically-administered amylin (Lutz et al. 2001; Lutz et al. 1998b; Mollet et al. 2004). Another brain region studied in terms of amylin is the hypothalamus. Amylin infusion causes a satiety-like suppression of feeding when infused directly into the hypothalamus (Chance et al. 1991), or into the third ventricle (Rushing et al. 2000), which borders the hypothalamus. Recently, amylin in the ventral tegmental area (VTA) has also been studied. Quantitative real-time PCR provided evidence for CT-R and RAMP-1 mRNA in the VTA. Furthermore, behavioral studies demonstrated that intra-VTA infusions of salmon calcitonin (sCT), a peptide hormone with similar affinity at AMY1 receptors to amylin

(Beaumont et al. 1993), reduced chow intake and sucrose solution intake, and transiently reduced locomotor activity. In addition, intra-VTA infusions of the AMY1 receptor antagonist AC187 increased chow intake and transiently increased locomotor activity. However, there were several caveats to this study. VTA tissue samples for quantitative real-time PCR were collected through micropunching, so it is possible that neighboring regions were included in the sample. Furthermore, the time courses for locomotor and food intake studies showed a delayed reaction to VTA microinfusions, suggesting that diffusion of the infusate into neighboring regions may have been responsible for the measured behavioral effects (Mietlicki-Baase et al. 2013). These caveats are analyzed more fully in the Discussion of this thesis.

Despite the fact that the Acb has high-affinity binding for amylin (indeed, one of the densest concentrations of high-affinity amylin biding in the brain), along with high expression levels of the two gene components that make up the high-affinity AMY1 amylin receptor, the function of amylin the Acb remains largely unexplored. Only one study has examined the infusion of amylin directly into the Acb. This study showed that intra-Acb amylin infusions reduce locomotor activity and food intake, effects that are reminiscent of functional dopamine antagonism (Baldo and Kelley 2001). Two studies (although not in the Acb) further illustrate the possibility that amylin acts to counterbalance the dopamine system, with intracerebroventricular amylin reversing the behavioral effects of systemic apomorphine (a non-selective dopamine receptor agonist) and amphetamine (Clementi et al. 1999; Clementi et al. 1996). Therefore, amylin is implicated as being a part of the complex system of neurotransmitters in the Acb that serves to modulate PPI, possibly through interactions with the dopamine system.

Nucleus accumbens: anatomical considerations

As discussed above, the nucleus accumbens (Acb), ventral tegmental area, and hypothalamus are amylin-sensitive sites. However, these sites are protected by the blood-brain barrier. While dense amylin binding is also found in circumventricular organs such as the subfornical organ, organ vasculosum lateralis terminalis (OVLT), and area postrema, there is no blood-brain barrier protecting these sites (Young 2005). Therefore, it is likely that amylin in the circulation is able to access these regions. To access the Acb; however, amylin must cross the blood-brain barrier. Accordingly, uptake studies show that amylin crosses the blood-brain barrier to collect in the striatum and other sites, with better brain penetrance than insulin (Banks and Kastin 1998).

As previously mentioned, the Acb plays a prominent role in PPI regulation (Swerdlow et al. 2001a; Swerdlow et al. 2008) and in the pathophysiology of schizophrenia (Grace 2000; Heimer 2000); thus, it is useful to review here key points of Acb anatomy. The nucleus accumbens (Acb) is situated within the ventral striatum and is a component of the "limbic" CSPT loop. The Acb has a critical role in motivation and goal-directed behavior and has therefore been proposed to function as an interface between the limbic and motor systems (Groenewegen and Trimble 2007; Morgane et al. 2005). The Acb has two main subdivisions, the core and the shell (Meredith et al. 2008; Zaborszky et al. 1985). The core has a central location in the Acb, with the shell surrounding the core on its medial and ventral sides (Heimer 2000).

The core and shell have a number of distinctions. In general, the core is more similar to the overlying dorsal striatum than to the shell. In fact, the core forms a continuum with the dorsal striatum, while the shell has very distinct neurochemical markers and output circuitry. A variety

of neurochemical markers (including AMY-Rs) are present in greater abundance in the AcbSh, including calretinin, substance P, and neurotensin, whereas calbindin binds more strongly in the core (Zahm 1999). In addition, there are differences in connectivity between the core and the shell. The shell but not the core innervates the ventromedial subcommissural ventral pallidum and the anterior lateral preoptico-lateral hypothalamic continuum (Thompson et al. 2000; Zahm 1999); the latter projection is thought to be important for the AcbSh's feeding-modulatory effects (Maldonado-Irizarry et al. 1995; Stratford and Kelley 1999; Will et al. 2003). In addition, the ventral hippocampus and amygdala primarily innervate the shell. While the shell receives dopaminergic innervation almost exclusively from the ventral tegmental area, the core receives additional dopaminergic innervation from the substantia nigra (Meredith et al. 2008). Therefore, the AcbSh is a unique site, even when compared to neighboring regions of the striatum.

It would be logical for the Acb to be a critical structure in the pathology of schizophrenia, considering its dense innervation of monoaminergic fibers and placement in the CSPT loop. The monoaminergic systems, particularly dopamine, are targeted by antipsychotic drugs and play a role in the modulation of PPI. The ventral striatum, including the Acb, is strongly innervated by dopaminergic fibers from the ventral tegmental area, and also has a high density of serotonergic inputs (Groenewegen and Trimble 2007). In fact, the shell contains the highest concentration of dopaminergic D3 receptors in the brain (Groenewegen and Trimble 2007; Heimer 2000). Interestingly, the Acb shows less developmental dopamine receptor pruning than other striatal regions (Teicher et al. 1995). Since amylin may modulate dopamine function (Baldo and Kelley 2001; Clementi et al. 1999; Clementi et al. 1996), it is of potential clinical relevance that CT receptors and RAMP-1 proteins are densely localized within the Acb.

It is important to note that the AcbSh has well-established behavioral functions beyond the regulation of PPI, notably, the modulation of motivational state. In particular, a number of studies have suggested that the AcbSh has a specialized role in regulating food intake and the "hedonic liking" of food rewards. Stimulation of GABA receptors (Stratford and Kelley 1997) or blockade of glutamate receptors (Maldonado-Irizarry et al. 1995) in the medial AcbSh but not other striatal territories robustly increases feeding (Basso and Kelley 1999; Kelley and Swanson 1997). A mapping study has shown that there is a location within the rostrodorsal AcbSh that is a cubic millimeter in size and that plays a functional role in opioid-induced increases in unconditioned orofacial hedonic reactions to sucrose; however, stimulation of μ-opioid receptors in the entire AcbSh increased food intake (Pecina and Berridge 2005). In addition, glutamatergic and GABAergic stimulation within the AcbSh stimulates food intake, and GABAergic stimulation amplified hedonic "liking" in the anterior medial AcbSh (Faure et al. 2010; Richard et al. 2013). The role of AMY-Rs in regulating these appetitive-motivational functions is poorly understood; however, the only study that has been done showed that amylin infusions into the AcbSh dose-dependently suppresses food intake (Baldo and Kelley 2001). In summary, the AcbSh in particular has unique traits in comparison to other regions within the striatum, including particularly strong high-affinity amylin binding (Sexton et al. 1994; van Rossum et al. 1994) and the presence of a hedonic "hotspot" (Pecina and Berridge 2005). The functional implications of this overlap of amylin binding and μ-opioid-mediated responses are poorly understood.

Ingestive behavior:

Ingestive behavior is critical to the survival of organisms, since it helps to maintain energy homeostasis. Under ideal conditions, orexigenic hormones and anorectic hormones exist in proper balance. To maintain the proper amount of metabolic fuel availability, homeostasis involves the ability of an animal to alter food intake, body weight, adiposity, and energy expenditure. This enables an organism to store excess energy during periods of high food availability, and to use bodily energy stores in times of shortage or starvation (Schneider et al. 2013).

There are many hormones in the body that help to maintain energy homeostasis. Food intake in mice and rats is influenced by a variety of chemical messengers. Hormones such as leptin, ghrelin, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), insulin, amylin, orexin/hypocretin, neuropeptide Y, kisspeptin, and enkephalins act to regulate food intake (Lutz 2010; Schneider et al. 2013). Likewise, there are many brain regions involved in the regulation of ingestive behavior, including but not limited to the hypothalamus, nucleus tractus solitarius (NTS), area postrema (AP), parabrachial nucleus (PBN), nucleus accumbens (Acb), ventral tegmental area (VTA), and amygdala (Kelley et al. 2005; Schneider et al. 2013).

Although food is important for maintaining energy homeostasis, evidence suggests that ingestive behavior is not purely under homeostatic control. Since food and other palatable substances have a hedonic quality to them, animals may eat even when satiated. This can lead to dysregulation of ingestive behavior, especially when highly palatable, calorie-rich food is abundantly available. As discussed above, the nucleus accumbens in particular is thought to be a "hedonic hotspot" that enhances the pleasurable aspects of palatable food intake (Baldo and

Kelley 2007; Kelley et al. 2002; Kelley et al. 2005; Pecina and Berridge 2005). This could conceivably contribute to obesity. Obesity is a severe problem in our society (Malik et al. 2013). It is estimated to affect 36% of people in the United States alone (Flegal et al. 2012). Health risks of obesity include type 2 diabetes, cardiovascular disease, and some types of cancer (Danaei et al. 2009).

Another potential area of clinical relevance is in binge-type eating disorders (bulimia nervosa, binge-eating disorder). The endogenous mu-opioid system is implicated in binge-type eating disorders; there is evidence that opioid-blocking drugs reduce bingeing, with a possible site of action in the striatum (Cambridge et al. 2013; Davis et al. 2009; Marrazzi et al. 1995). In individuals with comorbid obesity and moderate binge-eating, a μ-opioid antagonist reduced striatal responses to images of food, and also reduced motivation to view food images (Cambridge et al. 2013). Hence, in exploring amylin's actions in the Acb, it is important to study the multiple behavioral processes and neuromodulatory systems important in Acb functions.

The opioid system:

Opium has been used as a psychoactive substance for millennia, and investigations into its mechanisms of action has led to the discovery of a complex endogenous system of opioid peptides that can function as neurotransmitters. In the 1970s, researchers discovered three main categories of opioid peptides that exist within the body. These endogenous opioid peptides include enkephalins, endorphins, and dynorphins (Cox et al. 1976; Goldstein et al. 1979; Hughes et al. 1975). Decades after opioid peptides were successfully cloned, receptors for the opioid peptides were identified, and included delta, kappa, and mu receptors (Chen et al. 1993; Evans et

al. 1992; Kieffer et al. 1992; Wang et al. 1993; Yasuda et al. 1993). These receptors have a wide distribution throughout the central nervous system (Mansour et al. 1995; Mansour et al. 1988). Delta and mu receptors have preferential binding for enkephalins and endorphins, while kappa receptors display a high affinity for dynorphins. μ-opioid receptors are sensitive to morphine and its alkaloid analogs, and may mediate the effects of these drugs in the central nervous system (Goldstein and Naidu 1989; Rothman et al. 1990). All three types of opioid receptors are G-protein coupled receptors that inhibit the formation of cyclic AMP (cAMP) through adenylyl cyclase activity (Al-Hasani and Bruchas 2011). Opioid peptides have a variety of functions, including modulating endocrine, cardiovascular, gastrointestinal, respiratory, thermoregulatory, and immune activity. They also play a role in stress and emotional responses, locomotor activity, learning and memory, pain regulation, reward, and ingestive behavior (Bodnar 2013).

Mu opioid receptors in the accumbens:

Mu-opioid receptors are present throughout the striatal complex but exhibit a particularly dense distribution within the nucleus accumbens shell (Mansour et al. 1995; Mansour et al. 1988). Within the nucleus accumbens, electron microscopy studies have shown that μ-opioid receptors are located on the dendrites of GABAergic medium spiny neurons, and on a small number of axon terminals (Gracy et al. 1997; Pickel et al. 2004; Svingos et al. 1996; 1997). In the nucleus accumbens, opioid receptors are thought to play a critical role in reward and ingestive behavior, with the AcbSh being a "hedonic hotspot." In rats, stereotyped orofacial reactions of "liking" elicited by sucrose were enhanced by μ-opioid stimulation selectively within the rostrodorsal portion of the medial AcbSh, although μ-opioid stimulation throughout

the entire AcbSh increased food intake (Pecina and Berridge 2005). Based on the ultrastructural localization of μ-opioid receptors, this could occur via the regulation of medium spiny neuron activity.

Morphine, which has a higher affinity for μ-opioid receptors than for delta or kappa opioid receptors, induces hyperphagia when infused into the Acb (Bakshi and Kelley 1993b; Evans and Vaccarino 1990; Mucha and Iversen 1986). An analysis of opioid peptides with differing affinities for the opioid receptor subtypes indicated that the μ-opioid receptor type in the Acb is particularly important for opioid-induced feeding effects (Bakshi and Kelley 1993a). Since opioid receptors in the Acb are thought to be critical for the hedonic value of rewards such as food, it is fitting that intra-Acb morphine preferentially increases the intake of palatable foods (Evans and Vaccarino 1990). Antagonists at Acb opioid receptors selectively decrease intake of preferred flavors and palatable substances such as sucrose (Bodnar et al. 1995; Kelley et al. 1996; Woolley et al. 2006). Intra-Acb naloxone, a non-selective opioid receptor antagonist that has slightly higher affinity at μ-opioid receptors than kappa or delta opioid receptors, decreases palatable feeding selectively, suggesting that μ-opioid receptors are important for this response (Kelley et al. 1996). As with morphine, more highly selective Acb μ-opioid receptor agonists can increase intake of preferred flavors and palatable substances such as fat, sucrose, saccharin, or saline (Woolley et al. 2006; Zhang et al. 1998; Zhang and Kelley 1997; 2002). Hence, investigations into the mechanisms by which μ-opioid receptor agonists in the AcbSh increase food intake have provided insights into the neural regulation of food intake and reward. The interesting overlap between amylin binding and the μ-opioid "hedonic hotspot" provides

tantalizing evidence that, in addition to PPI modulation, amylin signaling could also regulate appetitive behaviors.

Synthesis:

The overarching goal of the present thesis was to explore the roles of AMY-Rs in two crucial functional domains of the AcbSh: 1. PPI regulation; 2. food intake modulation. By testing amylin's effects in divergent AcbSh-mediated behavioral processes, we aimed to assess the specificity versus generalizability of amylin's putative modulatory role in the Acb. Moreover, these two functional domains have high translational relevance for a wide variety of psychiatric illnesses, especially schizophrenia, binge eating, and obesity.

First, given that high-affinity amylin binding, likely at AMY1 receptors, is present in the medial nucleus accumbens shell and to a lesser extent in neighboring regions (Sexton et al. 1994; van Rossum et al. 1994), that stimulation of these receptors results in behavioral changes resembling functional dopamine antagonism (Baldo and Kelley 2001), and also that AcbSh dopamine antagonism is important for the clinical efficacy of antipsychotics (Onn and Grace 1995), we sought to examine whether AcbSh AMY1 receptor stimulation could have antipsychotic properties. AMY1 manipulation may provide advantages in regional selectivity over current antipsychotics due to its unique anatomical distribution. Existing literature suggests that AMY1 receptors are relatively exclusively located within the striatum. In the work present in this dissertation, we aimed to examine the distribution of AMY-R genes with greater resolution. If AMY1 receptors are indeed densely expressed within the AcbSh but in few other brain regions, then treatment with amylinergic compounds might cause fewer extrapyramidal

side effects that are characteristic of typical antipsychotics, which cause extrapyramidal side effects through dopamine receptor blockade in neighboring striatal and basal ganglia sites. Furthermore, since amylin has been proposed to act as a satiety signal (Lutz 2010), amylin would be unlikely to cause weight gain, another common side effect of some antipsychotics (Newcomer 2005). Reduction in aversive side effects could increase rates of medication compliance among patients with schizophrenia, reduce mortality from diseases that are exacerbated by obesity, and greatly improve quality of life for these individuals.

Because amylin in the AcbSh also regulates food intake, and yet only one study (Baldo and Kelley 2001) had directly investigated the effects of amylin on ingestive behavior in rats, we sought to explore the effects of amylin on several types of ingestive behaviors. In particular, given the striking overlap of AMY-R binding with the reported μ-opioid "hotspot" for food reward, we were interested in possible interactions between AcbSh AMY1 receptors and AcbSh μ-opioid receptors. Mu-opioid receptor stimulation in the AcbSh potently increases food intake even in satiated rats, and it preferentially increases intake of palatable foods and solutions. Exploring potential interactions between AcbSh amylin receptor stimulation and μ-opioid receptor stimulation could provide insight into the mechanism by which amylin affects food intake.

To summarize, this dissertation was designed to explore the antipsychotic potential of AcbSh AMY1 receptor stimulation along with interactions with dopamine receptors, as well as potential interactions of AcbSh AMY1 receptor stimulation and AcbSh μ-opioid receptor stimulation on ingestive behavior. An outline of the specific data chapters is included below.

Chapter 2: Antipsychotic-like actions of the satiety peptide, amylin, in ventral striatal regions marked by overlapping calcitonin receptor and RAMP-1 gene expression

In this chapter, we investigated whether amylin infused into the nucleus accumbens shell (AcbSh) of rats could reverse PPI deficits induced by systemic amphetamine (AMPH). We also investigated whether the AMY1 receptor antagonist AC187 would disrupt PPI when infused into the AcbSh of rats, and if so, whether or not these PPI disruptions could be reversed by the typical antipsychotic drug and D2 dopamine receptor antagonist, haloperidol. As a site control, we examined whether AMY1 had the same PPI effects when infused into the dorsal striatum (DS). Finally, to examine receptor-specificity of amylin's reversal of the AMPH-induced PPI effect, we infused a cocktail of amylin and AC187 into the AcbSh and measured whether or not this cocktail affected PPI deficits generated by systemic AMPH.

In addition, we performed a systematic and thorough exploration of gradients of expression of the RAMP-1, RAMP-2, RAMP-3, CT, and CL mRNA at multiple striatal levels of the brains of experimentally naïve adult male rats. Since RAMP-1 and CT compose the high-affinity AMY1 receptor, we examined overlapping expression of RAMP-1 and CT mRNA.

Chapter 3: Endogenous amylin receptor signaling in the nucleus accumbens negatively modulates μ-opioid-driven feeding

Here, we explored potential interactions between AcbSh AMY1 receptors and μ-opioid receptors in their control of feeding behavior. First, we investigated whether AcbSh amylin could reverse hyperphagia induced by AcbSh DAMGO, a mu-opioid receptor agonist. Four different doses of amylin were tested in order to characterize the dose-effect function of this peptide. A site control in the anterior dorsal striatum (ADS) was also included. In addition, we investigated the ability of AcbSh amylin to reduce hunger-driven feeding in rats that had been food-deprived for 18 hours, and the ability of AcbSh amylin to reduce the intake of palatable food (in this case, a 10% sucrose solution). Finally, we examined the ability of the AMY1 receptor antagonist AC187 in the AcbSh to alter AcbSh DAMGO-induced hyperphagia in rats that had been food-deprived for 18 hours. Since endogenous amylin is released prandially, we performed AC187 experiments in rats that had been "prefed" for 30 minutes following the 18-hour fasting period (prefeeding presumably caused prandial amylin release), and compared these results to those of food-deprived rats that had not been "prefed" (when amylin levels were presumably low).

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Chapter II:

Antipsychotic-like actions of the satiety peptide, amylin, in ventral striatal regions marked by overlapping calcitonin receptor and RAMP-1 gene expression

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ABSTRACT

Amylin is a calcitonin-related, insulin-modulatory peptide that produces satiety through brainstem-localized receptors; however, its effects in forebrain are poorly understood. The nucleus accumbens shell (AcbSh) exhibits among the densest concentrations of high-affinity amylin binding; nevertheless, these receptors have not been explored beyond one study showing dopamine antagonist-like effects of intra-Acb amylin on feeding behaviors (Baldo and Kelley, 2001). Here, we investigated whether intra-Acb amylin signaling modulates prepulse inhibition (PPI), a measure of sensorimotor gating deficient in several illnesses including schizophrenia (SZ). Intra-AcbSh amylin infusions in rats (0, 30, 100 ng) reversed amphetamine (AMPH)-induced PPI disruption without affecting baseline startle; dorsal striatal amylin infusions had no effect. Coinfusion of AC187 (20 ug), a selective antagonist for the high-affinity amylin receptor (AMY1-R), completely blocked the ability of amylin to normalize AMPH-induced PPI disruption, showing the specificity of AcbSh amylin effects to the AMY1-R. Under conditions of high endogenous amylin tone (during rats' dark cycle), intra-AcbSh AC187 on its own disrupted PPI in a haloperidol-reversible manner (0.1 mg/kg). Finally, *in situ* hybridization revealed marked anatomical gradients for both receptor activity-modifying protein-1 (RAMP-1) and calcitonin receptor (CT-R) gene expression in striatum (co-expression of these genes yields AMY1-R), with highest overlap in the medial AcbSh, where amylin and AC187 modulated PPI. Thus, AMY1-R may be a potential target for the development of putative antipsychotics or adjunct treatments that oppose metabolic side effects of current medications. Moreover, AMY1-Rs may represent a novel way to selectively modulate tonic Acb activity in regions that support dopaminergic effects.

INTRODUCTION

While existing treatments for schizophrenia (SZ) offer some relief, serious side effects, including weight-gain and Type-2 diabetes liability (De Hert et al., 2011), are seen with numerous second-generation antipsychotics (SGA). These effects increase morbidity and reduce quality of life and treatment compliance. Hence, an important goal is to develop drugs devoid of these effects, or to identify adjunct treatments that potentiate antipsychotic actions while opposing adverse metabolic consequences of SGAs.

One candidate with such dual actions may be amylin, a peptide hormone co-released with insulin, which is best known for its roles in glucose regulation and satiety (Castillo et al., 1995; Lutz, 2012). Amylin agonists improve glycemic control and produce weight loss in Type- 2 diabetes (Roth et al., 2009; Singh-Franco et al., 2011), effects that would, in principle, counteract SGA-related metabolic side effects. Moreover, the distribution of central nervous system binding suggests that amylin could target brain regions known to mediate antipsychotic effects. Among the densest sites of amylin binding in the entire brain is the nucleus accumbens shell (AcbSh) (Beaumont et al., 1993; Sexton et al., 1994; van Rossum et al., 1994; Christopoulos et al., 1995), a structure that mediates numerous SZ-related deficits including prepulse inhibition (PPI). The only study to explore intra-Acb amylin actions showed that the peptide decreased exploratory activity and, at higher doses, attenuated hunger-driven feeding (Baldo and Kelley, 2001); the activity suppression resembled functional dopamine (DA) antagonism. Because successful antipsychotics act partly through D2 receptor blockade (Boyd and Mailman, 2012), stimulation of AcbSh-localized amylin receptors may represent a novel means of producing antipsychotic-like effects.

Amylin receptors are formed as complexes of calcitonin receptor (CT-R) and calcitonin-like receptor (CL-R) genes in conjunction with three identified receptor activity modifying proteins (RAMPs 1-3), which modulate CT-R and CL-R affinity (Young, 2005; Sexton et al., 2006). A particularly high-affinity receptor subtype for amylin is the amylin1 receptor (AMY1-R), which arises from the combination of the CT-R with RAMP-1; hence areas of AMY1-R expression are characterized by dense overlapping cellular expression patterns of CT-R and RAMP-1 mRNA. One of the few CNS sites with very high CT-R and RAMP-1 overlap is the AcbSh; this area of overlapping gene expression also matches the region that is delineated as a high-affinity binding site by the endogenous ligand amylin (Poyner et al., 2002). Notably, AMY1-R labeling is absent from regions just outside of AcbSh (like neighboring striatum), suggesting that this receptor may represent a potential 'tool' for modulating activity specifically within the AcbSh.

Here, we assessed the effects of intra-AcbSh amylin infusions using the PPI paradigm. PPI represents an index of sensorimotor gating, a preattentional form of information filtering deficient in schizophrenia (Braff and Light, 2004). The reversal of psychotomimetic-induced PPI deficits is a well-validated predictive tool for identifying drugs with antipsychotic efficacy, and is well known to be mediated in part through actions within the AcbSh (Geyer, 2006; Swerdlow et al., 2008). Hence, we tested the ability of intra-AcbSh infusions of an agonist (amylin) and antagonist (AC187) for the AMY1-R to modify basal PPI and PPI deficits induced by the psychotomimetic, amphetamine (AMPH). We also conducted *in situ* hybridization analyses to obtain a detailed striatal mapping of all amylin receptor-family genes (previous work has only looked at a very limited range of sections through the striatum), with a particular interest in

delineating the boundaries of densest co-expression of CT-R and RAMP-1 genes. We found that amylin infusions into the medial AcbSh, where the highest levels of overlapping CT-R and RAMP-1 expression were seen, reversed AMPH-induced PPI disruptions without any effects in neighboring sites (caudate) that were devoid of AMY1-R gene expression. Moreover, blockade of AMY1-R under conditions of high endogenous amylin tone led to a significant PPI disruption that was reversible by the D2 receptor antagonist haloperidol. These results demonstrate antipsychotic-like actions of amylin and modulation of DA activity via AcbSh AMY1-R signaling. This receptor subtype may therefore represent a viable target for the development of a new class of antipsychotic-like drugs that oppose the adverse metabolic side effects associated with many current antipsychotic medications; AMY1-R may also represent a powerful tool for regulating AcbSh DA tone in an anatomically selective manner.

MATERIALS & METHODS

Subjects. Fifty-one male Sprague-Dawley rats (300-400 g, Harlan Laboratories, Madison, WI) were pair-housed in clear cages in a temperature- and light-controlled vivarium with lights on from 0700-1900 hrs. All experiments except Experiment 5 were conducted between 1000-1500 hrs, and Experiment 5 was between 2100-0000 hrs. Facilities and procedures complied with animal use and care guidelines from the National Institutes of Health of the USA, and were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

Surgery and Drugs. Rats were anesthetized with isoflurane, and bilateral cannulae were implanted stereotaxically into either accumbens shell (AcbSh; coordinates in mm from bregma

with nosebar at +5mm: +3.2 AP; ±1.0 LM; -5.2 DV) or caudate (CPu; nosebar at -3.3mm: +1.6 AP; ±2.4 LM; -1.7 DV). Injectors extended 2.5mm beyond cannulae tips. Amylin (Bachem, Torrance, CA) and amphetamine (AMPH) (Sigma, St. Louis, MO) were dissolved in sterile isotonic saline, and AC187 (Tocris, Ellisville, MO) in sterile dH2O.

Startle and PPI testing. Testing occurred in startle chambers (San Diego Instruments, La Jolla, CA) that consisted of a Plexiglas cylinder within a sound-attenuated cabinet; a piezoelectric unit at the base of the cylinder detected vibrations and transmitted these signals via a computer interface unit to provide a measure of startle magnitude. Whole-body startle responses to a 120-dB white noise burst (40 msec; termed a "pulse") either alone or preceded 100 msec by "prepulses" (20-msec long) that were 3, 9, or 15 dB above background noise (65 dB) were thus recorded. Startle magnitude was the average of all pulse-alone trials, and PPI for each prepulse intensity was calculated as a percent score: *%PPI=100-{[(startle for prepulse+pulse trial)/ (startle for pulse alone trial)] x 100}.* Details of the session and equipment can be found in Alsene et al., 2011.

Experimental design: Six experiments using separate groups of rats were conducted employing counterbalanced within-subjects designs; at least 2-3 days separated consecutive test days. Intracranial microinfusion doses were given in a volume of 0.5 ul/side. *Experiment 1:* Rats received intra-AcbSh amylin (0 or 30 ng; N=6) immediately before AMPH (0 or 1.75 mg/kg, subcutaneously (SC)), and 5 min later were tested for PPI. *Experiment 2*: Identical to Experiment 1, but rats instead received a higher dose of intra-AcbSh amylin (0 or 100 ng; N=6-8/group).

Experiment 3: Rats (N=7) received haloperidol (0 or 0.1 mg/kg, SC) 15 min before intra-AcbSh infusions of the AMY1-R antagonist AC187 (0 or 20 ug) immediately before PPI testing. This experiment was performed during the dark cycle when endogenous release of amylin is highest in order to assess AC187 effects when basal signaling at AMY1-R would be greatest. *Experiment 4:* Same as Experiment 2, but with amylin (0 or 100 ng) infusions into CPu (N=8). *Experiment 5*: Identical to Experiment 4, but instead with a lower and higher dose of amylin into CPu (0, 30, or 300 ng; N=10). *Experiment 6:* Six naïve rats were anesthetized with isoflurane, decapitated, and their brains were flash-frozen and stored at -80ºC until cryostat sectioning and subsequent *in situ* hybridization analyses of amylin receptor genes.

***In situ* hybridization.** Brains were sliced into 20-µm sections, with five sequential sets of slices taken for each rat. *In situ* hybridization (with probes for RAMP-1, RAMP-2, RAMP-3, CT-R, and CL-R) was carried out using standard techniques. Methods used are identical to those described in (Schochet et al., 2008), with the following minor modifications. Phosphate-buffered saline (PBS) was used for the initial washes, and washes immediately following acetylation used 1x Tris-buffered saline (TBS). Sections were hybridized overnight with 150µl of [10,000Cts/µl] ^{35}S-labeled antisense riboprobe in a hybridization buffer (10 µM Tris, 1 µM EDTA, 0.3 M NaCl, 1x Denhardt's, 50% formamide, 10% Dextran sulfate, 500µg/ml tRNA, 50mM DTT). Following hybridization, slides were washed twice in 4x saline-sodium citrate (SSC) solution containing 2mM DTT, then incubated for 30 min in 20µg/ml of RNaseA in 10mM Tris-HCl, 0.5M NaCl (pH 8.0) at 37ºC.

Sections were exposed to phosphorimager screens (GE Health) for 4-23 days, depending upon signal intensity. Screens were scanned on a Typhoon scanner, and quantification of average optical density was performed using ImageQuant 5.2 software (Molecular Dynamics). Some sections with the RAMP-1 or CT-R probes underwent further processing for photographic emulsion autoradiography, with light-sensitive processing steps in a darkroom. Briefly, slides were dipped in heated (44ºC) liquid NTB-2 photographic emulsion (Kodak) and then air dried for 2 hrs before being covered with tinfoil and stored in a darkroom. Due to strength of labeling, RAMP-1 slides were exposed to emulsion for 3 days, with all others for 17 days. Afterwards, slides were brought to room temperature, developed for 5 min with D19 developer, washed with 32ºC water for 30 sec, exposed to fixer (Kodak) for 5 min, washed with 32ºC water for 15 minutes, and air dried.

Data Analysis. PPI data were analyzed with 3-factor analyses of variance (ANOVA)s (pretreatment x treatment x prepulse intensity), and startle data with 2-factor ANOVAs (pretreatment x treatment). With significant main effects or interactions, Bonferroni-adjusted t-tests were conducted for *posthoc* analyses. *In situ* data were analyzed with 2-factor ANOVAs (striatal level x region of interest (ROI)) for each gene, with Tukey's *posthoc* tests. Injector placements were confirmed by an experimenter that was blind to the behavioral data and treatment conditions; rats with placements deemed to fall outside of AcbSh were excluded from Experiments 1-3, and those with placements outside of the DLS were excluded from Experiments 4-5. Final sample sizes reflect these adjustments.

RESULTS

All experiments showed significant main effects of prepulse intensity, a standard parametric feature of PPI in which larger prepulse intensities elicit greater PPI (Alsene et al., 2011); for brevity, this is not repeated throughout the text.

1) Prepulse inhibition

Intra-AcbSh amylin reverses AMPH-induced PPI deficits via specific actions at the AMY1-R.

A main effect of AMPH on PPI was seen in both Experiment 1 [$F(1,5)=25.6$, $P<0.01$] and Experiment 2 [$F(1,12)=28.9$, $P<0.001$]. *Posthoc* analyses showed reductions in PPI by AMPH at all prepulse intensities (**Fig. 1**; $P<0.05$ – $P<0.001$). Neither dose of amylin affected PPI on its own [30-ng dose: $F(1,5)=3.3$, NS; 100-ng dose: $F(1,12)=2.9$, NS], but a pretreatment x treatment x prepulse intensity interaction for the higher dose [$F(2,24)=4.9$, $P<0.02$] that was followed by *posthocs* indicated that amylin significantly improved PPI in AMPH-treated rats ($P<0.05$) at multiple prepulse intensities (**Fig. 1b**). Similar comparison of means in the low-dose experiment also indicated that at the 3-dB prepulse intensity, there was a strong trend ($P<0.07$) for higher PPI levels in the Amy/AMPH versus the Veh/AMPH condition (**Fig. 1a**). Therefore, intra-AcbSh amylin, without altering basal PPI on its own, was found to partially reverse PPI deficits that were induced by systemic AMPH.

To ascertain the specificity of this amylin reversal to actions at the AMY1-R, an additional study was conducted in which a cocktail of amylin (100 ng) plus the highly selective AMY1-R antagonist AC187 (20 ug) was infused into AcbSh prior to systemic AMPH (1.75 mg/kg). The reasoning was that if AC187, which has 40-400-fold greater affinity for the AMY1-

R compared to any other in the calcitonin receptor family (Hay et al., 2005; Young, 2005), could prevent the PPI-restorative effect of amylin, then it could be concluded that amylin's ability to restore PPI in AMPH-treated rats is due to selective actions at the AMY1-R and not from nonspecific effects at other receptors. In this study, mean composite PPI values (PPI averaged across the three prepulse intensities since no significant interaction with this variable was seen) were: vehicle/vehicle = 49.17 ± 5.2; vehicle/AMPH = 23.5 ± 2.4; amylin&AC187 cocktail/vehicle = 52.9 ± 9.2; amylin&AC187 cocktail/AMPH = 26.4 ± 7.7. As before, there was a main effect of AMPH treatment showing a significant disruption of PPI by AMPH [$F(1,4)=9.3$, $P<0.05$]. The amylin&AC187 cocktail had no effect on PPI [$F(1,4)=0.3$, NS], nor was a pretreatment x treatment interaction seen [$F(1,4)=0.008$, NS], indicating that the cocktail did not alter the AMPH-induced PPI deficit. Therefore, intra-AcbSh infusion of the selective antagonist for the AMY1-R (AC187) completely abolished amylin's ability to reverse the AMPH-induced PPI deficit, suggesting that even at the 100-ng dose, amylin's PPI-restorative ability arises from actions specifically at the AMY1-R.

As one additional control, we also conducted a study examining the effects of this dose of AC187 on its own, to confirm that the above null result with the cocktail infusion was not due to individual AC187 effects on PPI. Thus, separate rats were tested with intra-AcbSh infusions of AC187 (0 or 20 ug) immediately before PPI testing (N=7) at the same time of day as the previous studies were conducted. No main effect of AC187 was seen on PPI [$F(1,6)=0.6$, NS] and there was no interaction of treatment with prepulse intensity [$F(2,12)=0.9$, NS]. The mean composite PPI values were: 41.03 ± 4.6 for vehicle, and 45.61 ± 5.0 for AC187. Hence, during the daytime, AC187 does not affect PPI on its own, but does completely prevent the PPI-

restorative actions of 100 ng amylin in the AcbSh. **Fig 1d** shows a representative injector placement in the AcbSh.

During conditions of high endogenous amylin release, AC187 disrupts PPI in a haloperidol-reversible manner. While the previous study indicated no effect of AC187 on PPI during the daytime, it should be noted that the light portion of the light-dark cycle likely represents the nadir of endogenous amylin tone, and thus antagonism of AMY1-R in AcbSh at that timepoint is unlikely to have much effect (since there is little endogenous ligand to block at that time). Several reports indicate that endogenous amylin release, which occurs with insulin release from pancreatic cells, is highest post-prandially; given that in rats, the dark portion of the light/dark cycle is when the highest levels of feeding occur, endogenous amylin tone would be highest during the dark phase (Arnelo et al., 1998; Ogawa et al., 1990; Qi et al., 2010). Therefore, it is possible that endogenous amylin tone in AcbSh does play a role in PPI, but that in order to adequately assess this hypothesis, one has to administer intra-AcbSh AC187 during the dark phase of the light/dark cycle.

Hence, Experiment 3 was run during the dark phase, and did reveal a significant main effect of AC187 on PPI [$F(1,6)=20.3$, $P<0.004$], with *posthocs* indicating that AC187 disrupted PPI at all prepulse intensities ($P<0.05$ – $P<0.01$; **Fig. 1c**). Haloperidol on its own had no effect [$F(1,6)=1.6$, NS], but did interact with AC187 treatment [$F(1,6)=6.9$, $P<0.039$]. *Posthocs* showed that haloperidol significantly improved PPI in AC187-treated rats ($P<0.05$) at multiple prepulse intensities (**Fig. 1c**). Thus, blocking AMY1-R at a time when endogenous amylin tone is relatively high produces a deficit in PPI that can be reversed by DA receptor antagonism,

suggesting that AcbSh AMY1-R signaling regulates PPI in part by modifying DA levels in AcbSh.

***Amylin in dorsal lateral striatum (DLS) has no effects*.** To determine the anatomical specificity of the amylin reversal of AMPH-induced PPI deficits, separate studies were conducted to evaluate a wide dose range of amylin infusions into the neighboring DLS. Because no significant interactions were found with prepulse intensity and any other factor, these data are presented as composite PPI scores (PPI values averaged across the three prepulse intensities). Once again, AMPH disrupted PPI, as indicated by significant main effects of treatment [$F(1,7)=9.2$, $P<0.02$ & $F(1,9)=41.2$, $P<0.001$] and subsequent *posthocs* ($P<0.05$) in these experiments (**Fig. 2a**). Neither the 100-ng amylin dose [$F(1,7)=0.05$, NS] nor 30- and 300-ng doses [$F(2,18]=0.5$, NS] in DS produced main effects on PPI. Amylin into DLS also did not interact with AMPH treatment at any dose [$F(1,7)=0.2$, NS & $F(2,18)=0.9$, NS], indicating that it did not change AMPH-induced PPI deficits. For simplicity of visual presentation, the Veh//Veh and Veh/AMPH bars have been averaged across the two experiments, since there was no significant difference between them [$F(1,16)=0.7$, NS], and all DLS PPI results are shown together in **Fig 2a**. A representative injector placement in the DLS is shown in **Fig. 2b**.

2) Baseline startle

In every experiment with AMPH, there was a significant main effect of AMPH treatment on baseline startle, with AMPH lowering this measure (Table 1). No effects of any other drug or any interactions with AMPH treatment were seen in any experiment. Therefore, AMPH reduced

baseline startle (an effect that we and others have seen previously (Alsene et al., 2010)), but in contrast to the PPI profile, the startle-reduction effect of AMPH was *not* reversed by stimulating AMY1-R in AcbSh, suggesting that the PPI-ameliorative effects of AcbSh amylin are specific to sensorimotor gating per se and not just an artifact of altering baseline startle responses.

3) Amylin receptor gene expression

Highest levels of RAMP-1 and CT-R gene expression overlap in the medial AcbSh where amylin and AC187 exert their PPI effects. Previous whole-brain surveys evaluated RAMPs and CT-Rs separately, with just 1 or 2 striatal sections not in anatomical registration across studies (Nakamoto et al., 2000; Oliver et al., 2001; Ueda et al., 2001; Becskei et al., 2004; Lee et al., 2008). We therefore conducted a systematic *in situ* hybridization study of all RAMPs and the two calcitonin receptor-subtype genes in serial striatal sections, focusing on levels through Acb. Gene expression analyses (optical density measurements) were conducted using 5 ROIs following a dorsal-ventral and medial-lateral progression through the striatum and allied regions. ROIs were placed in dorsolateral striatum (DLS), central striatum (CS), nucleus accumbens core (AcbC), medial nucleus accumbens shell (AcbSh), and olfactory tubercle (OT) (see **Fig. 3**). Mean optical density values within ROIs were analyzed at three anteroposterior levels of the striatum, with ROIs from each hemisphere averaged together. For RAMP-1, ANOVA showed significant differences among the ROIs across the three levels (ROI X level interaction, $F(8,45)= 2.5$; $P=0.023$). Expression (collapsed across anteroposterior level) was highest in the OT compared to all other regions ($Ps< 0.001$), and three levels (ROI X level interaction, $F(8,45)= 2.5$; $P=0.023$). Expression (collapsed across anteroposterior level) was highest in the OT

compared to all other regions (Ps< 0.001), and expression in medial AcbSh was higher than all other regions except OT (Ps< 0.001). AcbC showed higher levels of expression than DLS (P= 0.009). Individual analyses at each anteroposterior level revealed that the most posterior level of the AcbSh showed significantly less RAMP-1 expression compared to the anterior (P< 0.001) and middle (P=0.008). For CT-R (N=2), gene expression was limited almost exclusively to the AcbSh (ROI X level interaction: F(8,15)= 6.6; P< 0.001). Photographic emulsion autoradiography confirmed the strong signal seen in the medial AcbSh (overlapping with medial intra-AcbSh zones of strong substance P immunoreactivity, in side-by-side comparisons with substance P-immunostained, in-register sections; **Fig. 3**). At middle and posterior levels, some signal was seen in medial aspects of AcbC, although labeling was still strongest in AcbSh. As a control, labeling with a sense probe for the RAMP-1 gene was also conducted; as expected, no signal was detected with this manipulation (**Fig. 3**), indicating that the expression patterns seen with the above antisense probes were not due to nonspecific binding.

DISCUSSION

Intra-AcbSh infusion of amylin reversed the psychotomimetic effect of AMPH in the PPI paradigm; coinfusion of the highly selective AMY1-R antagonist AC187 completely prevented this reversal, indicating specificity of this phenomenon to the AMY1-R. Furthermore, blockade of AMY1-R with AC187 during a period of high endogenous amylin tone produced a PPI disruption that was reversible by the dopamine antagonist, haloperidol. These PPI effects were independent of baseline startle alterations, suggesting that AMY1-R regulation of basal or AMPH-disrupted PPI truly reflects modifications of sensorimotor gating and cannot be ascribed

simply to being an artifact of baseline startle changes. Furthermore, a systematic striatal *in situ* hybridization mapping study showed significant anteroposterior and dorsal-ventral intra-striatal gradients of CT-R and RAMP-1 gene expression (the molecular components of the high-affinity AMY1-R), with an overlapping zone in a circumscribed area of the medial AcbSh, where infusion of amylin-active compounds modulated PPI. To our knowledge, this is the first demonstration that endogenous amylin signaling specifically in the ventral striatum regulates schizophrenia-like information-processing deficits, likely through the modulation of dopaminergic activity.

The ability of amylin in AcbSh to normalize AMPH-induced PPI deficits demonstrates a novel peptide system that could be involved in SZ-like sensorimotor gating abnormalities. Our finding that AcbSh AC187 prevented the amylin reversal of PPI deficits under conditions when it did not alter PPI on its own strongly indicates that the amylin-PPI-reversal effect is mediated specifically by AMY1-R, since AC187 is highly selective for AMY1-R: AC187 is 40-fold more selective for amylin receptors as compared to calcitonin receptors, and 400-fold more selective for amylin receptors versus CGRP receptors (Hay et al., 2005; Young, 2005). Furthermore, in areas where little or no AMY1-R components were expressed (i.e., DLS), amylin infusions had no behavioral effects, which also shows that the AcbSh results cannot be due to diffusion of the peptide to other neighboring regions. Hence, the present findings provide strong evidence for the importance of AMY1-R in AcbSh as potential modulators of basic information-processing functions that are aberrant in SZ, and that compounds that stimulate AMY1-R could have potential antipsychotic-like effects in these paradigms.

To date, central amylin actions have been studied almost exclusively with regard to food intake and energy-balance regulation (Castillo et al., 1995; Rushing et al., 2001; Lutz, 2012). Amylin is released peripherally along with insulin from pancreatic beta cells, but acts centrally to produce satiety-like effects through first-stage neurons in the area postrema (AP) that transsynaptically suppress hypothalamic feeding centers via a parabrachial relay (Potes et al., 2010; Lutz, 2012). Amylin also modulates leptin's effects in the hypothalamus to produce synergistic effects on body-weight regulation (Roth et al., 2008; Turek et al., 2010). Beyond the AP and mediobasal hypothalamus, however, amylin's central effects are poorly understood, despite the fact that the Acb exhibits among the highest density of amylin binding sites in the entire brain (Sexton et al., 1994; van Rossum et al., 1994). Only one prior study examined intra-Acb amylin actions, showing a suppressive effect of exogenously administered amylin on feeding and associated exploratory activity (Baldo and Kelley, 2001); however this study did not assess the behavioral role of *endogenous* intra-Acb amylin signaling.

The present demonstration of a psychotomimetic-like PPI disruption with intra-AcbSh infusions of the amylin receptor antagonist, AC187, reveals, for the first time, a behaviorally relevant amylin "tone" at the level of the telencephalon. The endogenous ligand is unknown, but likely candidates include peripherally released amylin (uptake studies showed that amylin crosses the blood-brain barrier to collect in striatum and other sites, with better brain penetrance than insulin (Banks and Kastin, 1998)), or endogenous peptides of the amylin family, such as CGRP (van Rossum et al., 1997). This experiment was first attempted during the daytime, to match the experimental timepoint at which we observed the intra-AcbSh amylin reversal of AMPH; however, we failed to see an effect of AC187 on PPI during this timepoint. We

hypothesized that this was due to circadian fluctuations in endogenous amylin levels. Since amylin is released in response to feeding-related cues, circulating amylin levels are low when rats are naturally asleep; therefore, we repeated the experiment during the dark portion of the light/dark cycle (Butler et al., 1990; Moore and Cooper, 1991; Young and Denaro, 1998). Another study has found that systemic amylin had a relatively weak anorectic effect when administered during the middle of the dark phase, unless the rats had been food-deprived, which could indicate a ceiling effect due to already high levels of circulating amylin (Lutz et al., 1995). Our results were consistent with our hypothesis, since intra-AcbSh AC187 exerted an effect on PPI during the dark period but not during the light period of the light/dark cycle.

The reversal of the PPI-disruptive effect of intra-AcbSh AC187 by haloperidol suggests that amylin signaling in the Acb interacts with dopamine function. Prior studies have provided general evidence for amylin-dopamine interactions in the brain, although the specific sites of interaction are unclear. For example, intraventricularly administered amylin reduces the effects of systemically administered dopamine agonists on locomotor activity and sexual behavior (Clementi et al., 1996; Clementi et al., 1999), a D2 antagonist alters the satiety effect engendered by peripheral amylin, possibly via interactions in the NTS (Lutz et al., 2001), and amylin's feeding-inhibitory effects are augmented in D3 receptor knockout mice (Benoit et al., 2003).The present finding that haloperidol reversed AC187's PPI-disruptive effect indicates that dopamine signaling contributes to behavioral changes induced by amylin receptor blockade at the level of the AcbSh. A parsimonious explanation is that the interaction occurs at AMY1-Rs located postsynaptic to dopaminergic innervation, because CT-R and RAMP-1 mRNA is localized in AcbSh---indicating that the receptors are produced in intrinsic striatal neurons. For example,

amylin may reduce DA receptor activity in Acb medium spiny neurons either through membrane receptor-receptor interactions or intracellular signaling cascades, such that amylin blockade releases DA receptors from negative modulation and augments their responsiveness to baseline levels of dopamine release. Conversely, the enhancement of amylin signaling with exogenous agonists would augment negative dopaminergic modulation, accounting for the antipsychotic-like actions of amylin seen here. Further studies are required to identify the precise mechanism; nevertheless, the present results are the first to suggest tonic modulation of DA activity by amylin signaling in the Acb.

Binding of radiolabeled amylin is distributed heterogeneously throughout the brain, and early studies identified a circumscribed area of particularly high-affinity amylin binding in the medial parts of ventral striatum (Sexton et al., 1994; van Rossum et al., 1994; Aiyar et al., 1995). Molecular analyses revealed that binding affinity to amylin-family peptides is conferred by the various combinations among two receptor genes, CT-R and calcitonin-like receptor (CLR), and three RAMPs (RAMPs 1-3) (Christopoulos et al., 1999); intracellular co-expression of RAMP-1 and CT-R yields the high affinity AMY-1 subtype whose affinity profile for amylin and related peptides matches the high-affinity binding seen in the Acb (Poyner et al., 2002; Young, 2005). The presence of CT-R and RAMP-1 in striatum and Acb has been reported in whole-brain surveys (Nakamoto et al., 2000; Oliver et al., 2001; Ueda et al., 2001; Becskei et al., 2004; Lee et al., 2008), and here we extended these results by showing a systematic mapping and semi-quantitative analysis of these genes through multiple levels of the striatum. We confirm that RAMP-1 and CT-R are the only amylin-receptor family genes present in striatum in high abundance, and moreover show a gradient of RAMP-1 gene expression, with highest levels in a

restricted zone of medial AcbSh. Here it overlaps a remarkably circumscribed zone of intense CT-R expression. At rostral levels of the Acb, CT-R expression is seen almost exclusively in the medial shell, in registration with the classic marker of the core-shell boundary, substance P (Zahm and Heimer, 1993). Progressing caudally, CT-R expression is still highest in the medial shell, although some labeling is also seen in medial sectors of the core. CT-R gene expression is almost completely absent from lateral parts of the core, and completely absent from dorsal striatum.

Hence, the AMY1-R represents a substrate at which neural activity in the medial AcbSh, relative to the rest of striatum, can potentially be targeted with considerable precision. An interesting implication is that amylin-active agonists may represent adjunct treatments that potentiate antipsychotic actions in the ventral but not dorsal striatum, reducing the required antipsychotic dose and limiting motoric or other side effects mediated in the dorsal striatum. Beyond schizophrenia, the circumscribed distribution of AMY1-R gene components has implications for understanding the neurochemical modulation of behavioral processes mediated specifically by the medial shell, notably the enhancement of hedonic food reward (Pecina and Berridge, 2005).

The amylin analog, Pramlintide, has shown clinical effectiveness as an adjunct treatment for Type-2 diabetes, and in several studies shows promise as an anti-obesity drug (Singh-Franco et al., 2011; Roth et al., 2012). The present results suggest that this drug could potentially be repurposed as an adjunct treatment for schizophrenia patients for whom second-generation antipsychotics offer symptomatic relief, but who suffer from the well-known obesity and Type-2 diabetes side effects (De Hert et al., 2011). The present results suggest that amylin-like

compounds may not only reverse these side effects, but also improve antipsychotic drug actions. In this regard, amylin and similar compounds may have potential for development as a novel category of third-generation antipsychotics.

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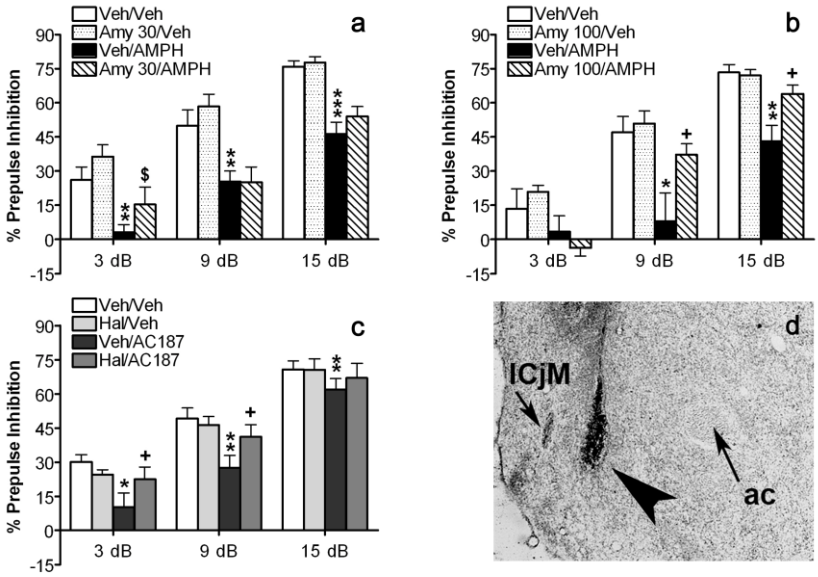

Figure 1: Effects on PPI of amphetamine (AMPH, 1.75 mg/kg) and intra-AcbSh amylin (Amy) - **a)** Amy, 30ng/0.5ul; **b)** Amy, 100ng/0.5ul. **c)** haloperidol (Hal, 0.1 mg/kg) and intra-AcbSh AC187 (20ug/0.5ul). **d)** Large arrows show representative injector tip placements within the AcbSh. *ac* = anterior commissure; *ICjM* = island of Calleja. Prepulse intensities are in dB above background. Values represent means ± SEM. *****P<0.05, ******P<0.01 versus corresponding Vehicle/Vehicle condition. +P<0.05 versus Veh/AMPH. **$** denotes a trend (P<0.07) versus Veh/AMPH.

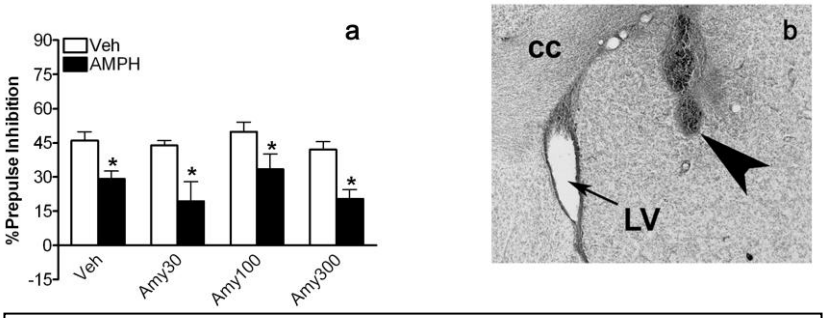

Figure 2: **a)** Effects of amphetamine (AMPH, 1.75 mg/kg) and intra-DLS amylin (Amy, 30, 100, 300ng/0.5 ul) on composite PPI (average of PPI values across all prepulse intensities). Values represent means ± SEM. *****P<0.05 versus corresponding vehicle/vehicle (Veh/Veh). **b)** Large arrows show representative injector tip placements within DLS, as indicated by the arrows. *LV* = lateral ventricle; *cc* = corpus callosum.

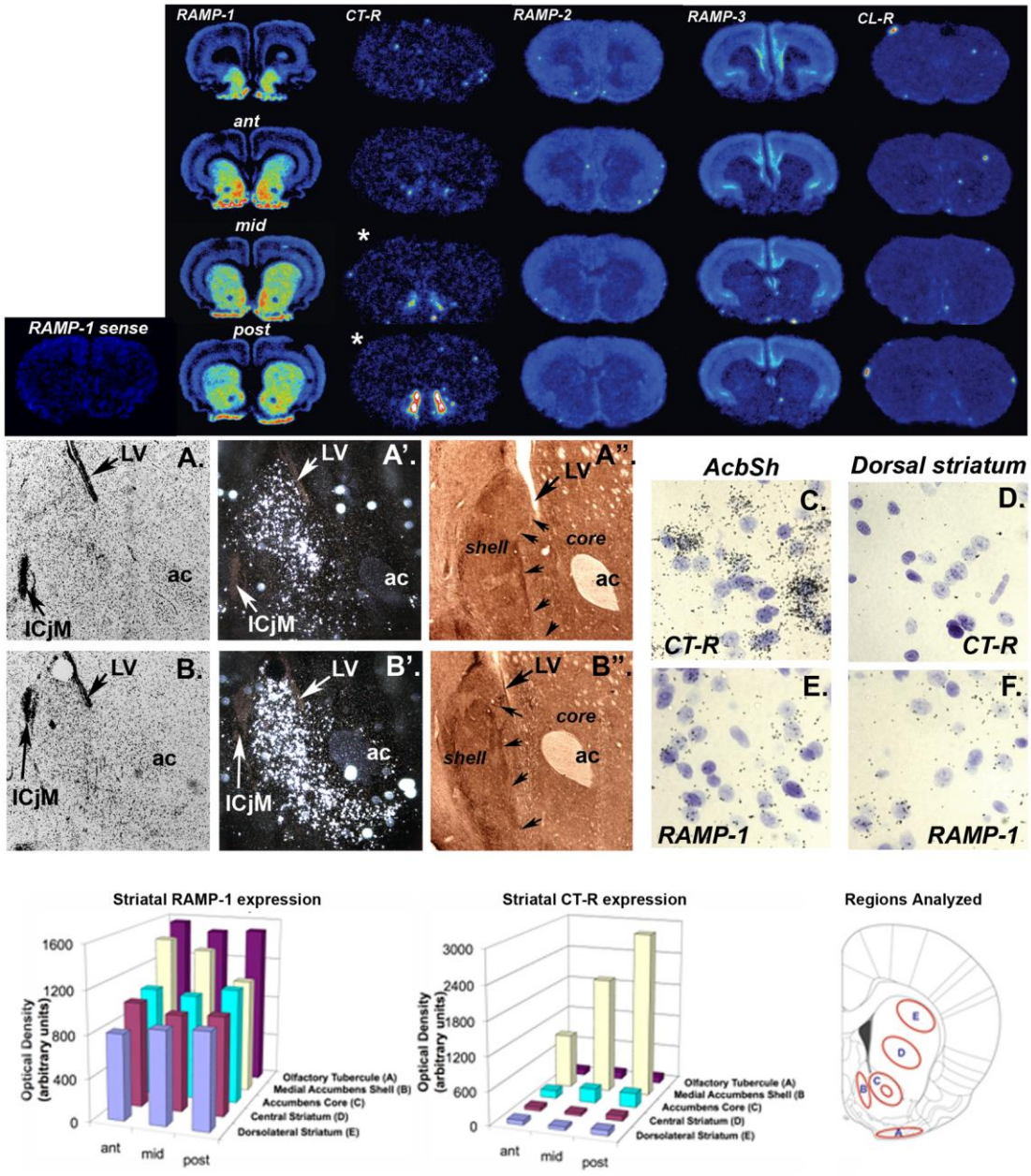

Figure 3: *Top panel*: Pseudocolor images showing hybridization of probes for RAMPs 1-3, CTR, and CL-R in the striatum. Warmer colors indicate greater labeling intensity; white shows highest intensity. *Middle panel*: Sections hybridized with CT-R probe, processed for photographic emulsion autoradiography (with Nissl counterstain) at two anteroposterior levels (see asterisks in the top panel). (A) and (B), brightfield images showing the Nissl staining; (A') and (B'), darkfield images of the same field of view, with silver grains showing CT-R labeling; (A'') and (B''), in-register sections processed for substance P immunoreactivity. Small arrowheads depict the core-shell boundary. (C-F), brightfield images of silver grains (appearing as black specks) for CT-R and RAMP-1 labeling and Nissl-stained cells (appearing purple). *Bottom panel*: Bar graphs depicting signal intensity of RAMP-1 and CT-R at multiple striatal levels. "Ant," "mid," and "post" correspond to levels shown in top panel. Abbreviations: ac=anterior commissure; LV=lateral ventricle; ICjM=Island of Calleja, major island.

Experiment	F-Ratio	Treatment	Startle
1	F(1,5)=83.5, P<0.001	Veh/Veh	322 ± 37
		Amy 30/Veh	394 ± 33
		Veh/AMPH	114 ± 11 **
		Amy 30/AMPH	113 ± 23 **
2	F(1,12)=12.3, P<0.004	Veh/Veh	449 ± 135
		Amy 100/Veh	447 ± 89
		Veh/AMPH	191 ± 48 *
		Amy 100/AMPH	270 ± 45
3	No significant main effects or interactions	Veh/Veh	578 ± 76
		Hal/Veh	595 ± 55
		Veh/AC187	517 ± 70
		Hal/AC187	601 ± 60
4 & 5	F(1,7)=27.1, P<0.01 F(1,9)=60.7,P<0.0001	Veh/Veh	453 ± 57
		Amy30/Veh	354 ± 34
		Amy100/Veh	561 ± 76
		Amy300/Veh	334 ± 30
		Veh/AMPH	219 ± 30 **
		Amy30/AMPH	192 ± 22 ***
		Amy 300/AMPH	203 ± 34 **
Amylin /AC187 cocktail study	F(1,4)=64.1,P<0.01	Veh/Veh	339 ± 41
		Cocktail/Veh	442 ± 63
		Veh/AMPH	114 ± 14 **
		Cocktail/AMPH	191 ± 34 *
AC187 daytime study	No significant main effects or interactions	Veh	419 ± 67
		AC187	407 ± 58

Table 1: Startle magnitude ± SEM, organized by experiment number. Veh = Vehicle, Amy = Amylin dose in ng/0.5ul, AMPH = amphetamine (1.75 mg/kg), Hal = haloperidol (0.1 mg/kg), AC187 = 20 ug /0.5 ul. *****P<0.05, ******P<0.01, *******P<0.001, compared to the corresponding Veh/Veh value. For visual simplicity, the data from Experiments 4 and 5 are combined, but statistical analyses were performed separately. F-ratios are for significant main effect of AMPH treatment for each study.

Chapter III:

Endogenous amylin receptor signaling in the nucleus accumbens opposes non-homeostatic μ-opioid-driven feeding after a meal

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Abstract

Amylin is a pancreatically released peptide that penetrates into the brain, and produces satiety-like effects via actions in the brainstem and mesencephalon. Little is known, however, about amylin's effects in the nucleus accumbens shell (AcbSh), where a circumscribed zone of intense amylin receptor (AMY-R) binding closely overlaps established mappings of a 'hotspot' for μ-opioid receptor (μ-OR) amplification of food reward. In the present study, the ability of intra-AcbSh AMY-R signaling to regulate μ-OR-driven feeding was explored. Amylin was administered with the μ-OR agonist, DAMGO, directly into the AcbSh of *ad-libitum*-maintained rats. Intra-AcbSh amylin dose-dependently reversed DAMGO-induced hyperphagia; a 3-ng dose reduced DAMGO-mediated feeding by nearly 50%. This dose was, however, completely ineffective at altering DAMGO-induced feeding in the anterior dorsal striatum (ADS). Intra-AcbSh amylin alone modestly suppressed 10% sucrose intake in *ad-libitum*-maintained rats, and chow in food-deprived rats, but a 30 ng dose was required. This result indicates that reversal of AcbSh DAMGO-induced feeding at a 10-fold lower dose was due neither to malaise nor motoric impairment. Finally, intra-AcbSh infusion of the AMY-R antagonist, AC187, significantly attenuated the ability of pre-feeding to reduce DAMGO-induced food intake, with no effects in non-prefed rats. Hence, endogenous AMY-R signaling potently regulates μ-OR-mediated appetitive responses at the level of the AcbSh. This negative modulation appears strongest after eating, when circulating amylin levels are highest, suggesting a novel pathway for peripheral-central integration in the control of appetitive motivation and opioid reward.

1. Introduction

Amylin is a 37-amino acid peptide that belongs to the calcitonin family, which also includes calcitonin, calcitonin gene-related peptide (CGRP), and adrenomedullin (Poyner et al. 2002; van Rossum et al. 1997). Originally isolated from an insulin-producing tumor (Westermark et al. 1987) and diabetic pancreas (Cooper et al. 1987), amylin is cosecreted with insulin from pancreatic beta cells in coordination with prandial stimuli (Ahren and Sundler 1992; Butler et al. 1990; Moore and Cooper 1991) . Once secreted, amylin counterbalances insulin's effects on glycogen synthesis and glucose uptake in muscle, and therefore plays an important role in glycemic control (Cooper et al. 1988; Leighton and Cooper 1988; Molina et al. 1990).

In addition to these metabolic effects, amylin also regulates food intake via actions at multiple levels of the central nervous system (CNS). Amylin penetrates into the brain at least as well as insulin, and accumulates in sites throughout the neural axis (Banks and Kastin 1998; Butler et al. 1990; Moore and Cooper 1991). Because CNS amylin receptors (AMY-Rs) show regional differences and localization to discrete neural pathways and structures, it is hypothesized that amylin and related peptides play a role in neuroregulation (Beaumont et al. 1993; Christopoulos et al. 1995; Sexton et al. 1994; van Rossum et al. 1994). Accordingly, amylin infusion causes a satiety-like suppression of feeding when infused into the lateral ventricle, third ventricle, hypothalamus, and ventral tegmental area (VTA) (Bouali et al. 1995; Chance et al. 1991; Lutz et al. 1998a; Mietlicki-Baase et al. 2013; Morley and Flood 1991; Rushing et al. 2000). Perhaps the most extensively studied site for amylin's feeding modulatory actions is the area postrema; blockade of area postrema AMY1 amylin receptors and lesions

specific to the area postrema both attenuate the anorectic effect of systemically-administered amylin (Lutz et al. 2001; Lutz et al. 1998b; Mollet et al. 2004).

Far less is known about amylin's feeding-modulatory effects in the telencephalon, despite the fact that one of the densest concentrations of high-affinity amylin binding sites, and expression of component genes encoding the high-affinity amylin receptor (AMY1), is found in the medial nucleus accumbens shell (AcbSh). This zone of intense amylin receptor (AMY-R) binding conforms remarkably well with the circumscribed medial AcbSh area from which intense feeding responses are elicited by GABA or μ-opioid receptor (μ-OR) stimulation (Bakshi and Kelley 1993a; Stratford and Kelley 1997; Zhang et al. 1998; Zhang and Kelley 1997; 2002), moreover, the established "hotspot" for amplification of hedonic taste reactions by μ-OR stimulation closely overlaps with AMY-R distribution (Pecina and Berridge 2005). Hence, AcbSh-localized AMY-Rs are well-positioned to modulate food intake and hedonic taste reward via interactions with multiple neuromodulator systems, including the μ-opioid system.

To date, only one study (Baldo and Kelley 2001) has investigated the role of AcbSh-localized AMY-Rs in regulating feeding behavior; this study showed that exogenously administered amylin in the 30-100 ng range suppressed feeding. Nevertheless, the interaction of AMY-Rs with other Acb-localized neuromodulator systems, and, importantly, the role of endogenous AMY-R signaling in the AcbSh, remains unknown. Hence, in the present study, interactions between AMY-Rs and μ-ORs were studied, both in the AcbSh where dense AMY1 binding is found, and the anterior dorsal striatum (ADS), devoid of AMY-R binding but where μ-ORs also modulate feeding (Bakshi and Kelley 1993b; DiFeliceantonio et al. 2012). We also examined the effects of AMY-R blockade on μ-OR-driven feeding, during either a food-deprived

state (when circulating amylin is low) or immediately after a pre-feeding session (when circulating amylin levels are high) (Arnelo et al. 1998; Ogawa et al. 1990), to explore whether an endogenous 'tone' of AMY-R signaling at the level of the AcbSh regulates the behavioral functions of μ-ORs.

2. Methods

2.1 Subjects

Subjects in all experiments were male Sprague-Dawley rats, obtained from Harlan (Madison, WI), weighing 300-325 g on arrival at the laboratory. The rats were housed in clear polycarbonate cages (9.5-in. width x 17-in. length x 8-in. height), with cob bedding, in a light- and temperature- controlled vivarium. Animals were maintained under a 12:12-h light-dark cycle (lights on at 7:00 AM). Food and water were available ad libitum, except as indicated for the various experiments. Animals were handled daily to reduce stress. Testing occurred between 1200-1800 h. All facilities and procedures were in accordance with the guidelines regarding animal use and care put forth by the National Institutes of Health and were supervised and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

2.2 Surgical Procedure

Rats (weighing 300-325 g at the time of surgery) were anesthetized with isoflurane gas and then secured in a Kopf stereotaxic frame. Bilateral stainless steel cannulae (10-mm long, 23 gauge) were implanted according to standard stereotaxic surgical procedures. Cannulae were aimed bilaterally either at the accumbens shell (AcbSh) or at the anterodorsal striatum (ADS) using the

atlas of Paxinos and Watson (1998). All surgeries were flat skull with the nosebar set to 3.3 mm below interaural zero. For the AcbSh, the surgical coordinates from bregma were: +3.2 mm anteroposterior (AP); ±1.0 mm lateromedial (LM); and -5.2 mm dorsoventral (DV) (with injectors extending an additional 2.5 mm beyond cannulae tips for a final DV coordinate of -7.7). For ADS surgeries, the surgical coordinates from bregma were: +1.6 mm AP; ±2.4 mm LM; and -1.7 mm DV (with injectors extending an additional 2.5 mm beyond cannulae tips for a final DV coordinate of -4.2). Cannulae were fixed in place with dental acrylic (New Truliner, Skokie, IL) and anchoring skull screws (Plastics One, Roanoke, VA). Wire stylets (10-mm long, 30 gauge) were placed in the cannulae to prevent blockage. Animals were given an intramuscular injection of penicillin (0.3 ml of a 300,000 U/ml suspension; Phoenix Pharmaceuticals, St. Joseph, MO), placed in a warm recovery cage, returned to their home cages on awakening, and given a recovery period of no less than 5 days (with daily health checks) before behavioral testing commenced.

2.3 Drugs

Amylin (Bachem, Torrance, CA) and ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) (DAMGO) (Bachem) were dissolved in sterile isotonic saline, while AC187 (Tocris Bioscience, Ellisville, MO) was dissolved in sterile dH2O. The 0.25 µg/0.5 µl/side dose of DAMGO was chosen because it has been shown to elicit robust feeding in satiated rats (Perry et al. 2009). The dose of the AMY1 antagonist AC187 (Hay et al. 2005) was chosen because it was effective at altering prepulse inhibition in our lab (Baisley et al., 2013). In other literature, a dose of 30 µg but not 10 µg delivered into the central nervous system was sufficient to increase food intake in rats (Lutz et al. 1997; Mollet et al. 2004; Rushing et al. 2001); the present experiment used an AC187 dose

of 20 µg per side. All three drugs were infused directly into specific brain regions in accordance with the experimental designs. For microinfusions, injectors (connected via tubing to a microdrive pump) extended 2.5 mm past cannulae tips, and delivered drugs at 0.32 µl/min over 1 min 33 sec, with a 1-min post-infusion period before reinsertion of stylets and placement of rats into testing chambers.

2.4 Experimental Design

In all experiments, testing was conducted in wire-bottom polycarbonate cages with rat chow pellets and water freely available. A sheet was placed under the cages to collect food spillage. Chow and water intake were measured after a 30-min test period. Prior to all the experiments, rats were habituated to the testing cages to minimize stress.

2.4.1 Interactions between amylin and DAMGO in the AcbSh

Effects of amylin were tested in two dose ranges (0, 1, 3 ng and 0, 10, 30 ng). These two dose ranges were tested in separate groups of rat (n=8 for the low-dose range; n=10 for the high-dose range) to minimize the number of infusions per experiment. For both experiments, rats were surgically implanted with cannulae aimed at the accumbens shell. Rats were infused with saline or 0.25 µg/0.5 µl DAMGO into the AcbSh 10 minutes before testing. 5 minutes before testing, rats received a second infusion of saline or amylin into the AcbSh. DAMGO dose and amylin dose were both within-subjects factors; hence, each rat received all amylin doses with or without DAMGO. Treatments were counterbalanced across test days according to Latin square designs.

2.4.2 Interactions between amylin and DAMGO in the ADS

The same basic protocol as Experiment 1 was followed, except N=6 rats were implanted with cannulae aimed at the ADS instead of the AcbSh. ADS coordinates were chosen based on prior literature describing a μ-opioid-sensitive zone just dorsal to the anterior Acb (Bakshi and Kelley 1993b; DiFeliceantonio et al. 2012). Rats received DAMGO (0, 0.25 μg) and amylin (0, 3 ng) according to a within-subjects, counterbalanced design. The 3 ng dose was chosen because it robustly decreased DAMGO-induced feeding in the AcbSh.

2.4.3 Effects of intra-AcbSh amylin (without DAMGO) on hunger- and palatability-induced feeding

Eight rats were used to test the effects of intra-AcbSh amylin in sucrose intake in *ad libitum*-maintained rats. Prior to experimentation, the rats were exposed daily for 2 days to a 10% sucrose solution until they consumed a consistent amount over two 30-minute test sessions spaced 4 hours apart. Once stable intake was achieved, rats were given intra-AcbSh infusion of amylin (0, 3, 10, 30 ng/0.5 μl/side) administered according to a Latin square design. Amylin was infused 5 minutes before testing, whereupon rats were placed into the testing cages for 30 minutes with free access to chow, water, and a bottle containing 10% sucrose. Intake was measured after the 30-minute testing period had ended.

Seven of the 8 rats in this experiment also underwent testing for intra-AcbSh amylin effects on hunger-driven chow intake. In this part of the experiment, rats were food-deprived for 18 h prior to each testing period, given intra-AcbSh amylin (0, 3, 10, 30 ng/0.5 μl) infusions, and placed

into the testing cages for 30 minutes with rat chow and water present. The 2 experiments (sucrose intake and hunger-driven chow intake) were performed in a counterbalanced order, with half the rats receiving sucrose first, and the other half, hunger/chow intake first.

2.4.4 Effects of AC187 on DAMGO-induced feeding, with or without pre-feeding.

Seven rats were surgically prepared with cannulae aimed at the AcbSh. After recovery, rats underwent behavioral testing every other day for a total of 8 test days. All rats were food-deprived for 18 h before each of these testing days. On each testing day, rats were either given a 30-min "prefeeding" session, or given no prefeeding session, whereupon they received intra-AcbSh infusions of DAGMO and AC187. DAMGO (0, 0.25 µg/0.5 µl) was infused bilaterally into the AcbSh 10 minutes before testing, followed 5 min later by an infusion of AC187 (0, 20 µg/0.5 µl). For rats exposed to the prefeeding session, infusions were given after the prefeeding session, and the time between the prefeeding and feeding session was 15 min. Following drug infusions, both prefed and non-prefed rats were tested in a 30-min feeding session. Each rat received all treatment combinations (mock/mock, DAMGO/mock, mock/AC187, DAMGO/AC187) under both prefed and non-prefed conditions, according to a within-subjects, Latin-square design, for a total of 8 treatment conditions per rat.

3. Results

3.1 Intra-AcbSh amylin potently reduces intra-AcbSh DAMGO-induced chow intake

In the low-dose study, there was a significant main effect of DAMGO on food intake [$F(1,7)=55.535$, $P<0.001$], with DAMGO increasing food intake (see Figure 1). In the lower dose

range, there was no main effect of amylin [$F(2,7)=3.042$, NS]; however, there was a significant interaction between DAMGO and amylin [$F(2,14)=4.817$, $P<0.05$], with amylin dose-dependently reversing the effects of DAMGO on food intake. Posthoc tests (paired t-tests with a Bonferroni correction for multiple comparisons) revealed that there were significant differences between the Saline/Saline and DAMGO/Saline conditions; as well as between the DAMGO/Saline and DAMGO/Amylin 3-ng conditions; but not between the Saline/Saline and Saline/Amylin 1-ng conditions; the Saline/ Saline and Saline/Amylin 3-ng conditions; nor between the DAMGO/Saline and DAMGO/Amylin 1-ng conditions. These results demonstrate that the 3 ng/0.5 µl dose of amylin in the AcbSh, which did not affect feeding on its own, significantly reversed DAMGO-induced feeding.

Similarly, in the high-dose study, DAMGO also had a main effect on food intake [$F(1,9)=17.870$, $P<0.01$] (see Figure 2). There was also a significant main effect of amylin [$F(2,9)=7.036$, $P<0.01$], and a significant DAMGO x amylin interaction [$F(2,18)=6.567$, $P<0.01$], with amylin dose-dependently reversing the effects of DAMGO on food intake. Posthoc tests (paired t-tests with a Bonferroni correction for multiple comparisons) revealed that there were significant differences between the Saline/Saline and DAMGO/Saline conditions; between the DAMGO/ Saline and DAMGO/Amylin 10-ng conditions; and between the DAMGO/Saline and DAMGO/Amylin 30-ng conditions; but not between the Saline/Saline and Saline/Amylin 10-ng conditions nor between the Saline/Saline and Saline/Amylin 30-ng conditions. Thus, 10 and 30 ng of amylin in the AcbSh did not suppress feeding non-specifically in ad libitum-maintained rats, yet these doses significantly reversed DAMGO-induced hyperphagia.

3.2 Intra-ADS amylin does not alter intra-ADS DAMGO-induced chow intake

It has been shown that, outside the Acb, a zone within the ADS also subserves μ-opioid-driven feeding (Bakshi and Kelley 1993b; DiFeliceantonio et al. 2012). We replicated these effects, obtaining a main effect of DAMGO in the ADS [$F(1,5)=39.749$, $P<0.01$] on food intake (see Figure 1 inset). In contrast to the AcbSh, there was no significant main effect of 3-ng amylin in the ADS on food intake [$F(2,5)=1.997$, NS]. There was also no significant interaction between ADS amylin and DAMGO on food intake [$F(2,10)=1.476$, NS]. Therefore, the dose of amylin that reduced the effects of DAMGO on food intake by nearly 50% in the AcbSh was ineffective at reducing DAMGO-induced feeding in the ADS.

3.3 Intra-AcbSh amylin modestly decreases hunger- and palatability-driven feeding

There was no main effect of AcbSh amylin on sucrose intake [$F(3,7)=1.902$, NS] (see Figure 3). However, planned comparisons showed a significant difference between the Saline condition and the Amylin 30-ng condition, with the Amylin 30-ng condition slightly suppressing sucrose intake ($P<0.01$). AcbSh amylin had a significant main effect on food intake in food-deprived rats [$F(3,6)=4.190$, $P<0.05$] (see Figure 4). Similarly to sucrose intake, posthoc tests showed a significant difference between the Saline and Amylin 30-ng conditions, but not between the Saline and Amylin 10-ng conditions.

3.4 Intra-AcbSh AC187 partly reverses the ability of prefeeding to suppress DAMGO-induced food intake

As expected, rats that were prefed ate less than rats that were not prefed [main effect of prefeeding: $F(1,6)=24.816$, $P<0.01$], and DAMGO had a significant main effect on food intake [$F(1,6)=268.220$, $P<0.0001$] (see Figure 5). Moreover, there was a significant DAMGO x amylin interaction [$F(1,6)=6.121$, $P<0.05$]. Fisher's PLSD tests revealed significant differences between the DAMGO/mock and prefed/DAMGO/mock conditions ($P<0.0001$), with the prefed rats exhibiting less DAMGO-induced feeding. Most interestingly, there was a significant difference between the prefed/DAMGO/mock condition compared to the prefed/DAMGO/AC187 condition ($P<0.05$), with rats in the latter condition eating more, thus demonstrating that blocking AMY-Rs in prefed rats partly reverses the ability of prefeeding to diminish μ-opioid-driven feeding responses. For other means comparisons, see Fig. 5 legend.

4. Discussion

These results show for the first time a potent modulatory influence of AMY-R signaling on μ-OR-mediated responses at the level of the AcbSh. Our results demonstrate that stimulating AMY-Rs with exogenously administered amylin reduces DAMGO-induced feeding at doses considerably lower than those required to even modestly diminish either hunger-associated chow intake or palatable feeding (sucrose drinking). Moreover, blockade of AMY-Rs partly reversed the ability of prefeeding to suppress intake engendered by intra-AcbSh DAMGO. Together, these results reveal a potent negative modulation of μ-ORs by both exogenous and endogenous AMY-R signaling, and show for the first time a role of endogenous AMY-R ligands in post-meal feeding modulation at the level of the AcbSh.

In the present study, the lowest effective dose of exogenously administered amylin to reduce DAMGO-driven feeding in the AcbSh was 3 ng. Hence, the potency for amylin to reverse

μ-OR-mediated feeding is comparable to that seen at lower levels of the neural axis; for example, 7 ng/rat of amylin infused into the lateral ventricle (Lutz et al. 1998a) and a 3.5 ng/rat dose into the third ventricle, immediately adjacent to the hypothalamus acutely reduced feeding (Rushing et al. 2000). We also found that the 3-ng amylin dose, strongly effective at reducing DAMGO-induced feeding in the AcbSh, was ineffective at altering μ-OR-driven feeding in the ADS. It has been shown that μ-OR stimulation outside the Acb, in more anterior striatal regions, increases feeding (Bakshi and Kelley 1993b; DiFeliceantonio et al. 2012). However, these striatal territories possess neither AMY-R binding nor expression of AMY-R-component genes. Therefore, our results indicate that DAMGO-induced hyperphagia is only reduced when amylin is infused into regions rich in AMY-R receptors.

Interestingly, we found that a relatively high dose of intra-AcbSh amylin (30 ng) was required to produce a very modest decrease of sucrose intake. This potency is consistent with the one prior report of intra-Acb amylin infusion on hunger-associated chow intake (Baldo and Kelley 2001) as well as results shown here with hunger-driven feeding. Initially, this may seem counterintuitive, because palatable feeding presumably engenders μ-opioid peptide release (DiFeliceantonio et al. 2012), and we found in the present study that intra-AcbSh amylin potently reduced food intake elicited by exogenous μ-opioid administration. However, it is worth considering that while intra-AcbSh DAMGO infusions affect μ-ORs only in that structure, sucrose drinking may recruit μ-opioids in multiple redundant sites; indeed, hyperphagia induced by intra-Acb DAMGO is dependent on the activation of a distributed network of brain regions (Will et al. 2003). Therefore, amylin actions (in the dose range tested) in the AcbSh may not be sufficient to reduce sucrose solution intake beyond the modest degree seen here. Accordingly,

Kelley et al. (1996) found that intra-Acb infusions of relatively high doses of naloxone or naltrexone effectively reduced sucrose drinking, but only by about 20%. Moreover, while 30 µg/0.5 µl of intra-AcbSh naloxone did not significantly reduce chow intake, there was a trend towards a reduction of about 15%. Hence, the present results with amylin are not inconsistent with these opioid antagonist findings, in the sense that intra-Acb stimulation of AMY-Rs, or blockade of opioid receptors, both modestly reduced, but did not eliminate, sucrose intake and hunger-driven feeding (Kelley et al. 1996).

To explore the role of endogenous AMY-R signaling, we tested the ability of prefeeding to suppress AcbSh DAMGO-induced hyperphagia either with or without intra-AcbSh infusions of the AMY-R antagonist, AC187 (Hay et al. 2005). AcbSh AC187 partly reversed the ability of prefeeding to suppress DAMGO-induced food intake. These results suggest that levels of the endogenous AMY-R ligands that negatively modulate µ-OR responses fluctuate according to prandial stimuli. Amylin is secreted along with insulin, in response to feeding and macronutrient flux (Ogawa et al. 1990; Qi et al. 2010). Plasma amylin levels are significantly higher 30 minutes after the onset of feeding (Arnelo et al. 1998). Thus, we reasoned that prefed rats would have higher amylin levels than the non-prefed rats and that this elevated amylin 'tone' would, perhaps, partly underlie the satiety-like reduction of opioid-driven feeding after a meal. The food-deprived rats that were not prefed likely had lower levels of amylin. Hence, the lack of AC187 effect in non-prefed rats (DAMGO-treated or otherwise) could reflect a paucity of available ligand, and, consequently, only low levels of endogenous AMY-R signaling to block. Note that it is also possible that the lack of AC187 potentiation in DAMGO-treated hungry rats was due to a ceiling effect rather than to fluctuations in the level of endogenous ligand. However, close

examination of intake levels in individual rats shows that roughly half of the rats ate more during the DAMGO/AC187 condition relative to DAMGO alone, including the rat exhibiting the greatest degree of DAMGO-induced feeding, while the other half ate less. This pattern would tend to argue against the idea that there was no room to move upward under the DAMGO/AC187 condition.

Another possibility (though not mutually exclusive) is that the endogenous AMY-R ligand is CGRP. There are appreciable densities of CGRP-like immunoreactive fibers in the Acb, and relatively high densities of CGRP binding (van Rossum et al. 1997). CGRP binds to the Acb-localized AMY-R, albeit with considerably less affinity than amylin (Beaumont et al. 1993); hence, it is possible that either CGRP, amylin, or a combination of both ligands participate in μ-OR modulation through AMY-Rs. Further studies are needed to clarify this issue. Regardless, the present results clearly indicate for the first time that there is a negative modulating interaction between endogenous AMY-R and μ-opioid systems, which is best revealed immediately following a meal.

The mechanism underlying AMY-R and μ-OR interactions is presently unknown. However, it is interesting to consider that the high-affinity AMY-1 receptor is a G-protein coupled receptor that increases intracellular cAMP levels, and that μ-ORs are coupled to G(i)-proteins, which decrease intracellular cAMP levels (Al-Hasani and Bruchas 2011; Morfis et al. 2008). Therefore, it is possible that the AMY-Rs may negatively modulate μ-ORs via interactions between post-receptor cAMP-dependent transduction pathways.

Clinically, our results may be relevant to disorders such as binge-eating disorder and bulimia nervosa. Mu-opioid signaling in the CNS is implicated in both disorders; accordingly,

there is considerable evidence that opioid-blocking drugs (including selective μ-OR antagonists) reduce bingeing, with a possible site of action at the Acb (Cambridge et al. 2013; Davis et al. 2009; Marrazzi et al. 1995). In individuals with comorbid obesity and moderate binge-eating, a μ-opioid antagonist reduced striatal activation to images of food, measured with fMRI, and also reduced motivation to view food images (Cambridge et al. 2013). This is perhaps consistent with the suggestion that "surges" of striatal μ-opioid release accompany palatable feeding (DiFeliceantonio et al. 2012). A theoretical framework has been proposed stating that μ-OR signals in the Acb may extend feeding beyond the point that caloric intake is physiologically necessary (Kelley et al. 2005). Hence, given the present results, AMY-R-modulatory drugs may be useful treatments for excessive non-homeostatic palatable feeding, as occurs in pathological conditions such as obesity and binge-type eating disorders.

In summary, this is the first study to examine interactions between AcbSh μ-opioid receptors and amylin. We find that AMY-R signaling enacts robust negative modulation on μ-OR-mediated responses, highlighting a novel receptor-based mechanism with which to modulate central μ-OR signaling in multiple 'disorders of appetitive motivation.'

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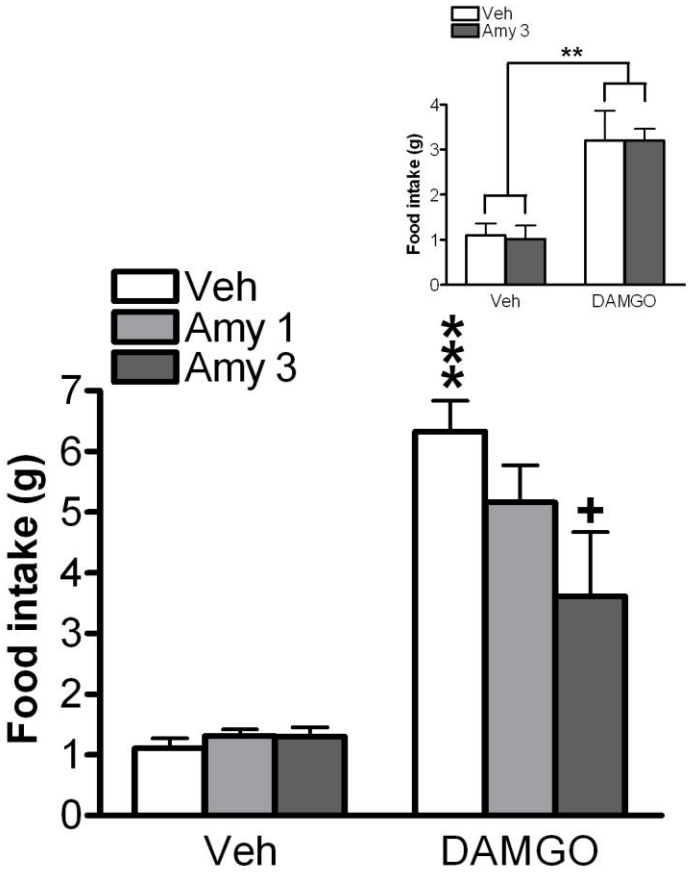

Figure 1: The effects of intra-accumbens shell (AcbSh) vehicle (Veh) or 0.25 µg/0.5 µl DAMGO vs. intra-AcbSh Veh, 1 ng/0.5 µl or 3 ng/0.5 µl amylin (Amy) on chow intake in grams (g) during 30 minutes. Values represent means ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to Veh/Veh. +P<0.05, compared to Veh/DAMGO.
Inset: The effects of intra-anterior dorsal striatum (ADS) vehicle (Veh) or 0.25 µg/0.5 µl DAMGO vs. intra-accumbens shell Veh, 3 ng/0.5 µl or 10 ng/0.5 µl amylin (Amy) on chow intake in grams (g) during 30 minutes. **P<0.01 between Veh and DAMGO groups.

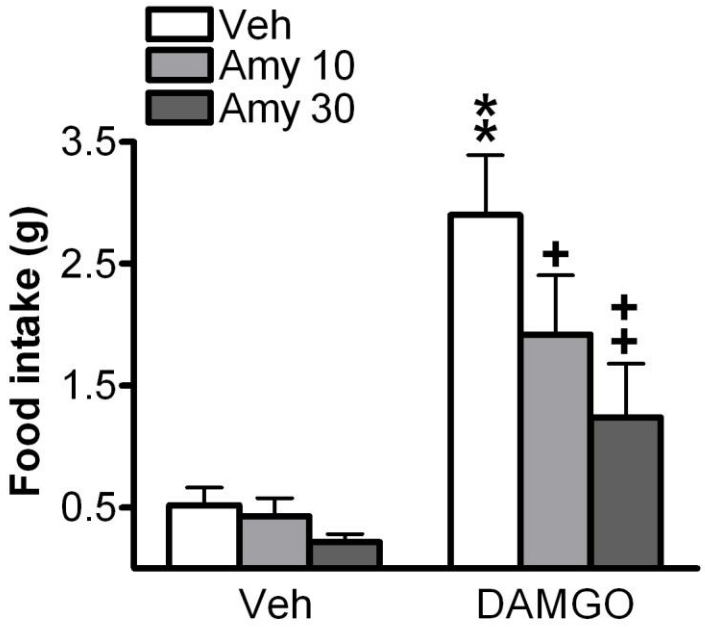

Figure 2: The effects of intra-accumbens shell (AcbSh) vehicle (Veh) or 0.25 µg/0.5 µl DAMGO vs. intra-AcbSh Veh, 10 ng/0.5 µl or 30 ng/0.5 µl amylin (Amy) on chow intake in grams (g) during 30 minutes. Values represent means ± SEM. *P<0.05, **P<0.01, compared to Veh/Veh. +P<0.05, ++P<0.01, compared to Veh/DAMGO.

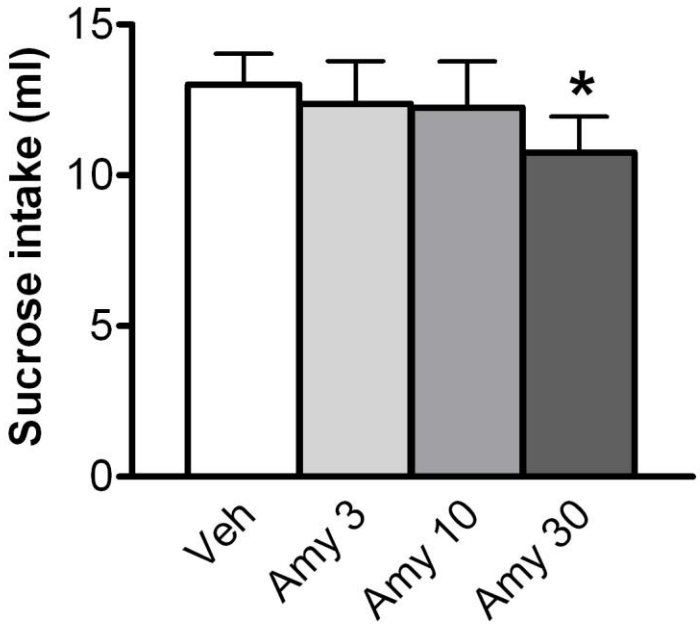

Figure 3: The effects of intra-accumbens shell (AcbSh) vehicle (Veh) or 3, 10, or 30 ng/0.5 µl amylin (Amy) on 10% sucrose intake during 30 minutes. Values represent means ± SEM. *P<0.05 compared to Veh condition.

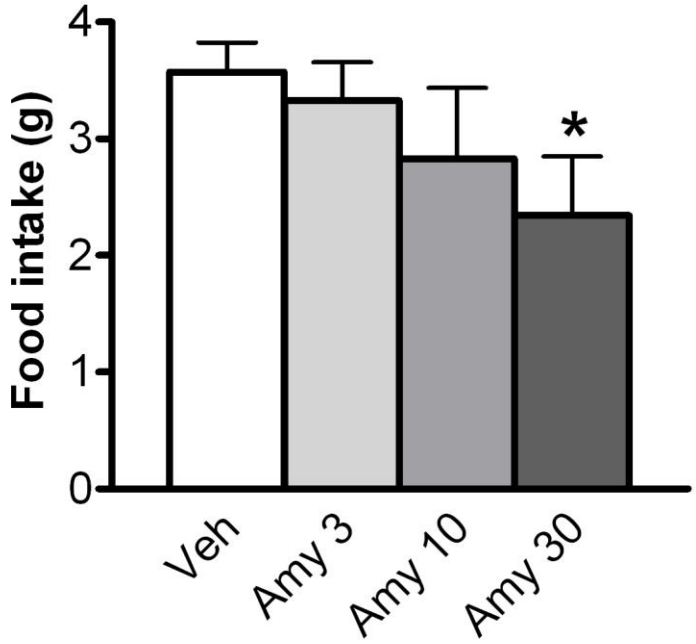

Figure 4: The effects of intra-accumbens shell (AcbSh) vehicle (Veh) or 3, 10, or 30 ng/0.5 µl amylin (Amy) on chow intake in grams (g) by 18 hr food-deprived rats during 30 minutes. Values represent means ± SEM. *P<0.05

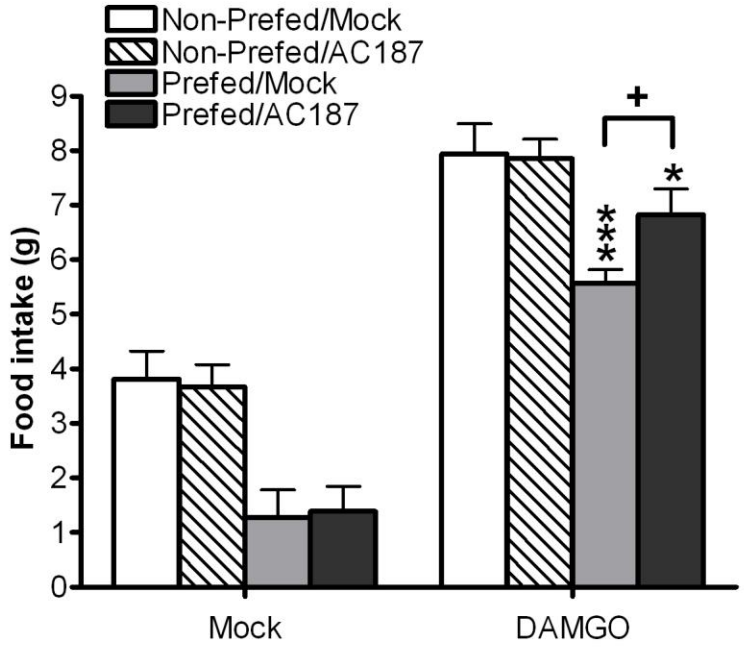

Figure 5: The effects of intra-accumbens shell (AcbSh) mock infusions (mock) or 0.25 µg/0.5 µl DAMGO vs. intra-AcbSh mock or 20 µg/0.5 µl AC187 on chow intake in grams (g) during 30 minutes. All rats were food-deprived for 18 hr. Non-prefed rats were given treatments before their first 30 minutes of eating. Prefed rats ate chow for 30 minutes, were given treatments, and then were tested for 30 minutes. Values represent means ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to Non-Prefed/DAMGO/Mock condition. +P<0.05 between the Prefed/DAMGO/Mock and Prefed/DAMGO/AC187 conditions.

Chapter IV:
Discussion

Summary of findings

The work presented in this dissertation enhances our understanding of the behavioral effects of amylin in the nucleus accumbens shell (AcbSh). In Chapter 2, we found that in rats, 100 ng/0.5 µl amylin in the AcbSh partially reverses prepulse inhibition (PPI) deficits created by systemic amphetamine (AMPH). Since PPI has high predictive validity for whether or not a compound has antipsychotic properties (Geyer 2006; Swerdlow et al. 2008), this suggests that amylin in the AcbSh may act as an antipsychotic. In contrast, amylin in the dorsal striatum (DS) did not reverse PPI deficits created by systemic AMPH, even at doses as high as 300 ng/0.5 µl. A cocktail of amylin (100 ng) and the AMY-R antagonist, AC187 (20 µg) co-administered into the AcbSh did not reverse PPI deficits induced by systemic AMPH, which provides evidence that amylin's reversal of AMPH effects is mediated by AMY-Rs. Furthermore, the AMY1 antagonist AC187 (Hay et al. 2005) impaired PPI, which suggests that an endogenous amylinergic 'tone' is present in the AcbSh, and that this tone modulates PPI. Systemic administration of the antipsychotic and D2 dopamine receptor antagonist, haloperidol, partially reversed the AC187-induced PPI disruption, which suggests that amylin in the AcbSh interacts with the dopamine system.

In Chapter 2, we also confirmed and extended the knowledge of the anatomical distribution of AMY1 receptor gene component distribution in the brains of adult male rats by showing a systematic mapping and semi-quantitative analysis of these genes through multiple levels of the striatum. Only RAMP-1 and CT-R, but not other related genes such as RAMP-2, RAMP-3, or CL-R, showed dense expression of mRNA within the AcbSh. RAMP-1 mRNA, but not CL-R mRNA, was present in the dorsal striatum (DS). Since RAMP-1 and CL-R are required

to form an AMY1 receptor (Poyner et al. 2002), we can conclude that the AcbSh is likely to have the highest density of AMY1 receptors within striatal levels of the rat brain. Also, while it was unclear purely from our behavioral studies that amylin in the AcbSh was acting at the high-affinity AMY1 receptors and not other receptors with an affinity for amylin, we can more reasonably conclude from our receptor mRNA mapping study that AcbSh amylin was most likely acting at this high-affinity subtype.

These findings suggest that amylin has potential as an antipsychotic target. Because our data suggest that AMY1 amylin receptors are densely localized within the AcbSh and not other striatal regions, this reduces the possibility that amylin as a novel antipsychotic treatment would create side effects by acting at AMY1 receptors in neighboring regions. Thus, amylin, in contrast to first-generation antipsychotics, could avoid extrapyramidal side effects. Also, the amylin analog Pramlintide has shown clinical effectiveness as an adjunct treatment for Type-2 diabetes, and in several studies shows promise as an anti-obesity drug (Roth et al. 2012; Singh-Franco et al. 2011). Therefore, amylin treatment could counteract the metabolic side effects and weight gain that are characteristic of second-generation antipsychotics (De Hert et al. 2011). In conclusion, amylin may not only be a novel antipsychotic compound, but could also avoid many deleterious side effects that are present in existing treatments.

In Chapter 3, we found that in adult male rats, AcbSh amylin at doses as low as 3 ng/0.5 μl significantly reduced hyperphagia generated by the μ-opioid receptor agonist DAMGO infused into the AcbSh. DAMGO also engenders hyperphagia when infused into the neighboring anterior dorsal shell (ADS), but amylin in the ADS did not significantly reverse this effect. Moreover, we found that a ten-fold higher dose (30 ng) of AcbSh amylin was required to reduce

intake of a 10% sucrose solution or of chow intake in rats that had been food-deprived for 18 hours, with absolutely no effect of the 3-ng amylin dose. Hence, the potent reversal of DAMGO's effects by amylin was not the result of non-specific performance effects (e.g., malaise or motoric impairment). Collectively, these findings demonstrate robust interactions between AcbSh AMY1 receptor stimulation and AcbSh μ-opioid receptor stimulation.

In our final experiment of Chapter 3, we tested DAMGO-induced hyperphagia, both in rats that had been food deprived for 18 hours ("non-prefed rats"), and in rats that had been food deprived for 18 hours, allowed to feed for 30 minutes, then tested for food intake for an additional 30 minutes ("prefed rats"). Prefed rats ate significantly less than the non-prefed rats, and prefeeding also suppressed DAMGO-induced food intake. Intra-AcbSh infusions of AC187 partly reversed the ability of prefeeding to suppress DAMGO-induced hyperphagia. Intra-AcbSh AC187 was inactive in non-prefed rats, either with or without DAMGO. Because amylin is secreted postprandially (Ogawa et al. 1990), the "prefeeding" paradigm likely stimulated amylin secretion in prefed rats. The fact that AcbSh AC187 enhanced DAMGO-induced hyperphagia in prefed rats suggests that there is an endogenous AcbSh amylinergic tone associated with post-meal satiety that regulates μ-OR-mediated behavior. Combined with the observation that AcbSh amylin potently reduces DAMGO-induced hyperphagia, this also shows that AcbSh AMY1 receptors and μ-opioid receptors interact to regulate feeding.

Integration of findings

In these chapters, we have presented evidence for the first time regarding the existence of an amylinergic 'tone' within the AcbSh that is relevant in regulating two different behaviors.

Using the AMY1 antagonist AC187 (Hay et al. 2005), we have shown that blockade of AcbSh AMY1 receptors disrupts PPI, and also amplifies DAMGO-induced hyperphagia in "prefed" rats. These demonstrations of psychotomimetic-like PPI disruption and enhanced DAMGO-induced hyperphagia with intra-AcbSh infusions of AC187 reveal a behaviorally relevant amylinergic tone at the level of the telencephalon that opposes both dopamine- and opioid-mediated behaviors. While the endogenous ligand that generates this tone is unknown, possible candidates include peripherally released amylin (uptake studies showed that amylin crosses the blood-brain barrier to collect in the striatum and other sites, with better brain penetrance than insulin (Banks and Kastin 1998)), or endogenous peptides of the amylin family, such as CGRP, which is present in the Acb (van Rossum et al. 1997). Because effects were most prominent when feeding levels were high (dark cycle, Chapter 2; post-meal, Chapter 3), this suggests that the endogenous ligand responsible for this effect fluctuates according to feeding status. The likely candidate is amylin.

In addition to the discovery of an endogenous AMY-R tone, this dissertation also proposes a new behavioral role for amylin. This is the first study to examine the effects of amylin on PPI. At this point, it is unclear how AcbSh amylin interacts with other neurotransmitter systems to regulate PPI. Our finding that AcbSh amylin reverses PPI deficits generated by systemic AMPH suggests that AcbSh amylin may interact with dopamine, norepinephrine, and/or serotonin, since AMPH is an indirect agonist for these three neurotransmitters. In addition, our finding that haloperidol, a D2 dopamine receptor antagonist, reverses PPI deficits caused by AcbSh AC187 suggests that there may be an interaction between AcbSh AMY1 receptors and the dopaminergic system in which AMY-R signaling dampens dopamine function.

AC187 released dopamine receptors from this negative modulation, thus causing a mild dopamine-agonist-like PPI disruption, reversible with haloperidol.

Chapter 3 contains the second study to directly examine the role of AcbSh amylin on feeding behavior, and the first study to examine possible interactions between AcbSh AMY1 receptors and AcbSh μ-opioid receptors. Our findings suggest that there is an interaction between AcbSh μ-opioid receptors and AMY1 receptors; specifically, AMY1 receptors in the AcbSh appear to negatively modulate AcbSh μ-opioid receptor function.

There is limited literature regarding amylin in the accumbens, but our results are consistent with other studies. Our findings in Chapter 2 regarding the localization of AMY1 receptor component mRNA expression are consistent with the existing literature. Several studies have shown that the Acb exhibits among the highest density of amylin binding in the whole brain (Aiyar et al. 1995; Sexton et al. 1994; van Rossum et al. 1994), which is consistent with our finding that the AcbSh in particular has dense expression of both CT-R mRNA and RAMP-1 mRNA. The presence of CT-R and RAMP-1 in the striatum and Acb have been reported in whole-brain surveys (Becskei et al. 2004; Lee et al. 2008; Nakamoto et al. 2000; Oliver et al. 2001; Ueda et al. 2001); however, there is limited prior knowledge of the topography of expression within the striatum. We have confirmed and extended earlier findings by providing a detailed, semi-quantitative analysis of multiple genes within the striatum, and a detailed exploration of the striatal gradients of their expression. Indeed, our use of substance P as a marker of the core-shell boundary (Zahm and Heimer 1993) reveals that CT-R mRNA expression is found almost exclusively within the medial AcbSh.

Proposed mechanism

To briefly review, our data show that the indirect dopamine agonist AMPH reduces PPI; an effect which is reversed by AcbSh amylin. In addition, stimulation of AcbSh mu-opioid receptors by DAMGO increases chow intake, which is also reversed by AcbSh amylin. Hunger- and palatability-induced feeding are reversed by AcbSh amylin as well, although more modestly. As for antagonist studies, nighttime AcbSh AC187 reduces PPI, an effect that is reversed by the D2 antagonist haloperidol. AcbSh AC187 also enhances AcbSh DAMGO-induced hyperphagia, but only in prefed rats. Based on these findings, the most parsimonious explanation is that AcbSh amylin signaling negatively modulates the effects of D2 receptor and mu-opioid receptor activation in the AcbSh. This raises the questions regarding the mechanism through which this negative modulation occurs.

Where are the amylin receptors?

Are they on striatal interneurons?

Approximately 5% of AcbSh neurons are classified as interneurons (Meredith 1999). Interneurons would be one possible location of AMY1 receptors in the AcbSh. However, it is important to examine the *in situ* hybridization data generated in Chapter 2 to consider the likelihood of this location of AMY1 receptors. Our emulsion data show an intense band of CT-R mRNA expression in the AcbSh. Nissl counterstaining shows a large percentage of cells co-expressing CT-R and RAMP-1 mRNA within one small region of the AcbSh. If AMY1 receptors were localized on interneurons, which only account for 5% of AcbSh neurons, it is unlikely that we would observe the patterns of mRNA expression seen in our studies. We would be more

likely to observe discrete puncta of RAMP-1 and CT mRNA in the AcbSh, rather than the relatively dense, uniform AcbSh RAMP-1 and CT mRNA expression that we observed. Therefore, it is not consistent with our findings that AMY1 amylin receptors would be localized exclusively on AcbSh interneurons.

Are amylin receptors on dopaminergic nerve terminals?

Another possibility is that AcbSh amylin receptors are located on dopaminergic nerve terminals. The AcbSh receives dense innervation of dopaminergic fibers originating from the ventral tegmental area (VTA) (Groenewegen and Trimble 2007). Since we have demonstrated in Chapter 2 that AcbSh AMY1 receptor stimulation negatively modulates the effects of the indirect dopamine agonist AMPH on PPI, a presynaptic mechanism through which AMY1 negatively modulates dopamine release represents a compelling hypothesis.

Indeed, it has recently been suggested by Mietlicke-Basse et al. (2013) that the components for AMY1 amylin receptors are expressed within dopaminergic neurons in the VTA. Quantitative real-time PCR studies performed in micropunched tissue from the VTA produced evidence for high densities of CT-R and RAMP-1 mRNA within the VTA. Furthermore, behavioral evidence from this study shows that salmon calcitonin (sCT), a peptide with similar affinity to amylin at AMY1 receptors (Beaumont et al. 1993), reduces chow intake and 15% sucrose solution intake following infusion into the VTA. At face value, this evidence is consistent with AMY1 receptor components being expressed in the VTA, and perhaps then being transported from VTA dopaminergic cells to nerve terminals in the AcbSh.

As attractive as this hypothesis is, however, closer examination of the evidence presented in the work by Mietlicke-Basse et al. (2013) demonstrates several problems with this model of AMY1 receptor localization. The micropunching technique employed in this study may have considerable caveats due to lack of precision. Preliminary data generated in our lab characterize RAMP-1, CT-R, and tyrosine hydroxylase (TH) mRNA expression in rat brain levels that include the VTA (see figure A). TH was used as a marker for dopaminergic cells within the VTA. In contrast to Mietlicke-Basse et al. (2013), in a series of in-register sections, we found virtually no evidence for an overlapping distribution of RAMP-1, CT-R, and TH mRNA, suggesting that RAMP-1 and CT-R mRNA are not found in great abundance within the VTA. However, a zone containing dense levels of CT-R mRNA expression was seen

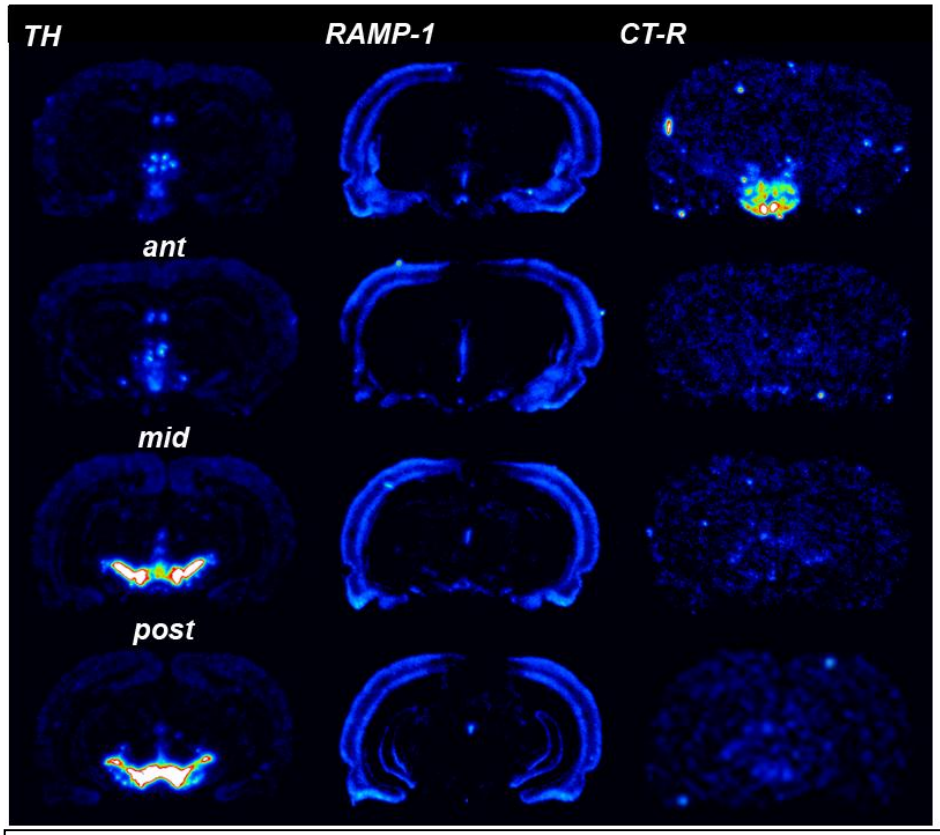

Figure A: Pseudocolor images showing hybridization of probes for tyrosine hydroxylase (TH), RAMP-3, and CT-R in the ventral tegmental area (VTA) and neighboring regions. Warmer colors indicate greater labeling intensity; white shows highest intensity. ant = Anterior, mid = Middle, post = Posterior.

approximately 80 microns anterior to the VTA, which corresponds to the premammillary area of the hypothalamus. Therefore, it is possible that the CT-R mRNA expression measured in the Mietlicke-Basse study was from the posterior basal hypothalamus and not the VTA.

Careful investigation of the behavioral data in the Mietlicke-Basse paper further calls into question whether observed effects were due to a putative VTA-localized AMY-R. While sCT infusion into the VTA produced significant reductions in chow intake and sucrose solution intake, these effects were not observed until at least 40 minutes after the VTA microinfusion occurred. This suggests that the site of sCT action may not have been located at the site of the injector tips, and that the peptide may have diffused to a different brain region in order to create the observed behavioral effects. It is possible that sCT reduced food and sucrose solution intake by acting at CT-Rs in the posterior basal hypothalamus, which in our hands, exhibits dense CT-R mRNA expression.

In addition, there are several problematic issues with the notion that VTA neurons produce AMY1 receptor components, having to do with the forebrain localization of AMY-R binding. VTA dopaminergic neurons innervate a number of other brain regions, including the Acb core, amygdala, and frontal cortex. The nucleus AcbSh, but not the core, amygdala, or frontal cortex show high-affinity amylin binding (van Rossum et al. 1994). It seems unlikely that VTA dopaminergic neurons produce AMY1 receptor components, yet transport them only to the AcbSh (particularly the medial portion), and not the core, amygdala, or frontal cortex. The substantia nigra, like the VTA, sends dopaminergic projections to a number of brain regions, including the superior colliculus and regions of thalamus. If AMY1 expression is an intrinsic property of dopaminergic projection neurons, then we would expect to observe high-affinity

amylin binding in areas to which the substantia nigra sends dopaminergic nerve terminals. However, the superior colliculus does not show high-affinity amylin binding (van Rossum et al. 1994). It is possible that the VTA but not the substantia nigra contains AMY1 receptor components that are transported to nerve terminals, and that the VTA only produces high quantities of AMY1 receptors on nerve terminals targeting the medial AcbSh; however, this does not appear to be the most parsimonious explanation. Therefore, it seems unlikely that AMY1 receptors are on dopaminergic nerve terminals in the AcbSh.

Are amylin receptors on medium spiny striatal neurons?

Medium spiny neurons (MSNs) are the predominant type of cell in the Acb, and they comprise at least 95% of Acb cells (Meredith 1999). MSNs are projection neurons that, upon stimulation, release GABA in areas such as the ventral pallidum. Our emulsion and *in situ* hybridization data show that mRNA for RAMP-1 and CT-R, the components of the AMY1 receptor, are abundantly expressed within the AcbSh. Furthermore, our images with Nissl staining show that there are dense concentrations of cell bodies with overlapping CT-R and RAMP-1 mRNA expression within a small region of the AcbSh. Since MSNs are so abundantly localized within the AcbSh, the hypothesis that AMY1 amylin receptors are localized on MSNs is consistent with these findings. We find this to be the most parsimonious explanation.

Because ultrastructural evidence from electron microscopy studies provides direct evidence that mu-opioid receptors and dopaminergic D2 receptors are located on MSNs in the AcbSh (Delle Donne et al. 1997; Gracy et al. 1997; Pickel et al. 2004; Svingos et al. 1996;

1997), our proposed mechanism explanation revolves around coordinated amylin receptor actions with dopaminergic and mu-opioid receptors, all located on AcbSh MSNs. In terms of intracellular signaling, is well-established that D2 dopaminergic receptors and mu-opioid receptors are coupled to G(i)-proteins, which inhibit cyclic AMP (cAMP) formation through inhibition of adenylyl cyclase (Al-Hasani and Bruchas 2011). There is also evidence that stimulation of AMY1 receptors increases intracellular cAMP, possibly through G(s)-protein coupling (Morfis et al. 2008).

This mechanism could account for multiple results in this thesis. In the case of AMPH and intra-AcbSh amylin, AMPH may stimulate D2 receptors on MSNs by stimulating dopamine release from presynaptic VTA projection neurons. It is well-established that D2 receptors reduce intracellular cAMP through a G(i)-protein-coupled pathway. A putative increase in MSN cAMP levels by AMY-R signaling would oppose this D2-mediated effect. The same principle would apply to AcbSh D2 and AMY1 receptor blockade, with AC187 preventing an endogenous ligand from stimulating the AMY1 receptor, thereby reducing tonic AMY1-receptor-mediated effects on cAMP production and "magnifying" D2-based effects. AcbSh D2 blockade would then normalize D2-mediated inhibition of cAMP production. Hence, AcbSh D2 blockade and AMY1 blockade would have opposing effects on intracellular signaling, accounting for the behavioral results in Chapter 2.

A similar dynamic would affect mu-opioid receptors and AMY1 receptors located on MSNs in the AcbSh. Mu-opioid stimulation would reduce intracellular cAMP via a G(i)-protein-mediated pathway, and AMY1 receptor stimulation would increase intracellular cAMP. In the prefed condition, AMY1 receptor blockade could inhibit an endogenous, prandially-released

ligand from stimulating intracellular cAMP formation via the AMY1 receptor. This would indirectly enhance the effects of intra-AcbSh mu-opioid receptor stimulation, which inhibits intracellular cAMP formation through G(i).

Amylin/opioid interactions: further mechanistic considerations

One point to consider is the modest effect of intra-AcbSh amylin on palatability-driven feeding. While the necessity of 30 ng/0.5 µl of amylin to significantly reduce chow intake is consistent with another study (Baldo and Kelley 2001), the necessity of a considerably higher dose to reduce 10% sucrose intake relative to the dose needed to reduce DAMGO-induced feeding is initially counterintuitive, since palatable feeding is thought to crucially involve µ-opioid receptors in the AcbSh. However, it is worth considering that while intra-AcbSh DAMGO infusions have a specific effect on µ-opioid receptors in the AcbSh, palatable feeding is mediated by additional, redundant systems. Palatable feeding does increase enkephalin signaling in the AcbSh (DiFeliceantonio et al. 2012), but it also engages other substrates in the AcbSh, and within other areas. Therefore, AcbSh amylin may reduce µ-OR effects at only one node (albeit an important node), leaving other systems unaffected. Accordingly, Kelley et al. (1996) found that it was necessary to infuse a high dose (30 ug/0.5 µl) of the opioid receptor antagonist naloxone into the AcbSh in order to reduce sucrose intake. Even at the highest dose tested, sucrose intake was reduced by only 25% - not entirely eliminated. While 30 µg/0.5 µl of intra-AcbSh naloxone did not significantly reduce chow intake, there was a trend towards a reduction of about 15%. Hence, our results are not as divergent with the findings of Kelley et al. (1996) as it might at first seem.

Importantly, the fact that AcbSh amylin modestly reduced hunger- and palatability-driven feeding serves as a control for the intra-AcbSh DAMGO-driven feeding experiment. Since AcbSh amylin in the food deprivation and sucrose intake studies did not cause a drastic reduction with either type of feeding, this suggests that the amylin-induced marked reduction in feeding in the AcbSh DAMGO-driven feeding experiment was not due to malaise or locomotor impairment in the animals.

The same principle may apply to our finding with prefed rats that were given intra-AcbSh AC187 but not intra-AcbSh DAMGO. Again, food intake (as occurred during prefeeding) stimulates a number of pathways throughout the neural axis. It is possible that we did not observe an effect in rats that were prefed and infused with AcbSh AC187 but no DAMGO, because blockade of AcbSh AC187 only modified prefeeding-induced satiety effects at one receptor within one brain region. The existence of multiple, redundant pathways could ensure that satiety endures despite disruption of amylinergic signaling in the AcbSh. In contrast, in prefed rats receiving DAMGO, the stimulation of AcbSh μ-ORs exogenously ensured that the 'excess' feeding above baseline was due to μ-OR signaling specifically in the AcbSh. Here, the reversal of the prefeeding effect could be interpreted as the blockade of an AMY1-mediated satiety mechanism that counteracts μ-OR-generated feeding.

Another possibility is that there was a ceiling effect, and that non-prefed rats given DAMGO/AC187 could not feasibly eat more than 8 grams in the allotted 30-minute testing period. However, rat-by-rat analysis disputes this hypothesis. Roughly half of the rats ate more during the DAMGO/AC187 condition, including the rat that ate most with DAMGO alone, and the other half ate more during the DAMGO/Mock condition. This suggests that at least a subset

of rats were not at a theoretical "ceiling" for feeding. Instead, these results are consistent with our model of an enhanced amylin tone being present immediately after feeding, when amylin levels should be highest.

Nighttime/prefeeding effects of AC187

It is important to note that the AC187 experiment in Chapter 2 took place at night, during the dark period of the rats' light/dark cycle. This experiment had originally been attempted during the light period, but we encountered mixed, inconclusive results. The nighttime experiment was proposed due to the fact that amylin is released prandially (Ogawa et al. 1990), and that the rats are more likely to eat during the dark period of their cycle, when they are naturally awake. Again, this would maximize the chances of testing AC187 during a time of physiologically relevant AMY-R tone. Note that it is not definite from our data that the endogenous ligand responsible for the amylinergic tone is actually amylin. Other potential candidates would include calcitonin gene-related peptide (CGRP) and calcitonin, both of which bind to AMY1 receptors. Calcitonin exhibits similar binding affinity to amylin at AMY1 receptors, while CGRP has lower affinity (Beaumont et al. 1993). In order to be consistent with our results, it makes sense for the endogenous ligand to be secreted prandially, and to accumulate in the striatum, similarly to amylin (Banks and Kastin 1998; Ogawa et al. 1990).

Interestingly, CGRP has been shown to influence Acb μ-opioid receptor expression, and morphine has been shown to influence CGRP receptor component expression (Yan and Yu 2013a; b). Indeed, sustained morphine exposure stimulated release of CGRP (Yan and Yu 2013a). These effects took place after 48 hours, suggesting that it could have been a

counteradaptive mechanism to compensate for elevated morphine levels. Together, these results hint that CGRP and μ-opioid receptors may have opposing effects. This is consistent with our proposed mechanism, since we propose that AMY1 receptors and μ-opioid receptors have opposing intracellular effects on cAMP within medium spiny neurons. If Acb μ-opioid receptors are being repeatedly stimulated by morphine, then medium spiny neurons may upregulate RAMP1 expression and thereby potentially upregulate AMY1 receptors.

Since CGRP is an agonist at AMY1 receptors (Beaumont et al. 1993; Young 2005), it is possible that AC187 results seen in this thesis are partly due to CGRP-mediated AMY-R signaling. This could be tested in future studies using microdialysis to recover samples from the AMY1 before and after feeding, and analyze the dialysate using mass-spectrometry to detect multiple calcitonin-related peptides.

Clinical relevance

Multiple lines of evidence suggest that there are interactions between the Acb circuitry that modulates PPI and the Acb circuitry that modulates ingestive behavior. These potential interactions would have important implications for a number of psychiatric disorders, including schizophrenia, binge-eating disorder, and bulimia nervosa. There is evidence that opioid-blocking drugs reduce bingeing, with a possible site of action at the Acb (Cambridge et al. 2013; Davis et al. 2009; Marrazzi et al. 1995). In individuals with comorbid obesity and moderate binge-eating, a μ-opioid antagonist reduced striatal responses to images of food, and also reduced motivation to view food images (Cambridge et al. 2013). Normally, satiety-inducing peptides such as amylin within the AcbSh may inhibit feeding after a meal. However, μ-opioid

signaling in the AcbSh in response to palatable feeding may trigger ingestion of palatable food even in satiated conditions, especially if AMY1 signaling in the AcbSh is disrupted.

There are links in the literature between non-homeostatic eating, metabolic dysfunction, and schizophrenia. For example, patients with schizophrenia often display metabolic dysfunction even before treatment with antipsychotics (Kirkpatrick et al. 2012; Thakore et al. 2002; Verma et al. 2009). Although it is possible that patients with schizophrenia may have difficulties maintaining a healthy diet and exercise regimen due to the debilitating nature of the disorder, it is also possible that schizophrenia involves disruptions in metabolic functions. For example, patients with schizophrenia have a higher tendency than healthy control subjects to consume diets that are rich in fat (Dipasquale et al. 2013). One study has even shown that a chronic high fat diet lowers PPI in mice (Labouesse et al. 2013). Another study has shown an indirect link between hyperphagia, excess fat deposition, and PPI deficits in mice (Pacheco-Lopez et al. 2013). Again, this underscores the fact that amylin may have multi-dimensional beneficial effects in psychiatric disorders including schizophrenia.

Interestingly, our studies with amylin may represent an initial foray into a larger field where peptide endocrine factors, which are extensively and somewhat exclusively known for their roles in peripheral metabolism, can be repurposed as tools to modify higher-order CNS motivational and cognitive functions. It is interesting to consider another possible example of this dual functionality. It has been found that levels of leptin, a peptide hormone known for its role in ingestive behavior, are increased in some patients with schizophrenia (Jin et al. 2008). When administered intracerebroventricularly (ICV) in rats, leptin decreases dopamine levels in the Acb (Krugel et al. 2003). Isolation-rearing is a paradigm that is proposed to create a valid

model of schizophrenia in rodents (Domeney and Feldon 1998). In addition, ICV leptin ameliorates PPI deficits that are found in rats that were reared in isolation, suggesting that leptin may have an antipsychotic effect (Dashti et al. 2013). Db/db mice, which have an autosomal recessive point mutation in the gene that encodes the leptin receptor, display multiple metabolic dysfunctions, such as progressive hyperglycemia, hyperphagia, and obesity. These mice also have been reported to show PPI deficits (Sharma et al. 2010). It is possible that leptin may be elevated in patients with schizophrenia as a compensatory mechanism against heightened dopamine transmission in the accumbens.

Conclusions

In conclusion, the work in this dissertation has shown for the first time that a tonic amylinergic tone within the nucleus accumbens shell modulates both PPI and ingestive behavior, and that these findings may have relevance for a variety of psychiatric disorders including schizophrenia, bulimia nervosa, and binge-eating disorder. Our findings support a model where AMY1 receptors, μ-opioid receptors, and D2 receptors within the AcbSh are all localized on medium spiny neurons, and where AMY1 receptor stimulation has intracellular effects that negatively modulate the intracellular effects of μ-opioid receptors and D2 receptors. The broader implications of the present work may be in the identification of new tools that, based on their actions upon the amylin system, may have clinical effects in multiple psychiatric disorders.

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