Mesocorticolimbic Generation of Desire and Dread along a Rostrocaudal Gradient in Medial Shell of Nucleus Accumbens

by

Jocelyn Marget Richard

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Doctoral Committee:

Professor Kent C. Berridge, Chair
Professor J. Wayne Aldridge
Assistant Professor Brandon J. Aragona
Professor Israel Liberzon

In loving memory of Elizabeth Richard

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Abstract

Behaviors related to desire versus dread are generated by localized inhibitions along a rostrocaudal anatomical gradient within medial shell of nucleus accumbens of the rat. Either GABA receptor-mediated inhibitions, via microinjections of the $GABA_A$ agonist muscimol, or corticolimbic glutamate disruption, via microinjections of the AMPA antagonist DNQX, generate intense eating at rostral sites and fearful behaviors at more caudal sites. Importantly, local endogenous dopamine is needed for eating and fear generation by glutamate blockade. Additionally, environmental ambience can retune the valence of behavior produced by glutamate disruption, promoting appetitive behavior in a familiar environment, and promoting fearful behavior in a stressful environment. Here, I investigated what signals might contribute to desire versus dread. In Chapter 2 I found that behaviors generated by GABAergic inhibition of accumbens resist environmental retuning, and do not need endogenous dopamine. These results suggest that subcortical GABAergic generation of motivation is more robust, autonomous and anatomically biased than glutamatergic generation in accumbens. In Chapter 3 I found that only endogenous local signaling at D1 dopamine receptors is needed for generation of excessive eating by glutamate disruptions, whereas fear generation requires both D1 and D2 dopamine signaling simultaneously. Furthermore, when motivation valence generated by glutamate disruptions was flipped by manipulating environmental ambience, the roles of local D1 versus D2 signaling also switched dynamically to match the motivation valence generated at the moment. The experiments in Chapter 4 examined whether medial prefrontal cortex can modulate intense motivations generated by accumbens shell glutamate disruptions. I found that activation

of medial orbitofrontal cortex biased intense bivalent motivation in an appetitive direction by amplifying generation of eating behavior by middle to caudal accumbens disruptions, without altering fear. In contrast, activation of infralimbic prefrontal cortex powerfully and generally suppressed both appetitive eating and fearful behaviors generated by accumbens shell disruptions. As a whole, these experiments demonstrate that flips in motivational valence based on environmental cues may involve changes in dopamine signaling, or changes in top-down corticolimbic inputs. These findings carry important implications for the relationship between appetitive desire and aversive dread.

Chapter 1

Introduction

"It would be very surprising indeed if the brain were organized into spatially discrete units that conform to our abstract categorizations of behavior." (Valenstein, 1973)

Since Darwin, many researchers have studied the outward expressions and behaviors associated with particular motivational and affective states in order to understand their fundamental mechanisms and organization. In *The Expression of the Emotions in Man and Animals* (Darwin, 1872), Darwin argued that human emotional states can cause muscular discharges, including facial expressions but also other important changes throughout the body, which are inherited, evolved and rooted in similar, purposeful movements in animals. For instance, Darwin hypothesized that the baring of our teeth during anger may be rooted in our ancestors' use of their teeth in attack. Darwin described a variety of physical responses related to fear, which he described as both "the most depressing of all the emotions" which in some cases "induces utter, helpless prostration" but which can also act as "a powerful stimulant". The "expressions" that Darwin saw as tied to fear include the erection of the hair, muscle trembling, widening of the eyes and mouth, heart palpitations, dryness of the mouth, and labored breathing. While Darwin described both large variability of expression within an emotional category, and similarities of expression between different emotional categories (Barrett, 2011), his writings

were largely interpreted as supporting the existence of evolved, discrete emotional states, tied to particular sets of facial expressions (Allport, 1924; Ekman, 1992a, b).

Over the past half-century, many emotions theorists have embraced a "natural kinds," [1] or "basic emotions" view of emotions. Basic emotions theories have in common the guiding assumption that emotion categories (i.e. anger, fear, sadness) refer to states that are biologically basic and inherited, and that cannot be broken down into more basic psychological components (e.g. they are "natural kinds") (Darwin, 1872; Allport, 1922; Tomkins, 1962; Ekman, 1992b; Panksepp, 2000; Izard, 2007; Barrett, 2011; Ekman and Cordaro, 2011; Lindquist et al., 2012). Proponents of basic emotions theories emphasize the universal nature of basic emotional expressions and categories (Ekman, 1999), and have defined the basic categories of emotions based on a variety of criteria including facial expressions (Tomkins, 1962; Ekman, 1992b), common emotional language and schemas (Tomkins and McCarter, 1964; Johnson-Laird and Oatley, 1989), psychophysiological responses (Allport, 1922; Cacioppo et al., 2000; Rainville et al., 2006; Kreibig et al., 2007; Stephens et al., 2010) and brain structures and circuits (Panksepp, 2000; Hamann, 2012).

LeDoux (2012) has argued human emotions terms may not be the best way to conceive of the relevant, conserved functions that are also present in other animals. LeDoux argues that behavioral neuroscience researchers should instead focus on survival circuits, or "specific emotion/motivational circuits that are innately wired into the brain by evolution that mediate functions that contribute to the survival and well-being of the organism" (LeDoux, 2012, pg. 654). While he argues that there is limited support for the presence of dedicated neural circuits

[1] Sets or groupings of things based on natural properties and divisions, rather than groupings that are artificial or depend on humans. Members of a natural kind have particular properties in common and should be categorically distinct from, and non-overlapping with other natural kinds.

for basic emotions linked to human feeling states (fear, anger, happiness, sadness, disgust, and surprise), LeDoux suggests that there may be more evidence for neural circuits dedicated to particular survival functions, including circuits for defense, maintenance of energy and nutritional supplies, fluid balance, thermoregulation and reproduction.

Yet there are many examples in the brain of overlapping neural mechanisms for seemingly separate "survival circuits". For example, while studying motivated behaviors (including eating, drinking and gnawing) induced by hypothalamic stimulation in the 1960s, Valenstein and colleagues found that animals who reliably exhibited stimulation-bound behaviors towards one goal object (i.e. stimulation reliably induced eating of a chow pellet), would switch to exhibiting stimulation-bound behavior towards a different goal object (i.e. stimulation now reliably induced drinking of the water) when the preferred object was removed (Valenstein et al., 1968). This suggested that rather than trigger behaviors related to a particular survival circuit (drinking to ensure fluid balance or eating to maintain energy and nutritional supplies), the connection between hypothalamic stimulation and particular motivated behaviors was much more plastic (Valenstein et al., 1969). Perhaps rather than trigger very specific survival mechanisms, hypothalamic stimulation elicited more general states of enhanced appetitive motivation.

Overlapping neural and psychological mechanisms may extend to even seemingly opposite states, such as fear and desire. Why might we expect potential overlapping psychological and neural mechanisms for appetitive and aversive motivation? Overlapping mechanisms for the generation of fear and desire may lead to vulnerability to problems associated with both exaggerated incentive and fearful motivation in some individuals. For instance, "sign-tracking" rats, who exhibit greater propensity to attribute incentive motivational

value to a lever cue that predicts sucrose (Meyer et al., 2012), also show greater fear, as indicated by freezing, in response to an aversive cue (Morrow et al., 2011). The greater tendency to attribute incentive value to reward cues in sign-trackers has been linked to greater motivation to pursue drugs (Saunders and Robinson, 2011), and greater relapse to drug seeking in response to drug cues (Saunders and Robinson, 2010) and drug priming (Saunders and Robinson, 2011), as well as greater relapse to food seeking (Yager and Robinson, 2010) in these animals. Based on their greater freezing in response to an aversive cue, these animals may be similarly vulnerable to excessive fear, which may confer additional vulnerability to anxiety disorders such as post-traumatic stress disorder (Bush et al., 2007; Yehuda and LeDoux, 2007) or other disorders potentially involving exaggerated fear, such as paranoid schizophrenia (Kapur, 2003; Kapur, 2004).

In humans, amphetamine addicts (who may experience heightened incentive motivation) (Robinson and Berridge, 2001) can experience fearful "amphetamine psychosis" similar to paranoia seen in schizophrenia (Angrist et al., 1974; Kapur, 2003; Featherstone et al., 2007). Conversely, some schizophrenic patients exhibit higher or abnormal brain activations that encode appetitive incentive value which may lead to higher rates of both drug abuse, especially smoking, and obesity in patients with schizophrenia (Elman et al., 2006; Smith et al., 2009; Diaconescu et al., 2011). Additionally, stimuli that might usually produce motivation of one valence, may contribute to enhanced motivation of the opposite valence. For instance, the compulsive pursuit of rewards in drug addiction can be exacerbated by aversively motivating events, such as stress (Shaham et al., 2000). Stress can similarly generate excessive eating in sated individuals (Antelman et al., 1975; Morley et al., 1983). Furthermore, previous exposure to rewarding psychostimulants renders rats more sensitive to subsequent stressful events (Antelman et al.,

1980; Hamamura and Fibiger, 1993), in addition to increasing incentive motivation. Importantly, medications that may help with psychotic symptoms of schizophrenia by dampening the fear produced by delusions and hallucinations may actually aggravate negative symptoms by decreasing incentive motivation (Kapur, 2003). To better understand these phenomena is it not only important to understand where psychological and neural components of fear and desire diverge, but also where they overlap.

The role of the nucleus accumbens in desire and dread

Mogenson and colleagues drew attention to the potential role of the nucleus accumbens (NAc) in motivation and emotion when they described the NAc as a “limbic-motor interface" (Mogenson et al., 1980) where motivational and affective states are translated into action. The NAc can be divided into two major subregions, the core and the shell, based on differences in function, morphology, neurochemistry and connectivity (Zaborszky et al., 1985; Meredith et al., 1989; Voorn et al., 1989; Heimer et al., 1991; Jongen-Relo et al., 1993; Kelley and Swanson, 1997; Parkinson et al., 1999; Corbit et al., 2001; Meredith et al., 2008). In general, the NAc is most well-known for its role in reward, and is associated with the generation of appetitive motivational and affective states, including reward seeking, ingestion and pleasure (Ikemoto and Panksepp, 1999; Cardinal and Everitt, 2004; Kalivas and Volkow, 2005; Kelley et al., 2005a; Nicola, 2007; Sesack and Grace, 2010).

Yet, the NAc, and in particular its medial shell subregion, has also been implicated in aversive motivational and affective states related to fear, pain, stress, and disgust (Blackburn et al., 1992; Salamone, 1994; Horvitz, 2000; Reynolds and Berridge, 2001; Levita et al., 2002; Reynolds and Berridge, 2002; Scott et al., 2006; Kerfoot et al., 2007; Delgado et al., 2008; Levita et al., 2009; Carlezon and Thomas, 2009; Richard and Berridge, 2011a). Exposure to a

context previously paired with foot shock dramatically increases *c-fos* expression, a marker of neuronal activation, in the shell region of NAc (Beck and Fibiger, 1995). Human fMRI imaging also indicates that the NAc is activated in response to cues associated with both fear-inducing and disgusting pictures (Klucken et al., 2012), and that exposure to combat sounds selectively activates NAc in veterans with posttraumatic stress disorder (Liberzon et al., 1999). NAc dopamine signaling in particular has been of interest for its unexpected role in processes related to stress and aversive motivation (Blackburn et al., 1992; Salamone, 1994; Gray, 1995; Levita et al., 2002), despite its initially proposed role in pleasure and reward (Fouriezos and Wise, 1976; Wise, 1985; Ahn and Phillips, 1999; Hajnal et al., 2004).

Caudal medial shell of nucleus accumbens as a transition zone to extended amygdala?

Many neurochemical and hodological characteristics, including unique staining for neuropeptides, greater innervation by norepinephrine inputs (Berridge et al., 1997; Delfs et al., 1998; Park et al., 2010), and potential connectivity with extended amygdala nuclei (Heimer et al., 1991), have led to the suggestion that caudal shell is a corticostriatal transition zone that shares features with extended amygdala (Alheid and Heimer, 1988; Alheid, 2003; Zahm, 2006), which has received attention primarily for its role in aversive motivation and emotion (Davis, 1998; Koob, 2003; Aston-Jones and Harris, 2004). Based on these observations and others, functional differences along the rostrocaudal axis of NAc were predicted by Alheid (1999, pg. 650):

> Within the context of ventral striatum, [this pattern] suggests that a column of cells related to the amygdala traverses the caudal accumbens, and predicts that the rostral-to-caudal dimension in accumbens should be functionally differentiated. While differences along this dimension are less often reported, they nevertheless seem to exist. For example, only within the posterior shell region can the dopamine-evoked increase in dye coupling between accumbens neurons be blocked by clozapine, an effect postulated to be relevant to the drug's antipsychotic efficacy … Finally, in rats receiving electroconvulsive shock, dopamine receptor (D1 and D2) messenger RNA is acutely increased, but only in the posterior part of nucleus accumbens.

The functional differences described by Alheid, including the potential importance of caudal shell for the efficacy of antipsychotics (O'Donnell and Grace, 1993) and the localization of dopamine-related responses to aversive experience to caudal shell (Smith et al., 1995), provide some initial evidence for the importance of NAc shell in the generation of fear or aversive motivational states. If caudal NAc shell does represent an area where neuroanatomical and functional characteristics of extended amygdala and ventral striatopallidal systems overlap, then it may be uniquely suited to generate aversive motivation and affect.

Generation of unconditioned fear and desire along a rostrocaudal gradient in medial shell

Work conducted in our lab by Sheila Reynolds revealed that inhibitions of medial shell of NAc were capable of producing both intense appetitive eating, and intense, active fearful reactions, depending on the particular rostrocaudal location (Reynolds and Berridge, 2001, 2002, 2003, 2008). These experiments stemmed from attempts to replicate and expand upon studies conducted by Ann Kelley and colleagues, demonstrating that amino acid inhibition of medial shell by either corticolimbic glutamate blockade (via an AMPA/kainate glutamate antagonist) or GABAergic inhibition (via a $GABA_A$ agonist) can generate intense hyperphagia, causing rats to eat up to 10 times what they would normally under vehicle control conditions (Maldonado-Irizarry et al., 1995; Kelley and Swanson, 1997; Stratford and Kelley, 1997; Basso and Kelley, 1999; Kelley et al., 2005b). Eating induced by amino acid inhibitions is anatomically specific: similar eating cannot be elicited by inhibitions in dorsomedial striatum, NAc core or ventrolateral striatum (Kelley and Swanson, 1997). Subsequent experiments in our lab revealed that eating induced by amino acid disruptions was even more anatomically restricted than first described by Kelley and colleagues.

Most of the sites studied in the experiments conducted in the Kelley lab, even those intended to compare "anterior" and "posterior" regions of medial shell (Kelley and Swanson, 1997; Basso and Kelley, 1999), were located in what many would now classify as the rostral or front half of medial shell. Indeed, most microinjection studies from many labs around that time focused primarily on the rostral half of NAc (for example: Duvauchelle et al., 1992; Hyytia and Koob, 1995; Carlezon and Wise, 1996; Sills and Vaccarino, 1996; Sokolowski and Salamone, 1998; Burgdorf et al., 2001). The caudal half was left relatively unexplored until after 2000.

While Reynolds and Berridge (2001) found that GABAergic inhibition did generate intense eating when microinjections were given at more rostral locations (more than 1.4 mm ahead of bregma), progressively more caudal microinjection of a GABA agonist generated less eating, and at the most caudal locations eating was *suppressed*. Instead of generating eating, caudal inhibitions elicited a suite of defensive reactions. When the experimenter attempts to touch a rat who has received caudal shell inhibition, the rat is likely to emit audible distress vocalizations or calls, attempt to escape, and attempt to (and sometimes successfully) bite the experimenter (Reynolds and Berridge, 2001, 2002, 2003). Caudal inhibitions also generate a behavior known as defensive treading or burying, in which rodents use rapid forepaw movements to throw dirt or debris at a threatening stimulus or predator (i.e. rattlesnake, scorpion or shock prod) (Coss and Owings, 1978; Treit et al., 1981; Londei et al., 1998; Reynolds and Berridge, 2001).

Defensive treading as an active fearful response

Defensive treading has been studied as an active fear response in laboratory tests of shock prod burying, which is hypothesized to be a good model of both pathological and normal fear states (Treit et al., 1981; De Boer and Koolhaas, 2003). Work on defensive treading first

stemmed from a report by Hudson (1950), who found that if rats were provided with bedding material (woodshavings), they would bury shock-associated stimuli, rather than merely freeze or avoid the stimuli. Treit and Pinel (1978) further tested this phenomenon, and found that rats would readily bury a shock prod after only a single shock, and that this behavior was well directed towards the prod (and not randomly dispersed). In the wild, California ground squirrels have been reported to defensively tread at snakes threatening their burrow, kicking dirt or sand at the predator (Coss and Owings, 1978). Defensive treading generated by caudal shell inhibition in our laboratory setting tends to be directed towards the front of the cage (e.g. towards the experimenter, the cameras and the brightest part of the cage) or towards the corners of the cage where light tends to reflect brightly (Reynolds and Berridge, 2001). While the active defensive behaviors generated by caudal shell inhibition may not represent a classic or prototypical example of the *category of fear* normally studied in the fear literature, which generally tends to focus on the acquisition and expression of conditioned freezing and other learned responses (Fanselow and LeDoux, 1999; Lee et al., 2005; Maren, 2005), it does appear to represent a very specific, yet malleable, example of an aversively motivated behavior. By probing the NAc shell to directly turn on intense motivated behaviors, we are able to gauge just what NAc shell is capable of in terms of aversive versus incentive motivation. NAc shell fear-generating capabilities may have been generally overlooked in the past due to the focus of fear research on learning and recall of predetermined fearful behaviors.

Eating and fear produced by both corticolimbic glutamate blockade and GABAergic inhibition

NAc shell inhibition generated by either corticolimbic glutamate blockade (via the AMPA antagonist DNQX) or GABAergic inhibition (via muscimol) produces eating and fearful behaviors along a similar rostrocaudal gradient: rostral shell inhibitions generate intense eating

as reported by Kelley and colleagues, intermediate locations generate mixture of intense eating and fear, and the most caudal locations primarily produce intense fear (Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2008). These inhibitions likely generate intense motivated behaviors by disinhibiting downstream targets of NAc GABAergic projections including ventral pallidum, lateral hypothalamus and ventral tegmental area (Mogenson et al., 1983; Zahm and Heimer, 1990; Heimer et al., 1991; Lu et al., 1998; Usuda et al., 1998; Zhou et al., 2003), as well as potentially the substantia nigra pars compacta, pedunculopontine nuclei, and periaqueductal gray area (Zahm and Heimer, 1993), and areas of the extended amygdala (Heimer et al., 1991).

Importantly, changing the emotional ambience of the testing environment can retune the valence of behavior generated by corticolimbic glutamate blockade along the rostrocaudal gradient (Reynolds and Berridge, 2008). Testing in an aversive, stressful environment, with very bright lights and loud (80-86 decibel) rock music expands the zone where glutamate blockade produces fearful behaviors, such that most middle and rostral locations now produce intense fear in addition to eating (Reynolds and Berridge, 2008). In contrast, testing in the more familiar and comfortable environment of the rats' home room nearly eliminates defensive behaviors except in a very small region of caudal shell, effectively expanding the zone where DNQX produces purely appetitive behavior to more than 80% of shell (Reynolds and Berridge, 2008). The flexibility of motivational valence generated by the same exact neurochemical manipulation at the same exact sites in medial shell highlights the importance of understanding the psychological functions mediated by particular neural circuits, as a one-to-one relationship between neural circuits and specific survival functions is insufficient to explain the type of flexibility we see in NAc shell generation of fear and desire.

Rather than mediating rigid survival functions and their associated behaviors, brain structures implicated in motivation and affect may mediate more specific psychological components, or "limbic building blocks" that might be shared across emotions or survival functions. Some potential components include: sensory input from the body (James, 1884), core affect or affective valence (Wundt, 1897; Harlow and Stagner, 1932; Zajonc, 1980; Russell, 2003), arousal or activation (Schacter and Singer, 1962), conceptualization or attribution (Russell, 2003; Barrett, 2006), and motivational salience. In particular, we hypothesize that one critical component of both fear and desire is motivational salience (Berridge and Valenstein, 1991; Kapur et al., 2005; Faure et al., 2008; Puglisi-Allegra and Ventura, 2012).

Motivational Salience: Incentive and Fearful

The concept of incentive salience was proposed as an explanation for the ability of electrical stimulation of the lateral hypothalamus to both generate ingestion and support self-stimulation, without altering hedonic impact (Berridge and Valenstein, 1991). Any proposed psychological mechanism of stimulation-bound eating needed to be sufficiently general to support seeking and consumption of multiple reward targets (i.e. both food and water, Valenstein et al., 1968, 1969), but also needed to be able to interact synergistically with previous learning to promote seeking of particular preferred rewards or stimuli (Berridge and Valenstein, 1991). Theoretical work on incentive motivation by Bindra (1978) and others (Bolles, 1972; Toates, 1986; Dickinson, 1989) emphasized the importance of the incentive motivational aspects of reward cues and their ability to become salient and attractive based on synergistic combinations between previous learning and current physiological state. These important characteristics of incentive motivational cues suggested the existence of a psychological component that could meet the criteria described by Berridge and Valenstein (1991): incentive salience. The attribution

of incentive salience can produce 'wanting' or desire for stimuli that is separable from the experience of sensory pleasure associated with those stimuli. Exaggerated incentive salience, such as following "incentive sensitization", may lead to pathological attraction to, pursuit of, and consumption of rewards, such as in drug addiction (Robinson and Berridge, 1993, 2001, 2008) or binge eating (Gearhardt et al., 2009; Pelchat, 2009).

A similar mechanism may be involved in the attribution of motivational salience to fearful stimuli, which, once attributed with negative "incentive" properties, can lead to avoidance or fear. Just as reward cues can take on the motivational and sensory properties of the reward itself, so too can aversive cues that predict pain or punishment take on aversive or fearful motivational properties (Bindra, 1974, 1978; Puglisi-Allegra and Ventura, 2012). This "fear salience" may utilize overlapping or identical brain circuitry to that which generates incentive salience, to motivate defensive reactions towards and active avoidance of aversively-conditioned stimuli.

Dopamine as a neural mechanism of motivational salience

While dopamine has been (and still sometimes is) described as a pleasure neurotransmitter based on its responsiveness to pleasurable rewards and their cues and its importance in responding for rewards (Fouriezos and Wise, 1976; Wise, 1985; Ahn and Phillips, 1999; Hajnal et al., 2004), the bulk of available evidence indicates that dopamine is not important for pleasure associated with rewards per se (Peciña et al., 1997; Berridge and Robinson, 1998; Wyvell and Berridge, 2000; Leyton et al., 2002; Volkow et al., 2002; Peciña et al., 2003; Tindell et al., 2005). Instead, ongoing debate regarding the role of dopamine focuses primarily on its potential role in learning about rewards versus generating incentive salience. Learning based theories of dopamine function stem in large part from elegant studies

demonstrating prediction-error like signaling in dopamine neurons (Montague et al., 1996; Schultz, 1998). Delivery of an unexpected reward triggers a large increase in phasic dopamine signaling, which is purported to be critical for forming the association between a predictive cue and the reward; as learning is consolidated the phasic dopamine signal eventually transfers to the predictive cue (Schultz et al., 1997; Day et al., 2007). Recently, Flagel and colleagues (2011) demonstrated that this dopamine signal only transfers to the predictive cue when that cue is imbued with incentive salience, such as in sign-tracking animals (described above), who selectively approach and interact with a food-predicting lever cue, sniffing and gnawing on it as if it were food-like (Mahler and Berridge, 2009; Robinson and Flagel, 2009). The dopamine signal does not transfer to the cue in goal-tracking rats, who do not approach and interact with (and presumably do not attribute incentive salience to) the cue, even though they form a predictive association. Importantly, NAc dopamine signaling is critical for a sign-tracking response, but not a goal-tracking response (Saunders and Robinson, 2012). These results suggest that the role of NAc dopamine is primarily to attribute incentive salience or incentive motivational value to reward-related cues or stimuli.

In addition to its role in incentive motivation and salience (Ettenberg, 1989; Robbins et al., 1989; Blackburn et al., 1992; Robinson and Berridge, 1993; Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Cardinal and Everitt, 2004; Kalivas and Volkow, 2005; Cheer et al., 2007; Grace et al., 2007; Schultz, 2007; Wise, 2008; Flagel et al., 2011; Smith et al., 2011), mesocorticolimbic dopamine signaling may play a parallel role in aversive motivation or in the attribution of “fearful salience” (Oei and King, 1980; Blackburn et al., 1992; Salamone, 1994; Horvitz, 2000; Levita et al., 2002; Joseph et al., 2003; Ventura et al., 2007; Matsumoto and Hikosaka, 2009; Bromberg-Martin et al., 2010; Cabib and Puglisi-Allegra, 2012; Puglisi-Allegra

and Ventura, 2012). Early studies reported that electric foot shock stress selectively increases dopamine metabolism or utilization in NAc and prefrontal cortex (Thierry et al., 1976; Herman et al., 1982). Subsequent microdialysis experiments found that extracellular dopamine levels in NAc are increased by tail-shock stress (Abercrombie et al., 1989), restraint stress (Imperato et al., 1991), social defeat stress (Tidey and Miczek, 1996), and exposure to anxiogenic drugs (McCullough and Salamone, 1992). Mild footshock stress selectively enhances dopamine levels in the shell region of NAc (Kalivas and Duffy, 1995), and cues predicting shock increase dopamine levels selectively in the shell subregion of NAc as well (Pezze et al., 2001). Performance of a shock avoidance task, in which rats press a lever to delay a periodic shock, elevates NAc dopamine levels in a manner correlated with the number of avoidance responses (McCullough et al., 1993). Technical advancements including the use of shorter microdialysis periods (Young, 2004) and fast-scan cyclic voltammetry (Anstrom et al., 2009), indicate that these dopamine rises are in response to the aversive experience itself, and not a reward-related response indicating relief after the aversive experience is terminated.

In addition to encoding aversive experiences (described above) mesocorticolimbic dopamine projections have been implicated causally in aversively motivated behaviors. Potentiation of dopamine signaling, via amphetamine administration, enhances acoustic startle responding (Davis et al., 1975; Davis et al., 1986; Frankland and Yeomans, 1995; but see Schwienbacher et al., 2005), potentiates conditioned suppression (Miczek and Luttinger, 1978), retards fear extinction (Borowski and Kokkinidis, 1998) and increases the punishing effects (e.g. suppression of an instrumental response) of an aversively conditioned stimulus (CS) (Valentine and Barrett, 1981; Killcross et al., 1997). Similarly, suppression of dopamine signaling, either by systemic administration of dopamine antagonists (such as flupenthixol or haloperidol) or

destruction of dopamine neurons (by 6-OHDA lesions), decreases acquisition (Cooper et al., 1974) and expression of active avoidance of shock (Neill et al., 1974; McCullough et al., 1993), reduces the punishing effects of an aversive CS (Killcross et al., 1997), and decreases expression of fear-potentiated startle (Borowski and Kokkinidis, 1996). Selective dopamine depletion in NAc suppresses performance of both inhibitory and instrumental avoidance (Schwarting and Carey, 1985; McCullough et al., 1993). Though some studies have failed to find a role of dopamine in aversive motivation, Levita and colleagues (2002) hypothesize that dopamine may either be involved in a subregion specific manner (such as in medial shell of NAc where aversive dopamine responses are strongest) or in particular aspects of aversive motivation that need to be further defined.

Critically, both eating and fear induced by corticolimbic glutamate blockade (via DNQX) are also dependent on the presence of local endogenous dopamine, in that combining D1 and D2-like dopamine antagonists with the glutamate antagonist in the same microinjection prevents the generation of eating and fear (Faure et al., 2008). Corticolimbic glutamate and mesolimbic dopamine signals both may be important for the production of flexible motivational salience, which can be transformed into either the incentive salience of rewarding stimuli, or the negative or fearful salience of aversive stimuli (Berridge and Robinson, 1998; Kapur, 2003; Faure et al., 2008). But is mesolimbic dopamine also critical for GABAergic fear and eating?

Potential differences in GABAergic versus glutamatergic desire and dread generation

While corticolimbic glutamate blockade and GABAergic inhibition both produce a similar rostrocaudal gradient of eating and fear, they differ in important respects, including the types of neurochemical inputs they impact or mimic (Figure 1.2). The medial shell of NAc receives glutamate inputs from a variety of cortical and cortical-like areas, including agranular

(anterior) insular cortex, prelimbic and infralimbic regions of medial prefrontal cortex, midline thalamic nuclei, basolateral amygdala, and the subiculum of the hippocampus (Christie et al., 1987; Fuller et al., 1987; McDonald, 1991a). Preferential targeting of rostral or caudal subregions of medial shell by particular glutamate inputs may result in functional differentiation of rostral and caudal medial shell. Glutamate inputs from prefrontal cortex, middle rostrocaudal levels of the basolateral amygdala, septal or dorsal areas of the subiculum of the hippocampus, as well as the nucleus reunions and mediodorsal nucleus of the thalamus may preferentially target the rostral half of NAc (Phillipson and Griffiths, 1985; Groenewegen et al., 1987). In contrast, the ventral subiculum of the hippocampus and caudal portions of the basolateral amygdala may project preferentially to caudal NAc (Groenewegen et al., 1987; Brog et al., 1993; Friedman et al., 2002). Levita and colleagues (2002) have argued that functional rostral versus caudal NAc differences might arise in part due to convergence of inputs from septotemporal hippocampus and intermediate rostrocaudal amygdala in rostral NAc, and convergence of inputs from ventral hippocampus and caudal basolateral amygdala in caudal NAc (Groenewegen et al., 1999b; Groenewegen et al., 1999a).

In contrast, GABAergic inputs to the NAc shell largely arise from instrinsic or subcortical sources, including NAc shell GABAergic interneurons, axon collaterals from NAc shell medium spiny projection neurons, and afferents from ventral pallidum and ventral tegmental area (Sun and Cassell, 1993; Churchill and Kalivas, 1994; Vanbockstaele and Pickel, 1995). GABA afferents from ventral pallidum largely arise from rostral and medial ventral pallidum, as ventral pallidum largely projects topographically (medial to lateral) to NAc (Churchill and Kalivas, 1994). The caudal-dorsal extremity of the shell, just ventral to the lateral septum, sometimes referred to as the “septal pole” (Voorn et al., 1986), has been reported to also

receive projections from all divisions of the bed nucleus of the stria terminalis (of the extended amygdala macrosystem), the lateral and medial preoptic regions, medial hypothalamus, lateral habenula, medial and olfactory-related parts of amygdala (including the anterior amygdala), and the amygdalo-piriform and amygdala-hippocampal transition zones, in addition to projections from ventral pallidum and lateral hypothalamus (Brog et al., 1993). While their neurochemical content has not been defined, many of these caudal shell specific afferents, including from extended amygdala, are likely GABAergic (Swanson, 2005).

Previously, we showed that inhibition by either local disruptions of corticolimbic glutamate inputs, or mimicking subcortical GABA inputs to medial shell of NAc generate equivalent motivations expressed in behavior as appetitive eating and/or defensive treading mixtures, along the same rostrocaudal gradient in medial shell (Faure et al., 2010). But only GABA-related inhibitions by muscimol additionally produced corresponding changes in the hedonic impact of an affect-laden gustatory stimulus: muscimol microinjections in a small portion of rostrodorsal shell enhanced "liking" reactions such as lip licking, and decreased "disliking" reactions, such as gapes, in response to a bittersweet taste, whereas more caudal microinjections produced an aversive shift of enhanced "disliking" and decreased "liking" (Reynolds and Berridge, 2002; Faure et al., 2010). By contrast, glutamate-related disruptions by DNQX did not influence hedonic impact, having no effect on either 'liking' or 'disliking' for bittersweet sucrose-quinine taste (Faure et al., 2010). Because GABAergic inhibition of NAc shell is capable of generating changes in hedonic impact, in addition to the rostrocaudal gradient of eating and fear, and because dopamine signaling has been shown essentially irrelevant to hedonic impact (Berridge, 2007), I hypothesized that the effects of GABAergic inhibition would be separate from, and independent of local dopamine signaling (Chapter 2).

In addition to potentially being independent of local dopamine signaling, GABAergic eating and fear may be generally more robust and resistant to changes in other neural or psychological inputs. Muscimol microinjections stimulate $GABA_A$ receptors and might be expected to produce robust hyperpolarizations and reductions in the firing rate of local neurons, by allowing Cl- to enter the cells (Goetz et al., 2007), producing especially powerful inhibition of medium spiny neurons (Koos et al., 2004). By comparison, DNQX microinjections block glutamatergic AMPA receptors, and might be expected to similarly produce relative inhibitions of neurons containing glutamate receptors, by diminishing "up states," suppressing excitatory postsynaptic potentials (EPSPs), and reducing the number of action potentials produced (Meredith et al., 1993; Pennartz et al., 1994; Kiyatkin and Rebec, 1999; Meredith, 1999; O'Donnell, 1999; Suwabe et al., 2008; Jeun et al., 2009). However, important differences also exist between GABAergic and glutamatergic "hyperpolarizations". Muscimol may more potently hyperpolarize NAc neurons by acting on $GABA_A$ receptors located on somata and proximal dendrites (Sun and Cassell, 1993; Johnson et al., 1994; Behrends et al., 2002). DNQX may act more distally on medium spiny dendrites, blocking ionotropic glutamate signals at distant spines, where AMPA receptors are more likely to be found (Meredith et al., 1990; Sesack and Pickel, 1992; Johnson et al., 1994; Chen et al., 1998). More distal placement of glutamate receptors on the head of neuronal spines, compared to GABA receptors, also might dilute the intensity of inhibitory states at the soma and axon hillock induced by glutamatergic blockade, altering the degree of disinhibition passed on to output targets such as ventral pallidum, lateral hypothalamus or ventral tegmentum. All this implies that subcortical GABA inputs to NAc may achieve a greater potency of disinhibition of downstream targets to alter hedonic impact than blockade of glutamate inputs from predominantly cortical-related sources. This greater potency may produce

motivation that is more robust, anatomically biased, and resistant to changes in emotional ambience or dopamine blockade; this will be addressed in Chapter 2. If this is the case, then mechanisms for selecting appetitive versus aversive motivational valence from the same NAc shell site may be those that selectively interact with corticolimbic glutamate signaling; some potential mechanisms will be explored in Chapters 3 and 4.

Summary of the Present Experiments

The experiments described in this dissertation examined the generation of appetitive and defensive behavior along a rostrocaudal gradient in medial shell of NAc. Specifically, I sought to assess how seemingly oppositely valenced states, eating versus fear, can be generated by the same neurobiological manipulation, and what potential neurobiological inputs might select for eating versus fear, such as occurs during retuning by different emotional environments. I found that only eating and fear generated by corticolimbic glutamate disruption, and not by GABAergic inhibition, is sensitive to changes in emotional ambience and loss of local dopamine signaling. Additionally, I discovered that either altering the availability of different dopamine receptor subtypes (D1-like versus D2-like dopamine receptors) or activating different prefrontal inputs, can modulate the valence or intensity of appetitive and fearful motivated behaviors generated by glutamate disruption in medial shell of NAc.

Chapter 2: Eating and Fear Generated by GABAergic Inhibition in Nucleus Accumbens Shell Needs No Dopamine and Resists Environmental Retuning

My previous work helped show that local endogenous dopamine in NAc is needed for eating and fear generation by glutamate blockade (Faure et al., 2008). Our lab also showed that environmental ambience can retune the valence of behavior produced by corticolimbic glutamate

blockade at individual DNQX sites, promoting eating generation in a familiar, home environment, and promoting fear generation in a loud, bright environment. In this chapter, I sought to determine whether GABAergic generation of appetitive and fearful motivations would be similarly by retuned by environmental ambience and/or dependent on local dopamine. I found that fear and eating generated by direct GABAergic inhibition of NAc shell resists environmental retuning, and does not need endogenous dopamine. These results suggest that subcortical GABAergic generation of motivation is more robust and autonomous than glutamatergic generation in NAc, and that the neurobiological mechanisms that mediate environmental retuning of the eating and fear gradient may be those that interact preferentially with glutamate signaling, such as dopamine.

Chapter 3: Nucleus Accumbens Dopamine/Glutamate Interaction Switches Mode to Generate Desire Versus Dread: D1 Alone for Appetitive Eating but D1 and D2 Together for Fear

My initial work helped show that mesocorticolimbic dopamine inputs are required for glutamate blockade (via DNQX) to generate either eating or fear (Faure et al., 2008). In this chapter (Richard and Berridge, 2011b), my goal was to better characterize the role of dopamine in the generation of fear and feeding by determining relative roles of D1 and D2 receptors in DNQX-induced behaviors. I used microinjections of selective antagonist combinations to assess whether endogenous neurotransmission at D1 versus D2 dopamine receptor families was necessary for medial shell AMPA blockade to generate appetitive behavior (at rostral sites) and defensive behavior (at caudal sites). Only endogenous D1 receptor stimulation was needed for the intense eating generated by rostral shell AMPA blockade, while defensive behaviors produced by AMPA blockade in caudal shell required activity at both D1 and D2 receptors. Yet, rostrocaudal site so strongly biases the valence of DNQX-generated behavior that these results

might suggest that rostral shell function is predominated by D1 signaling whereas caudal shell might involve more D2 signaling. Therefore, I extended these findings by utilizing the flexibility of intermediate rostrocaudal sites, where environmental ambience can flip the valence of DNQX-induced behavior, to tease apart valence and rostrocaudal location. I found that the generation of defensive behavior (in a stressful environment) requires D2 receptors, even when generated at rostral sites, whereas hyperphagia produced at the same sites in the familiar environment was invulnerable to D2 blockade. Thus, NAc D1 and D2 receptors, and their associated neuronal circuits, play different and dynamic roles in enabling desire and dread to be generated by localized NAc glutamate disruptions in medial shell.

Chapter 4: Prefrontal Cortex Modulates Desire and Dread Generated by Nucleus Accumbens Glutamate Disruption

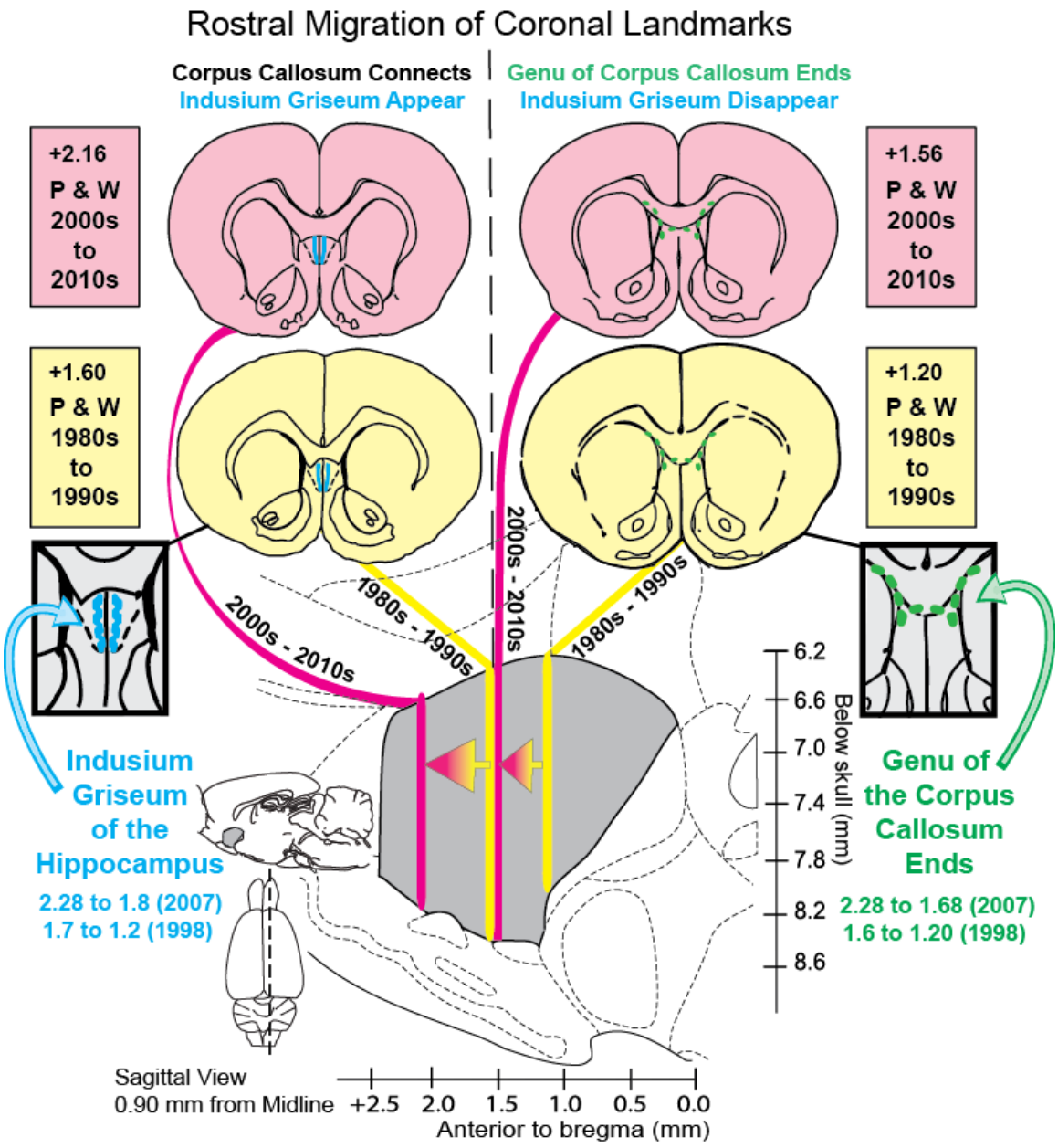
Corticolimbic circuits, including direct projections from prefrontal cortex to NAc, permit "top-down" control of intense motivations generated by subcortical circuits. In this chapter (Richard and Berridge, 2012), I asked whether medial prefrontal cortex can modulate intense motivations generated by subcortical NAc disruptions. I used simultaneous microinjections in medial prefrontal cortex regions and in NAc shell to examine whether the desire or dread generated by NAc shell disruptions is modulated by activation/inhibition of three specific regions of prefrontal cortex: medial orbitofrontal cortex, infralimbic cortex (homologous to area 25 or subgenual anterior cingulate in the human), or prelimbic cortex (midventral anterior cingulate). I found that activation of medial orbitofrontal cortex biased intense bivalent motivation in an appetitive direction by amplifying generation of eating behavior by middle to caudal NAc glutamate disruptions, without altering fear. In contrast, activation of infralimbic prefrontal cortex powerfully and generally suppressed both appetitive eating and fearful behaviors

generated by NAc shell disruptions. These results suggest that corticolimbic projections from discrete prefrontal regions can either bias motivational valence or generally suppress subcortically-generated intense motivations of desire or fear.

Chapter 5: New and Future Directions

To fully understand the NAc mechanisms that generate fear versus desire, it is critical to investigate the effects of inhibition of particular input or output pathways from NAc shell, as well as the role of particular neuronal types. Here, I describe pilot experiments examining the effects of NAc shell inhibitions generated by designer receptors exclusively activated by designer drugs (DREADDs), which are virally-expressed receptors that are activated by an endogenously inert ligand. DREADD-mediated inhibition of rostral NAc shell successfully generated intense eating, similar to eating generated by pharmacological inhibition. These results suggest that DREADD-mediated inhibition could be used to investigate the importance of inhibition of particular NAc shell neuronal types or efferent pathways in the elicitation of intense eating, though it may be less efficacious in investigations of defensive behavior. Additional experiments aimed at addressing the systems-level mechanisms of fear and desire are also discussed.

Figure 1.1. Rostral shift of anatomical markers in the coronal view. Sagittal illustration of how coronal landmarks have apparently shifted forward, in terms distance from bregma, as transferred from coronal planes in early editions of a popular brain atlas in 1980s and 1990s (yellow; Paxinos and Watson, 1998) to the more recent 2007 edition (pink; Paxinos and Watson, 2007). As one result, the coronal slice containing the rostral-most extent of the indusium griseum of the hippocampus where the corpus callosum is joined, would apparently move from 1.6 mm ahead of bregma (yellow; Paxinos and Watson, 1998) to approximately 2.16 mm ahead of bregma (pink; Paxinos and Watson, 2007). Concurrently, the slide just caudal to the genu of the corpus callosum and the indusium griseum appears to have moved from 1.2 mm ahead of bregma (yellow; Paxinos and Watson, 1998) to 1.56 mm ahead of bregma (pink; Paxinos and Watson, 2007).


Rostral Migration of Coronal Landmarks
Corpus Callosum Connects
Indusium Griseum Appear
Genu of Corpus Callosum Ends
Indusium Griseum Disappear
+2.16
P & W
2000s
to
2010s
+1.56
P & W
2000s
to
2010s
+1.60
P & W
1980s
to
1990s
+1.20
P & W
1980s
to
1990s
2000s - 2010s
1980s - 1990s
2000s - 2010s
1980s - 1990s
Indusium
Griseum
of the
Hippocampus
2.28 to 1.8 (2007)
1.7 to 1.2 (1998)
Genu of
the Corpus
Callosum
Ends
2.28 to 1.68 (2007)
1.6 to 1.20 (1998)
6.2
6.6
7.0
7.4
7.8
8.2
8.6
Below skull (mm)
Sagittal View
0.90 mm from Midline
+2.5
2.0
1.5
1.0
0.5
0.0
Anterior to bregma (mm)

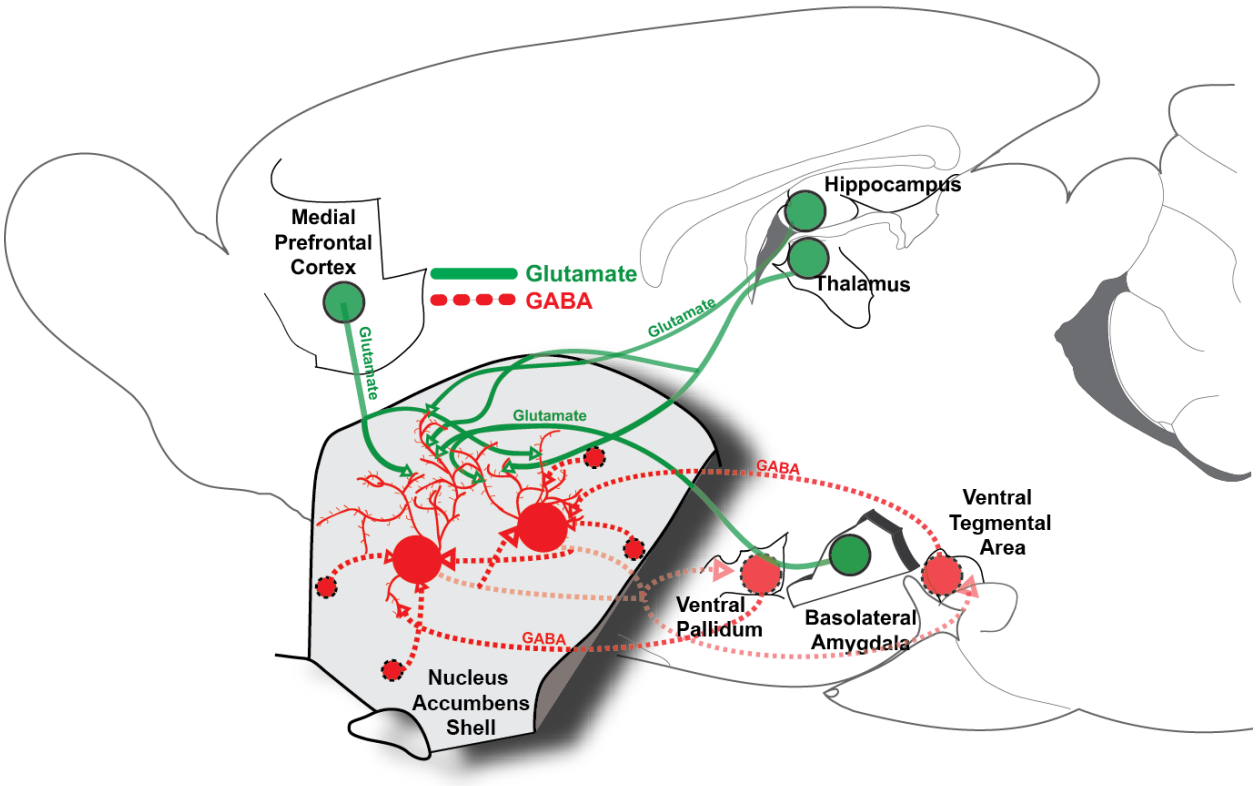


Figure 1.2. Glutamatergic and GABAergic NAc circuits. Green; glutamatergic inputs from medial prefrontal cortex, hippocampus, thalamus, and basolateral amygdala are shown entering the NAc, where they synapse on distal dendrites of medium spiny neurons in NAc. Red; GABAergic inputs from ventral pallidum and ventral tegmental area, as well as GABAergic interneurons and axon collaterals from other medium spiny neurons are shown synapsing onto proximal dendrites and soma of medium spiny neurons within NAc.

Chapter 2

Eating and Fear Generated by GABAergic Inhibition in Nucleus Accumbens Shell Needs No Dopamine and Resists Environmental Retuning

Introduction

Local inhibitions in medial shell of the nucleus accumbens (NAc) caused by either local blockade of AMPA glutamate transmission or augmentations of GABAergic inhibition can produce intense levels of either appetitive and/or fearful motivated behaviors organized along a rostrocaudal gradient resembling an affective "keyboard". That is, at rostral sites, microinjections of either AMPA antagonist DNQX or GABA agonist muscimol induce intense appetitive behaviors such as increased eating time and doubling of food intake (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1997). In contrast, at more caudal sites microinjection of these same drugs produce predominantly (for DNQX) or exclusively (for muscimol) fearful behaviors, including defensive distress vocalizations and escape attempts elicited by experimenter's touch and spontaneous emission of defensive treading/burying behavior, an anti-predator response which rodents use to throw debris at a localized threat (i.e. rattlesnake in the wild or shock prod in the laboratory) (Coss and Owings, 1978; Treit et al., 1981; Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2010).

While rostrocaudal keyboard patterns of intense motivations are generated by both GABAergic inhibitions and glutamatergic disruptions, these manipulations differ in important

respects. Psychologically, we have previously shown that subcortical GABA inhibition by muscimol generates intense hedonic or affective “liking” and disgust reactions to tastes in a rostrocaudal pattern that nearly parallels appetitive/fearful motivated behaviors (Reynolds and Berridge, 2002; Faure et al., 2010). By contrast, glutamate disruption fails to generate any affective ‘liking’ or disgust impact even when it generates comparably intense levels of appetitive/fearful motivated behaviors (Faure et al., 2010).

Here we were interested in comparing intense motivations generated by GABAergic versus glutamatergic NAc inhibitions on two other features, one neurobiological and the other psychological. A neurobiological feature of the glutamatergic keyboard is that endogenous local dopamine is necessary for DNQX microinjections to generate either desire or dread. That is, mixing dopamine D1/D2 receptor antagonists into a DNQX microinjection prevents the generation of intense eating or fear (Faure et al., 2008; Richard and Berridge, 2011b). However, dopamine does not mediate the hedonic impact of sweet (pleasure ‘liking’) or bitter (aversive disgust) tastes (Peciña et al., 1997; Berridge and Robinson, 1998; Peciña et al., 2003). Given that the GABAergic keyboard generates intense pleasure and disgust as well as motivation, one might infer that the GABAergic inhibition should not require dopamine to generate motivation. Is NAc GABAergic generation of appetitive or fearful motivations independent of local dopamine?

A psychological feature of the glutamate keyboard is that positive versus negative valence zones are retuned by current environmental ambience. In a familiar and comfortable home environment, more than 80% of the entire medial shell generates positive appetitive behavior while the fear-generating zone shrinks to a far-caudal 20%; but in a stressfully loud and bright environment DNQX instead generates mostly fearful behavior at 80% of sites in medial

NAc shell (Reynolds and Berridge, 2008; Richard and Berridge, 2011b). Is GABAergic generation of appetitive and fearful motivations similarly retuned by environmental ambience?

Here we examined whether endogenous dopamine is needed for GABAergic generation of intense motivations, and whether GABAergic motivation generation resists environmental retuning. We find that GABAergic motivation generation does not require local endogenous dopamine and resists environmental retuning to persist in purely anatomical determination for the valence of intense motivations.

Methods

Experimental Design

In order to assess the contribution of endogenous dopamine signaling to appetitive and fearful behavior generated by GABAergic inhibition we combined a D1 antagonist and D2 antagonist in the same microinjection with $GABA_A$ agonist muscimol. We separately assessed the influence of emotional ambience by comparing motivations generated by muscimol microinjections or DNQX microinjections in three different environments: "Stressful" (noisy and bright), "Standard" lab, and "Home" (a rat's own home-room: dark, quiet, familiar odor). Keyboard locations of desire-generation and dread-generation, and the neurobiological impact of dopamine blockade on GABAergic neuronal suppression, were mapped using measurements of the diameters of impact spread of drug microinjections obtained from Fos plume analyses on a separate group of rats.

Subjects

Male Sprague-Dawley rats [280 – 400 grams, total n = 95; dopamine dependence group, n = 18; environment shift groups, n = 59; Fos Plume group, n = 18] were housed on a reverse

12:12 light:dark cycle at ~21 ºC with *ad libitum* access to both food and water. All experimental procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Cranial cannulation surgery

All rats were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg) and pretreated with atropine (0.05 mg/kg) to prevent respiratory distress. After anesthesia induction, rats were placed in a stereotaxic apparatus (David Kopf Instruments), with the mouth bar set to 5.0 mm above intra-aural zero, so that cannulae could be angled to avoid puncturing the lateral ventricles. Bilateral stainless steel cannulae (14 mm, 23 gauge) were aimed 2 mm above points throughout the rostrocaudal extent of the medial shell, between coordinates anteroposterior (AP) +2.4 to +3.4 mm ahead of bregma, mediolateral (ML) +/- 1.0 mm from the midline, and dorsoventral (DV), +5.7 – 6.0 mm below skull. Cannulae were anchored to the skull using surgical screws and secured with dental cement; stainless steel obturators (28 gauge) were insert to prevent occlusion of the cannulae. Post-surgery, rats received cefazolin (75mg/kg) to prevent infection and carprofen (5 mg/kg) for analgesia. Rats were allowed to recover for 7 days before testing.

Drugs and intracerebral microinjections

Drug microinjections were always administered bilaterally in a 0.5 µl volume on test days spaced at least 48 hours apart. On test days, solutions were brought to room temperature (~21ºC), inspected to confirm the absence of precipitation, and infused at a speed of 0.3 µl per minute using a syringe pump attached via PE-20 tubing to stainless steel injectors (16 mm, 29 gauge) which extended 2 mm beyond the end of the guide cannulae into medial shell. Injectors

were left in place for 1 minute, and then obturators were replaced and rats were immediately placed in one of the testing chambers described below.

GABA/dopamine interaction experiment

For GABAergic muscimol microinjections in medial shell, rats (n=18) received a dose of muscimol (75 ng / 0.5 µl per side) previously shown to generate intense eating and fear (Faure et al., 2010). Muscimol was microinjected either alone or, to test the role of endogenous dopamine, in combination with two dopamine D1 and D2 antagonists as a mixture in the same microinjection: the selective D1 antagonist (SCH23390 *R*(+)-7-chloro-8-hydroxy-3-methyl1-phenyl-2,3,4,5,-tetrahydro-1H-3-benzazepine) at a dose of 3 µg/ 0.5 µl per side, plus the selective D2 antagonist raclopride (3,5-dichloro-N-{[(2S)-1-ethylpyrrolidin-2-yl]methyl}-2-hydroxy-6-methoxybenzamide) at a dose of 5 µg/ 0.5 µl per side. These doses of dopamine antagonists have been shown previously to prevent DNQX microinjections in NAc shell from producing eating and fearful behaviors (Faure et al., 2008). Each rat received 4 microinjections in counterbalanced order: 1) vehicle alone (ACSF), 2) muscimol alone, 3) dopamine antagonists alone (D1 & D2 dopamine antagonists without muscimol), and 4) muscimol plus D1 & D2 dopamine antagonists. Immediately after every microinjection the rat was placed in a chamber in a conventional laboratory testing room with normal daylight illumination conditions (white fluorescent light intensity 550-650 lux) and moderate intensity ambient noise (65 -70 decibels).

GABA versus glutamate environmental shift experiment

In order to compare environmental ambience modulation of intense motivations produced by glutamate or GABA NAc keyboards, rats were tested in three different emotional environments after either a DNQX or muscimol microinjection. DNQX was microinjected at a dose of 450 ng / 0.5 µl per side and muscimol was injected at a dose of 75 ng / 0.5 µl per side,

which were selected to produce comparable levels of motivated behaviors from previous studies (Faure et al., 2010). Some rats (n=29) received microinjections of either DNQX or its vehicle (50% DMSO and 50% .15 M saline) on separate days, prior to being tested twice each in the Home, Stressful and Standard environments, for a total of 6 test days and 6 microinjections per rats. Other rats (n=30) received microinjections of either muscimol or its vehicle (ACSF) prior to being tested twice each of the 3 environments, also for a total of 6 test days. The order of drug and environment conditions was counterbalanced in each group.

The Standard lab environment was identical to test conditions described for the dopamine blockade experiment. The Home environment test occurred in the rats' own home-room, and was characterized by dim red lighting (5-10 lux), low levels of ambient noise primarily consisting of only and static sound from the ventilations systems (65-70 decibels) and from other rats, and the familiar odors of the housing room. The Stressful environment was conducted in an unfamiliar room, featuring high-intensity sensory-stimulation of light and sound. Extra light was provided by additional incandescent lamps that were directed at the transparent test chamber (1000-1300 lux within the chamber). Loud, unpredictable sound was presented continuously through the test (raucous rock music from the continuous full-album soundtrack of "Raw Power" by Iggy & The Stooges [1973; Iggy Pop reissue 1997]; 80-86 decibels). In previous preference tests, rats have been shown to prefer the Home environment condition over the Standard environment condition and to prefer the Standard lab environment over the Stressful condition (Reynolds and Berridge, 2008).

Behavioral tests of unconditioned motivated behaviors

Prior to the first test day, rats were handled for 10 minutes per day for 3 days, and then were habituated to the testing procedure and apparatus for 1 hour each on 4 additional days. On

the 4th day of habituation rats received “mock” microinjections of vehicle before being placed in the chamber. On drug test days, rats received one of the microinjections described above and were placed immediately in a transparent testing chamber (23 x 20 x 45 cm) which contained pre-weighed food (~20g rats chow) and *ad libitum* water. The chamber always contained granular cob bedding (~ 3 cm deep) to allow expression of defensive treading behavior. Rats remained in the chamber for 60 minutes while behavior was videorecorded, to be coded later for analysis.

At the end of each session, rats were removed by the experimenter’s gloved hand using a standardized slow-approach hand motion in order to quantify any fearful distress calls, escape attempts or defensive bites elicited by human touch. Following a ~5 second approach towards the testing cage, the experimenter slowly reached towards the rat, taking ~2 seconds. Upon contact, the experimenter lightly brushed the side of the rat with gloved fingertips, taking ~1 sec, before lifting the rat from the chamber in a gentle movement that lasted ~2 sec. The observer recorded any a) attempts by the rat to escape when touched (e.g., frantic jumps or runs away from the hand), b) bites or attempts to bite the gloved hand, and c) audible distress vocalizations elicited by the approaching hand.

Behavioral coding of videorecorded behaviors

Videorecorded behaviors were scored offline by observers blind to drug conditions. The incidence of elicited fearful distress vocalizations, escape dashes, and bite attempts directed at the experimenter’s hand were scored when the rat was gently picked up at end of the test session (Reynolds and Berridge, 2003), at which time total grams of chow pellets consumed were also recorded. Behaviors emitted spontaneously and videotaped during the 1-hr test were subsequently scored for the total cumulative duration (seconds) for each of the following: eating

behavior (involving both appetitive approach and voluntary initiation of ingestion plus consummatory chewing and swallowing of food), drinking behaviors (licking from water spout), fearful defensive treading/burying behavior (defined as active spraying or pushing of bedding with rapid alternating thrusts of the forepaws, spatially directed generally towards the brightly lit front or corners of the cage) and grooming behavior (a stereotyped sequence described in Aldridge et al., 1993). Observers scored the total number for behaviors which tended to occur as discrete events, including appetitive behaviors such as food carrying (transportation of food pellets in the mouth) and food sniffs (sniffing near the food for at least 1 second), and two general motor activities: rearing (forepaws at least one inch off the floor) and cage crosses (forepaws and head cross the halfway point of the cage).

Histology

Following behavioral testing rats were euthanized with an overdose of sodium pentobarbital, their brains were removed and fixed in 10% paraformaldehyde for 1-2 days and in 25% sucrose solution for 3 days. In order to assess microinjection site locations, brains were sliced at 60 microns on a freezing microtome, and stained with Cresyl violet. Microinjections sites were mapped onto coronal slices from a rat brain atlas (Paxinos and Watson, 2007), which were then used to extrapolate the position of each site on a sagittal slice. This allowed to the presentation on the same maps of most of the rostrocaudal and dorsoventral extent of medial shell. Functional effects on appetitive and fearful behaviors were mapped using color-coding to express intensity of changes in motivated behaviors for individual behaviorally-tested rats. Symbols were sized to match the maximal diameter of Fos plumes as found here and previously (Faure et al., 2010; Richard and Berridge, 2011b). For rostral versus caudal statistical

comparisons, sites were classified as rostral shell if they were located more than 1.4 mm ahead of bregma, and as caudal if they fell behind this benchmark.

Fos-like protein immunohistochemistry

We reported previously that Fos plumes induced by drug microinjection shrank after a series of 6 repeated microinjections, indicating reduced drug impact on local neurons presumably due to gradual gliosis or necrosis surrounding the microinjection tip (Richard and Berridge, 2011b). Therefore we analyzed Fos in a separate group after only a single microinjection in order to measure maximum Fos plume diameters, in order to avoid potential mapping problems due to underestimation of impact spread. Rats used for Fos analysis (n=18) were anesthetized with an overdose of sodium pentobarbital and transcardially perfused 90 minutes after bilateral microinjection in NAc of 1) vehicle (n = 5), 2) muscimol (n = 6), 3) dopamine antagonists (n = 3), or 4) mixture (n = 3), or 5) no injection (n=1) for Fos plume analysis. Brains were removed and placed in 4% paraformaldehyde for 4 – 24 hours, and then transferred to 25% sucrose (in 0.1 M NaPB) for 3 days. Brains were sliced at 40 microns on a freezing microtome, and processed for Fos-like immunoreactivity using NDS, goat anti-cfos (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat Alexa Fluor 488 (Invitrogen, Carlsbad, CA) as described previously (Faure et al., 2008; Reynolds and Berridge, 2008; Faure et al., 2010). Sections were mounted, air-dried and coverslipped with ProLong Gold antifade reagent (Invitrogen).

Fos plume analysis

Immunoreactivity for Fos-like proteins was visualized using a Leica microscope equipped for fluorescent microscopy, using a filter with an excitation band at 480-505 nm for Fos-positive cells and images were taken using MCID Core software. For analysis of drug spread, Fos plume images were taken in the areas surrounding the microinjection with the most intense

areas of Fos expression, just caudal to the end of the injector tip, surrounding a small focal point of necrosis. Fos labeled cells were individually counted within successive blocks (50 µm x 50 µm), along 8 radial arms emanating from the center of the necrosis, with 10x magnification. Zones of Fos elevation (or "plumes") were assessed as described previously for muscimol and dopamine antagonist microinjections (Reynolds and Berridge, 2008). Fos plume sizes for DNQX microinjections were based on a previous study with this same dose (Richard and Berridge, 2011b).

Statistical analysis

The effects of dopamine blockade on muscimol-induced motivated behaviors were assessed using a three-factor mixed within- and between-subject ANOVA (muscimol x dopamine blockade x anatomical level [rostral versus caudal]). When significant effects were found, rats were split into rostral and caudal groups and additional one-way ANOVAs were conducted, including pairwise comparisons with Sidak corrections. For analysis of binomial data (vocalizations, escape attempts and bite attempts) Cochran's Q and McNemar's repeated measures tests were used. The effects of DNQX or muscimol in the three different emotional environments were compared using a three-factor mixed within- and between-subjects ANOVAs (drug x anatomical level [rostral versus caudal] x environment [Stressful versus Standard versus Home]) for all parametric behaviors. When significant effects were found, rats were split into rostral and caudal groups and additional one-way ANOVAs were conducted on the difference between vehicle and drug between the three environments, including pairwise comparisons with Sidak corrections. We also assessed the percent of rats which met criteria for appetitive, defensive or mixed valence behaviors, and tested whether that changed between the three environments for DNQX versus muscimol, using Cochran's Q and McNemar's repeated

measures test, and whether the proportion of rats that switched differed between DNQX and muscimol using Pearson's chi-squared test.

Results

Dopamine blockade experiment: dopamine is not needed for GABAergic motivation

Local blockade of dopamine D1 and D2 receptors failed to impair the ability of GABAergic muscimol microinjections to generate intense levels of motivated behaviors (5-times to 75-times above vehicle control levels): neither appetitive eating and food intake nor fearful active defense and escape behaviors were reduced by addition of dopamine antagonists, and muscimol-generated levels always remained high above vehicle levels (Figures 2.1 and 2.2). Muscimol microinjections in rostral shell generated robust tripling of food intake and a three-fold increase in time spent eating (Figures 2.1a and 2.2a and b; eating, interaction of muscimol by placement, $F(1, 14) = 14.51$, $p = 0.002$). The addition of dopamine antagonists did not reduce these intense appetitive behaviors generated at rostral sites, and eating remained intense, at more than double vehicle levels (Figures 2.1a and 2.2a; rostral shell, eating, interaction of muscimol and D1/D2 antagonists, $F(1,8) = <0.01$, $p = 0.996$). By comparison, caudal shell sites of muscimol microinjection with or without dopamine antagonists generated up to 60-fold increases in defensive treading behavior (interaction of muscimol by placement, $F(1, 14) = 6.15$, $p = 0.026$), as well as distress vocalizations, escape attempts and sometimes bite attempts in response to the experimenter (Figures 2.1c and 2.2d; vocalizations and escape attempts, $p < 0.001$). Dopamine antagonists did not alter the level of fearful defensive treading generated by muscimol at caudal sites, which remained at more than 50-fold vehicle levels (Figures 2.1c and 2.2b; interaction of muscimol and D1/D2 antagonists, $F(1, 6) = 0.86$, $p = 0.389$). Dopamine blockade

also had no effect on caudal distress vocalizations, escape attempts, or bite attempts observed after the mixture of muscimol and dopamine antagonists when compared to muscimol alone (Figures 2.1d and 2.2c; McNemar's Test, vocalizations, $p = 0.375$; escape attempts, $p = 0.180$; bite attempts, $p = 1.000$).

The persistence of intense GABAergic eating and fear after local dopamine blockade contrasts with the dramatic reduction of motivated behaviors generated by corticolimbic glutamate disruptions we previously reported to be caused by similar dopamine blockade (Faure et al., 2008; Richard and Berridge, 2011b). However, by themselves, microinjections of dopamine antagonists here (without muscimol) did suppress lower spontaneous baseline levels of appetitive eating and food intake below vehicle to nearly zero (Figure 2.1a and b; time spent eating, general effect of D1/D2 antagonists, $F(1,15) = 7.853$, $p = 0.014$; food intake grams consumed, average of 0.25 ± 0.68 g under D1/D2 antagonists vs 1.65 ± 1.28 g under vehicle; food intake, general effect of D1/D2 antagonists, $F(1,15) = 8.674$, $p = 0.012$).

GABAergic keyboard motivations are purely anatomically determined and resist change by environmental ambience

Muscimol microinjections in NAc shell generated similar keyboard patterns of intense motivated behaviors across all three environments, resisting environmental retuning (Figures 2.3 and 2.4). Muscimol generated intense appetitive behavior at sites that tended to occur largely in the rostral half of medial shell (main effect on eating, $F(1,27) = 6.309$, $p = .018$; interaction with placement, $F(2,27) = 5.672$, $p = .009$). At more caudal locations in medial shell, muscimol generated intense defensive treading behavior (main effect on treading, $F(1,27) = 4.511$, $p = .043$; interaction with placement, $F(2,27) = 6.388$, $p < .005$). Muscimol also generated intense defensive reactions to the experimenter at the end of the testing session, such that at least 46% of

rats emitted distress vocalizations to the experimenter, and at least 21% of rats attempted to escape in each environment (McNemar's Tests, vocalizations: Standard, $p = .000$, Stressful, $p = .004$, Home $p = .000$; escape attempts: Standard, $p = .031$, Stressful, $p = .008$, Home, $p = .031$). Environmental changes had no detectable influence on the intensity of muscimol-elicited appetitive eating (muscimol by environment: eating, $F(4,54) = 1.874$, $p = .128$) or the size of the appetitive zone in which eating was elicited (Cochran's $Q(2) = .400$, $p = .819$). Environmental changes also did not alter fearful escape attempts or distress vocalizations elicited by the approach of the experimenter's gloved hand (vocalizations, Cochran's $Q(2) = .500$, $p = .779$; escape attempts, Cochran's $Q(2) = .727$, $p = .695$), and altered only one category of fearful reaction: spontaneous defensive treading.

The dark, quiet and familiar Home environment virtually eliminated sites where muscimol generated defensive treading (McNemar's Tests, versus Standard, $p = .005$; versus Stressful, $p = .002$; Figure 2.5) and reduced the average intensity of muscimol elicited defensive treading ($F(1,26) = 8.551$, $p < .007$). However, the noisy and bright Stressful environment did not increase defensive treading behavior over the already intense levels produced by caudal muscimol microinjections in the Standard environment (muscimol by environment: $F(1,26) = .163$, $p = .690$), and failed to switch any rostral sites from generating purely appetitive eating to defensive treading (versus Standard, McNemar's Test, $p = 1.000$). Also, the reactive and even more extreme threat-evoked defensive reactions of distress vocalizations and escape attempts that were elicited by the experimenter's touch remained equally intense and unchanged across all three environments after caudal muscimol microinjections (distress vocalizations, Cochran's $Q(2) = .500$, $p = .779$; escape attempts, Cochran's $Q(2) = .727$, $p = .695$; Figure 2.6). Thus the GABAergic NAc keyboard pattern of rostral shell generation of intense eating, middle

generation of mixed eating/fear, and caudal generation of intense fear appeared stable, and determined purely by anatomical location of each GABAergic 'key' or microinjection that induced local hyperpolarization. The valence tuning of individual microinjection sites never shifted across environments. Only the behavioral intensity of spontaneous defensive treading for middle and caudal sites was at all influenced by environment, and only by being dampened in the presence of the familiar and comfortable Home conditions. All other aspects of intense motivated behaviors generated by muscimol microinjections were invulnerable to environmental changes.

By contrast to GABAergic inhibition, the keyboard pattern generated by DNQX microinjections was powerfully shifted both anatomically and in behavioral intensity generated at particular sites by changes in environmental ambience, as expected (Figures 2.3 and 2.4a; interaction with environment: eating, $F(2,44) = 7.359$, $p = .002$; treading, $F(2,44) = 4.985$, $p = .011$; distress vocalizations, Cochran's $Q(2) = 11.556$, $p = .003$; escape attempts, Cochran's $Q(2) = 6.500$, $p = .039$). The Stressful environment nearly doubled the size of the medial shell zone where defensive treading was generated, in comparison to the Standard environment, to more than 55% of sites (versus 31% in Standard), more than quadrupling the defensive zone in comparison to the Home environment (overall effect of environment on % defensive sites, Cochran's $Q(2) = 10.391$, $p = .006$; McNemar's Test, $p = .002$, Figure 2.5). Likewise, the Stressful environment doubled the zone in which DNQX produced attempts to escape from the experimenter's hand, compared to the Standard environment (to 7% of sites; McNemar's Tests, Lab versus Stressful, $p = .25$; Home versus Stressful, $p = .125$; Figure 2.6) and quadrupled the zone where DNQX produced reactive distress vocalizations (to 28%; McNemar's Tests, Lab versus Stressful, $p = .070$; Home versus Stressful, $p = .008$).

Conversely, the familiar Home environment nearly eliminated the fearful caudal zone where DNQX generated spontaneous defensive treading behavior (Cochran's $Q(2) = 10.391$, $p = .006$; difference between Stressful and Home environments, McNemar's Test, $p = .002$, Figure 2.5), and completely eliminated generation of reactive responses such as distress vocalizations and escape attempts to the experimenter's hand (McNemar's Test, Lab versus Home, $p = .500$, Stressful versus Home, $p = .008$; Figure 2.6). At the same time, the Home environment expanded the "appetitive zone" where DNQX produced purely appetitive behavior to more than 75% of medial shell (versus 31% in the Stressful environment; Figure 2.3), consistent with previous findings (Reynolds and Berridge, 2008). Testing the Stressful environment slightly, but non-significantly, reduced the intensity of DNQX-induced eating (DNQX by environment, $F(1,23) = 3.404$, $p = .078$).

Fos plume analysis of muscimol and dopamine antagonist microinjections

Mapping of effects was aided by measurements of drug impact spread for muscimol microinjections, as reflected in Fos plumes of about 0.5 mm in diameter. Microinjections of muscimol alone in NAc medial shell produced a small 0.15 mm radius excitatory plume center (volume = .014 mm^3), of elevated Fos expression (150% of vehicle levels), which was encompassed by a larger 0.24 mm radius (volume = 0.06 mm^3) inhibitory or "anti-plume" surround (Fos reduced to <75% of vehicle levels; Figure 2.8), similar to our previous reports (Faure et al., 2010). Dopamine D1 & D2 antagonists, combined together but without muscimol, produced a tiny excitatory center (volume = .005 mm^3) of 150% of vehicle level Fos expression up to .10 mm from the microinjection center, surrounded by a very large 0.41 mm radius robust inhibitory "anti-plume". The anti-plume contained a dense inhibitory middle layer of 0.16 radius (volume = .018 mm^3) of intense Fos suppression (< 50% of vehicle levels), contained within a

larger 0.41 mm radius (volume = .29 mm^3) but less intense anti-plume of moderate Fos suppression (50%-75% of vehicle levels), consistent with previous reports (Faure et al., 2008). Combining muscimol and dopamine antagonists in the same cocktail microinjection produced a similarly sized 0.4 mm radius anti-plume, containing even larger 0.31 mm radius middle layer of intense Fos suppression (volume = .135 mm^3), surrounded by a thinner shell (0.1 mm shell width; 0.4 mm total outer radius) of moderate Fos suppression (50%-75% of vehicle levels; total volume = .24 mm^3).

Discussion

Intense motivations generated in a keyboard pattern by microinjections of GABAergic muscimol in NAc appear to be more anatomically predetermined by rostrocaudal location in medial shell and more autonomous of afferent dopamine than the equivalent glutamatergic keyboard revealed by DNQX microinjections. GABAergic generation of intense appetitive eating and/or intense fearful behaviors was determined almost entirely by the rostrocaudal position of muscimol microinjections within medial shell, proceeded in the absence of endogenous D1 and D2 dopamine stimulation, and resisted environmental retuning by current emotional ambience. By contrast, glutamatergic generation of appetitive and/or fearful behaviors by DNQX, while also biased strongly by anatomical position, does require endogenous dopamine co-stimulation (Faure et al., 2008; Richard and Berridge, 2011b) and was much more potently retuned in valence generation by environmental changes.

Here, intense levels of eating and food intake generated at rostral sites by GABAergic muscimol microinjections did not need local endogenous D1 (or D2) dopamine receptor stimulation. Similarly, generation of fearful spontaneous defensive treading behavior and of

reactive frantic escape attempts and distress calls upon being touched persisted after local D1 and D2 receptor blockade. By contrast, we have previously reported that glutamatergic generation of eating requires endogenous D1 stimulation, and glutamatergic generation of fearful behaviors requires simultaneous dopamine stimulation at both D1 and D2 receptors in the same NAc location as the DNQX microinjection (Richard and Berridge, 2011b).

Similarly, here the GABAergic NAc keyboard psychologically resisted environmental re-tuning by the ambience changes that powerfully retuned valence-generating zones of the glutamatergic keyboard for DNQX. The sole environmental retuning effect observed for muscimol microinjections was that the comfortable Home environment nearly eliminated spontaneous defensive treading. However, reactive distress calls and frantic escape jumps away from the experimenter's hand at the end of the session remained unsuppressed in the Home environment after caudal muscimol microinjections, and those affective reactions elicited by human touch arguably reflect even more intense fear than defensive treading. That pattern suggests that at best the home environment suppresses moderate fear generation by caudal the GABAergic keyboard but does not suppress the more intense fear produced by the same caudal muscimol microinjections interacting with the potentially-threatening percept of an approaching hand. Additionally, even this degree of fear reduction in the dark Home environment (containing only dim red light at test, which rats cannot see) may alternatively be due more to removal of visual cues that otherwise seem to elicit and guide the direction of defensive treading in the Standard lab and Stressful environment after caudal microinjections. Those visual stimuli include the sight of the camera and experimenter in the room outside the transparent test chamber, and any glitter of reflected white light from the curved corners of the plexiglass chamber. In lighted Standard and Home environments, those stimuli normally attract defensive treading after caudal

shell microinjections of muscimol or DNQX, so that a rat typically threw bedding toward the reflecting corners or toward the transparent front wall of the chamber facing the room. Often a defensive mound of bedding was built between those stimuli and the rat as a consequence of persistently targeted defensive treading (Reynolds and Berridge, 2001). In the absence of those eliciting visual stimuli under darkness, conceivably even an actively fearful rat might not emit much spontaneous treading behavior. Especially considering that caveat, and that all other aspects of GABAergic keyboard generation of eating and fear remained intact across the three environments, we conclude that GABAergic generation of intense motivations is thus more robust and neuroanatomically pre-determined than similar motivations produced by AMPA blockade with DNQX (Faure et al., 2008; Reynolds and Berridge, 2008).

Subcortical autonomy resists retuning? Limits to top-down control

One possible explanation for why the GABAergic NAc keyboard for generating intense desire and/or dread is more anatomically pre-determined, more resistant to psychological retuning, and more autonomous of dopamine derives from the subcortical and intrinsic cellular nature of the neuronal signals produced by muscimol microinjection in NAc, compared to the corticolimbic nature of signal disruption mimicked by DNQX microinjections. That is, GABA signals would normally be delivered to spiny neurons in medial shell site either by neighboring intrinsic NAc GABAergic medium spiny neurons or interneurons, or by subcortical GABAergic afferent projections that arose from other deep brain structures such as ventral pallidum, extended amygdala (e.g., BNST), lateral hypothalamus, or brainstem (Brog et al., 1993; Sun and Cassell, 1993; Churchill and Kalivas, 1994; Vanbockstaele and Pickel, 1995; Meredith et al., 2008). Environmental ambience signals by comparison might ordinarily be delivered to NAc largely by ‘top-down’ corticolimbic glutamatergic projections from prefrontal cortex, or other

glutamatergic inputs from cortex-related structures such as basolateral amygdala, hippocampus or thalamus (Beckstead, 1979; Christie et al., 1987; Fuller et al., 1987; McDonald, 1991b). Intrinsic subcortical GABA signals therefore might be somewhat bottom-up and relatively autonomous from environmental modulation, or even from ambience-related catecholamine changes indirectly relayed from mesolimbic dopamine that interacts with glutamate signals in NAc. Subcortical autonomy is also consistent with the fact that GABAergic muscimol inhibition of NAc shell can additionally generate changes in hedonic 'liking' and disgust (which DNQX microinjections do not), as well as robust generation of eating and fear motivations (Reynolds and Berridge, 2002; Faure et al., 2010).

By this view, the resistance of GABAergic appetitive and fearful motivation to both changes in emotional environment and persistence after local dopamine blockade, reflect limitations of the effectiveness of top-down corticolimbic signals in modifying emotional experiences generated by more purely subcortical circuits (Beauregard et al., 2001; Russell, 2003; Davidson, 2004; Barrett et al., 2007a; Posner et al., 2007). The dopamine-independent nature of GABAergic eating and fear is consistent with subcortical GABAergic generation, which generates intense motivations (and hedonic impact) independent of these outside forces.

Physiological basis of GABAergic dopamine-independence

Why does GABAergic generation of intense eating or fear also persist without local dopamine, when glutamatergic generation cannot? In contrast to dopamine modulation of glutamate signals on post-synaptic NAc neurons (Cepeda et al., 1993; Calabresi et al., 1997; Brady and O'Donnell, 2004; Tecuapetla, 2010), post-synaptic interactions between GABA and dopamine may be less important to NAc neuronal impact. Although dopamine may modify pre-synaptic GABA release from NAc interneurons (Bracci et al., 2002; Centonze et al., 2002;

Tecuapetla et al., 2007; Towers and Hestrin, 2008), the post-synaptic hyperpolarizing effects of $GABA_A$ receptor activation on medium spiny neurons may not be powerfully modulated by dopamine. For example, dopamine transporter knock-down mice, which have increased levels of dopamine in the synapse, have normal GABA-receptor mediated synaptic currents onto medium spiny striatal neurons (Wu et al., 2007). Here muscimol microinjection would mimic post-synaptic GABA release effects by directly binding to $GABA_A$ receptors in medial shell, and hence may have acted downstream of dopamine modulation.

It is also possible that hyperpolarizations produced by GABAergic muscimol are more robust or intense at the neuronal level than relative inhibitions produced by DNQX glutamatergic blockade (Koos et al., 2004), which merely diminish "up states" to suppress EPSPs (Meredith et al., 1993; Pennartz et al., 1994; Kiyatkin and Rebec, 1999; Meredith, 1999; O'Donnell, 1999; Suwabe et al., 2008; Jeun et al., 2009). GABAergic signaling may more potently inhibit NAc medium spiny neurons by acting directly to open Cl- channels on somata or on proximal dendrites (Sun and Cassell, 1993; Johnson et al., 1994; Behrends et al., 2002; Goetz et al., 2007), whereas glutamatergic AMPA receptors are localized more distally at the ends of dendrite spines (Meredith et al., 1990; Sesack and Pickel, 1992; Johnson et al., 1994; Chen et al., 1998), allowing dopamine released on spines or dendrites to play a greater role.

Corticolimbic glutamate and mesolimbic dopamine signals in environmental retuning

Our findings may have particular implications for understanding the neurobiological basis of the retuning of DNQX generation of intense levels of desire and dread by shifts in emotional ambience. DNQX in medial shell primarily blocks the impact of excitatory glutamate release by corticolimbic projections from prefrontal neocortex, and from cortical-type forebrain structures (e.g., basolateral amygdala and hippocampus) or corticolimbic relays (e.g., thalamus

paraventricular nucleus) (Swanson, 2005; Wolf et al., 2005; Zahm, 2006; Belujon and Grace, 2008; Meredith et al., 2008; Kalivas, 2009). Potential mechanisms for environmental expansion/contraction of NAc zones for glutamatergic fear generation or appetitive generation include changes either in the pattern, intensity or anatomical distribution of corticolimbic glutamate afferent signals (Beckstead, 1979; Christie et al., 1987; Fuller et al., 1987; Sesack et al., 1989; McDonald, 1991b; Gill and Grace, 2011). Candidates also include psychologically-triggered release of neuromodulator signals, such as dopamine, norepinephrine, corticotrophin-releasing factor, serotonin, etc. (Chronister et al., 1980; De Souza et al., 1985; Voorn et al., 1989; Jacobs and Azmitia, 1992; Berridge et al., 1997; Delfs et al., 1998). In some cases, similar glutamatergic neurobiological events in medial shell neurons, regardless of rostrocaudal position, might allow generation of both the positive incentive salience of reward stimuli and the fearful salience of threat stimuli, depending on the nature of current mesolimbic dopamine inputs (Berridge and Robinson, 1998; Kapur, 2003; Berridge, 2007; Faure et al., 2008) that modulate glutamate signaling (Calabresi et al., 1997; Brady and O'Donnell, 2004).

Conclusions

Here, we report that GABAergic generation of intense motivations obeys very different rules than glutamatergic generation regarding dopamine and environmental retuning, even though both produce appetitive and fearful behaviors organized along the same rostrocaudal gradient. The GABAergic keyboard robustly generates intense eating and fearful motivations, solely determined by the anatomical position of the muscimol microinjection ‘key’, relatively immune to modulation by environmental ambience and free of any need for local dopamine inputs. By contrast, glutamatergic keyboard generation of intense eating and fear is easily

retuned by changes in emotional environment as well as highly dependent on endogenous dopamine.

This distinction between glutamatergic motivations that are modifiable via top-down and mesolimbic control versus robust GABAergic motivational states which are impenetrable by top-down control or dopamine, may have implications for psychiatric disorders and emotional well-being. Flips in the valence of pathologically intense motivational salience may occur more easily when they primarily involve NAc corticolimbic or glutamatergic motivational salience, rather than subcortical GABAergic mechanisms that are intrinsic to NAc. This may relate to why the incentive salience of amphetamine addiction can flip valence from appetitive to the fearful salience of amphetamine psychosis, or why schizophrenic patients can have not only heightened fearful salience of paranoia but may also show exaggerated incentive salience for appetitive stimuli (Elman et al., 2006; Featherstone et al., 2007; Jensen et al., 2008; Howes and Kapur, 2009). In contrast, GABAergic motivations generated in NAc-related circuits may be more anatomically coded, consistently valenced, hedonically laden, and less amenable to regulation by top-down control (Faure et al., 2010; Watson and Naragon-Gainey, 2010).

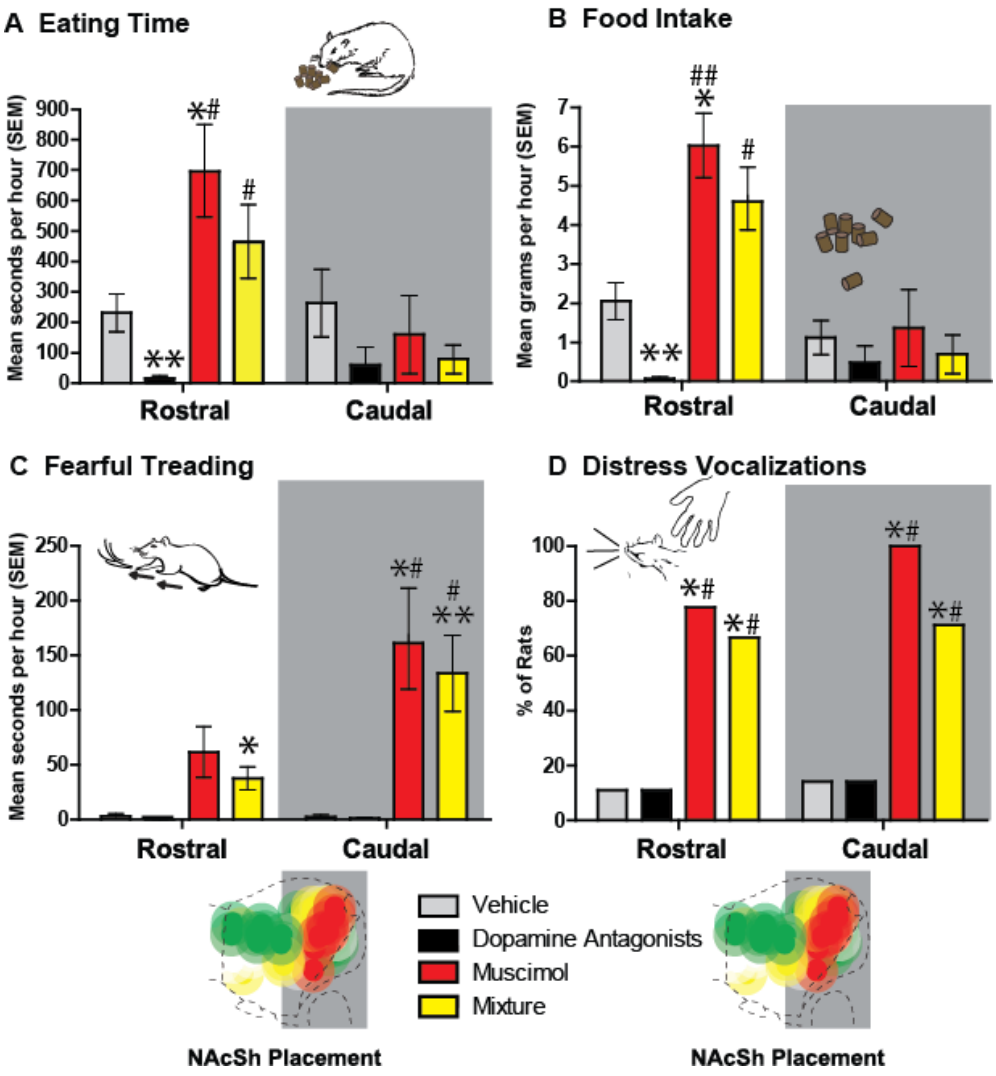
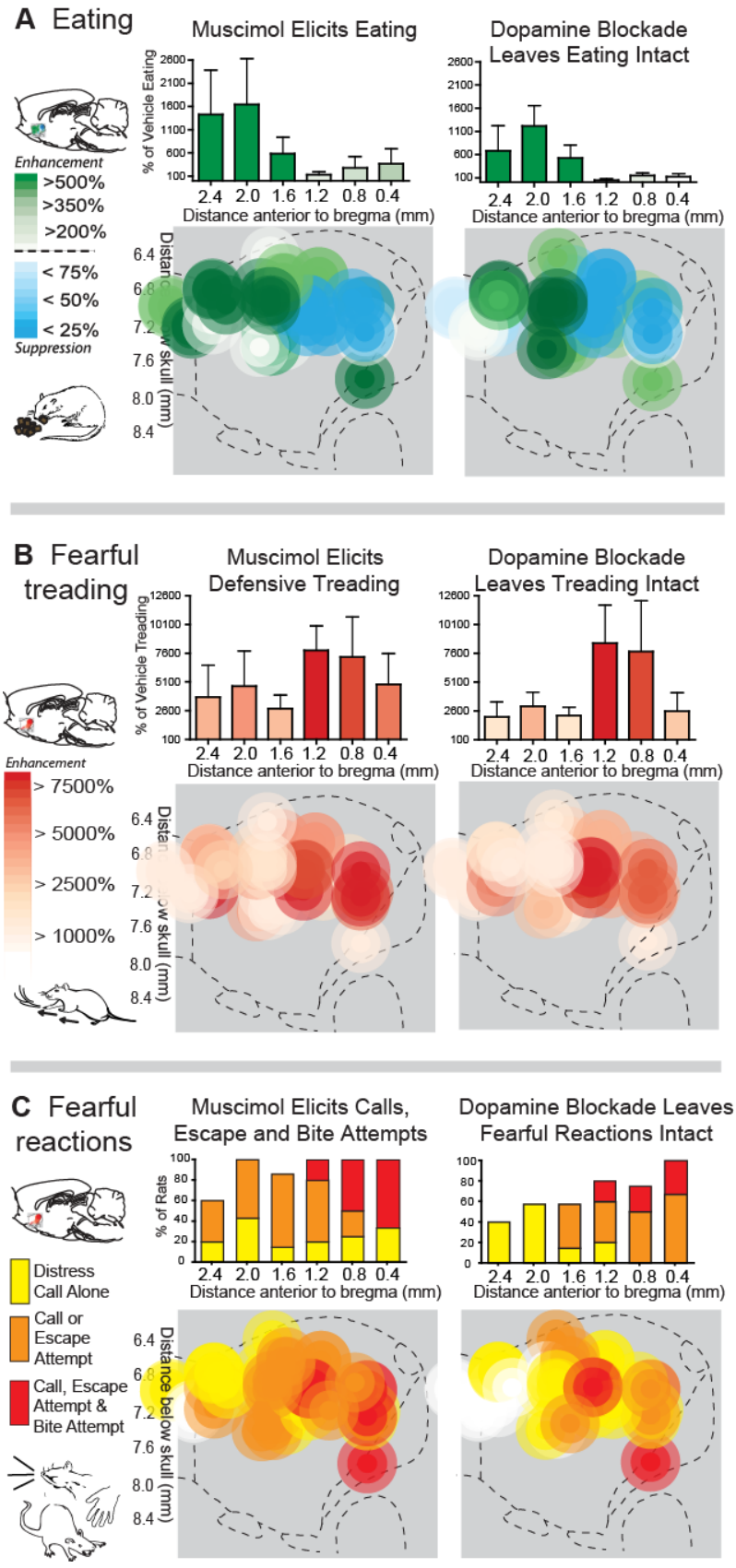


Figure 2.1. Summary graphs of the effect of local dopamine blockade on muscimol-generated eating and fearful behaviors. Time spent eating (A), amount of food intake (B), time spent defensive treading/burying (C), as well as incidence of distress vocalizations in response to human touch (D), elicited by vehicle (grey), dopamine antagonist combination of raclopride and SCH23390 (black), muscimol (red) and mixture of muscimol and dopamine antagonist (yellow) in rostral (n=9) and caudal (n=7) regions of medial NAc shell. Errors bars indicate SEM, * $p < 0.05$, ** $p < 0.01$ change from vehicle, # $p < 0.05$, ## $p < 0.01$ change from muscimol, pairwise comparisons using Sidak corrections for multiple comparisons (eating, food intake and defensive treading) or McNemar's test (distress vocalizations).

Figure 2.2. Effects of dopamine antagonism on muscimol-induced eating and defensive fearful behaviors. Fos plume maps (n=16) of the generation in medial shell of eating (A), defensive treading (B), and fearful calls, escape attempts and bite attempts (C) by muscimol (left) and a mixture of muscimol plus dopamine antagonists (right). Local dopamine blockade failed to prevent muscimol generated eating or fearful behaviors, despite its previously reported ability to prevent DNQX-induced eating and fear, and its generally suppressive effects. Histograms bars below the maps show behavior as percent of vehicle (eating, A; treading, B) or percent of subjects (calls, escape attempts and bite attempts, C) for each behavior at rostrocaudal level as marked along the medial shell (error bars = SEM).


A Eating
Muscimol Elicits Eating
Dopamine Blockade Leaves Eating Intact
% of Vehicle Eating
2600
2100
1600
1100
600
100
2.4 2.0 1.6 1.2 0.8 0.4
Distance anterior to bregma (mm)
Enhancement
>500%
>350%
>200%
< 75%
< 50%
< 25%
Suppression
Distance below skull (mm)
6.4
6.8
7.2
7.6
8.0
8.4
B Fearful treading
Muscimol Elicits Defensive Treading
Dopamine Blockade Leaves Treading Intact
% of Vehicle Treading
12600
10100
7600
5100
2600
100
Enhancement
> 7500%
> 5000%
> 2500%
> 1000%
C Fearful reactions
Muscimol Elicits Calls, Escape and Bite Attempts
Dopamine Blockade Leaves Fearful Reactions Intact
% of Rats
100
80
60
40
20
0
Distress Call Alone
Call or Escape Attempt
Call, Escape Attempt & Bite Attempt

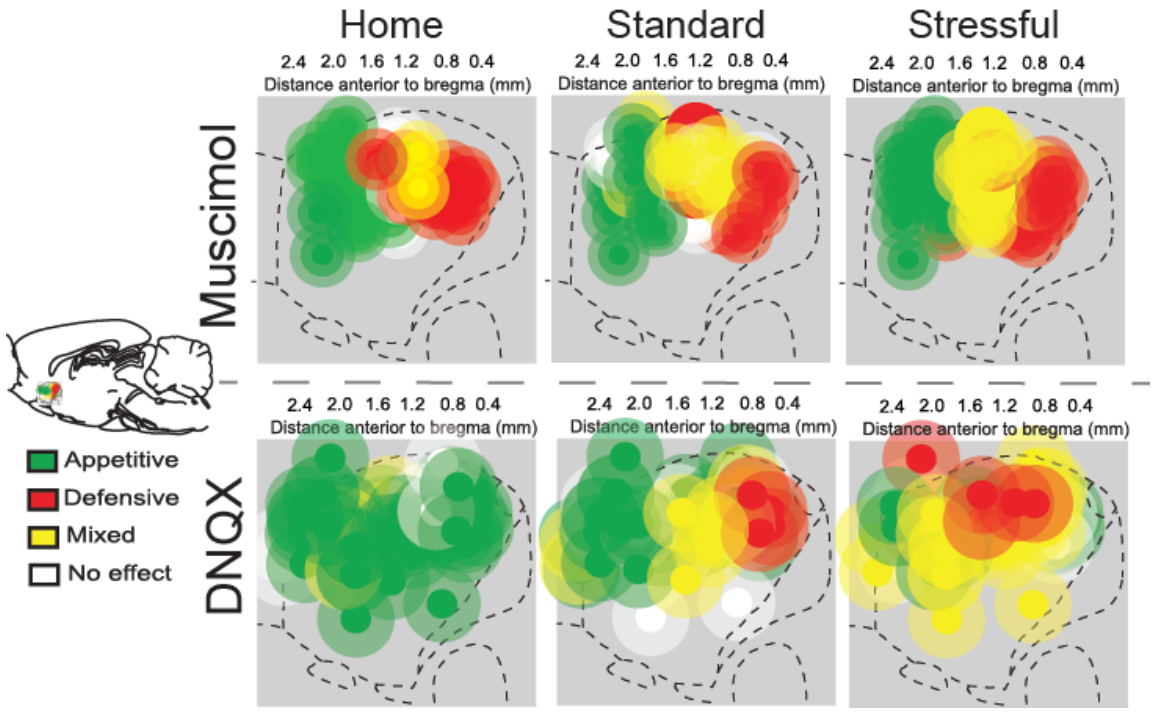


Figure 2.3. Summary maps of behavior and percent flipped by changes in ambience. Summary maps (A) show behavior produced by muscimol (top) or DNQX (bottom) in either the Home (left), Standard (middle) or Stressful (right) environments. Each subject (n=59) was designated as producing primarily appetitive (green symbols), defensive (red symbols) or mixed appetitive and defensive (yellow symbols) motivated behavior following DNQX microinjection. In a Standard environment, purely appetitive eating behavior and food intake (criteria for including a site was a >200% increase in eating) was primarily stimulated in rostral shell by DNQX and muscimol. Fearful distress calls, escape attempts and spontaneous emission of defensive treading-burying (criteria for including a site was a >500% increase in treading over vehicle levels, or emission of a defensive reaction to the experimenter) were primarily stimulated in caudal shell by DNQX. Mixed sites met criteria for both motivations. Testing in the Stressful environment shifted fearful behavior produced by DNQX into more rostral regions, whereas testing in the Home environment virtually eliminated all defensive behavior produced by DNQX. Muscimol produced a similar rostrocaudal gradient of eating and fear regardless of environment.

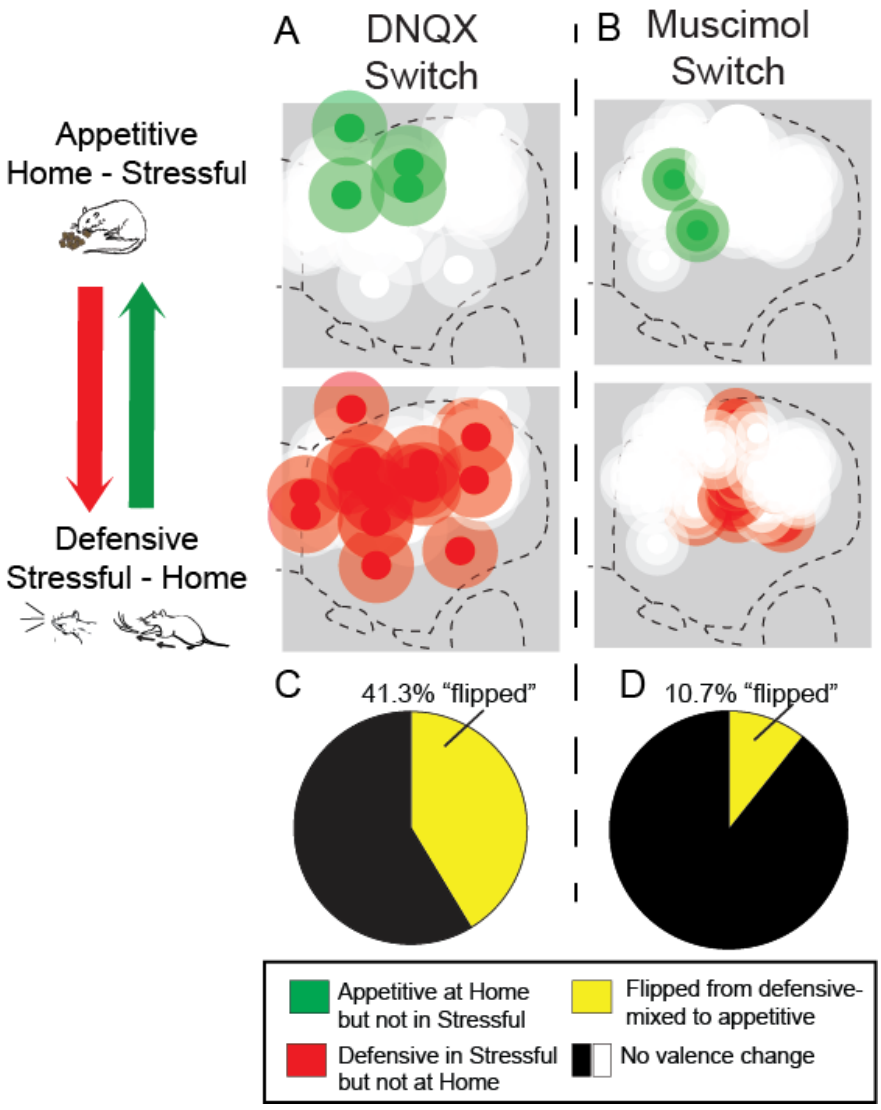


Figure 2.4. Maps of changes in motivational valence between the Home and Stressful environment. Summary maps show sites where DNQX (A) or muscimol (B) generated either intense eating behavior (top; green; > 200% of vehicle) in the Home environment but not in the Stress environment, or intense defensive behavior (bottom; red; > 500% of vehicle level treading or defensive reaction to the experimenter) in the Stressful environment but not in the Home environment. Sites mapped in white produced the same valence of behavior in both the Home and Stressful environment. Criteria for designating a site as appetitive was a >200% increase in eating, criteria for designating a site as defensive was a >500% increase in treading over vehicle levels, or emission of a defensive reaction to the experimenter. The percentage of rats that "flipped" between mainly defensive/mixed in the Stressful environment and purely appetitive in the Home environment was significantly greater in rats that received DNQX (C) than rats that received muscimol (D), and the number of rats who "flipped" when given muscimol was not significant.

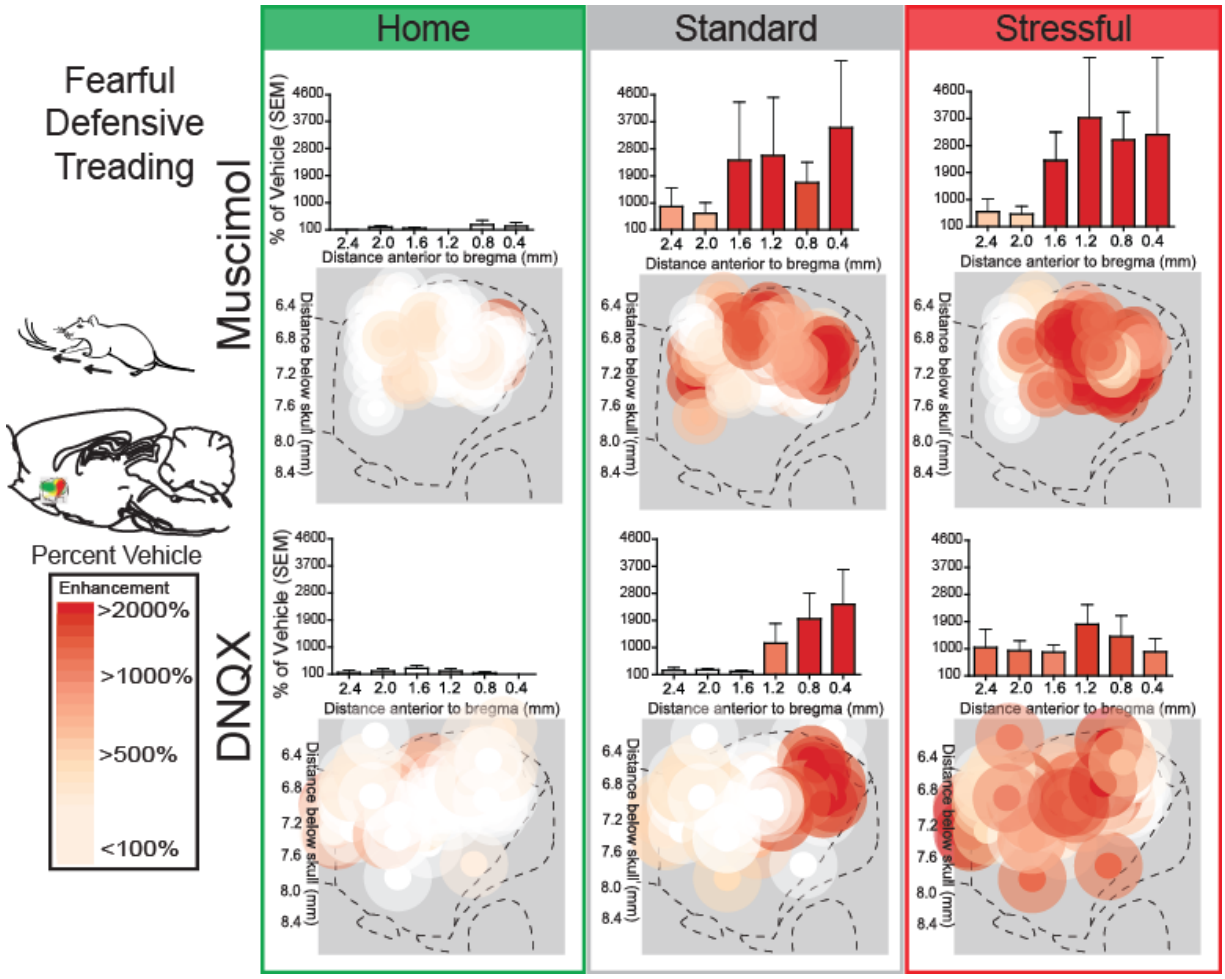


Figure 2.5. Effect of changing environmental ambience on defensive treading produced by DNQX or muscimol. Fos plume maps (n=59) of the generation of defensive treading by muscimol (top) or DNQX (bottom) in the Home (left), Standard (middle), or Stressful (right) environments. Testing in the Stressful environment had no effect on muscimol-generated treading, but the Home environment nearly eliminated muscimol-generated treading. This may be due to the removal of all visual cues that the animals usually tread toward. DNQX treading was produced at more rostral locations in a Stressful environment, but was nearly eliminated by testing in a Home environment. Histogram bars show treading as percent of vehicle at each rostrocaudal level as marked along the medial shell (error bars = SEM).

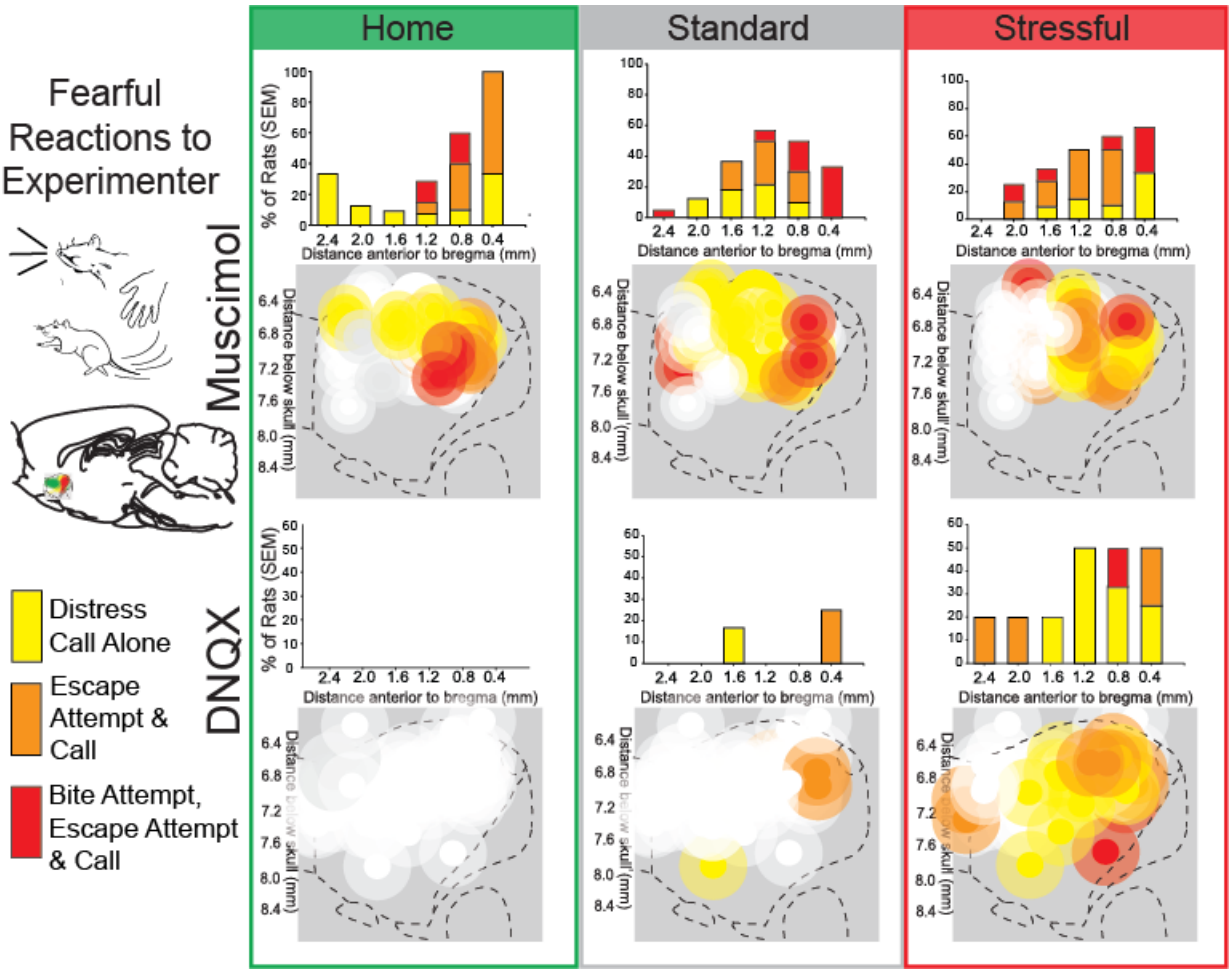


Figure 2.6. Effect of changing environmental ambience on defensive reactions to the experimenter produced by DNQX or muscimol. Fos plume maps (n=59) of the generation of defensive reactions to the experimenter by muscimol (top) or DNQX (bottom) in the Home (left), Standard (middle), or Stressful (right) environments. Changing environment between the familiar Home or the aversive Stressful environments had no effect on defensive reactions produced by muscimol, which were robustly generated regardless of environment ambience. In the Stressful environment, defensive reactions to the experimenter produced by DNQX treading were generated at more rostral locations in shell, but were nearly eliminated by testing in a Home environment. Histogram bars show percent of rats emitting distress calls (yellow), distress calls and escape attempts (red) and distress calls, escape attempts and bite attempts (red) at each rostrocaudal level as marked along the medial shell.

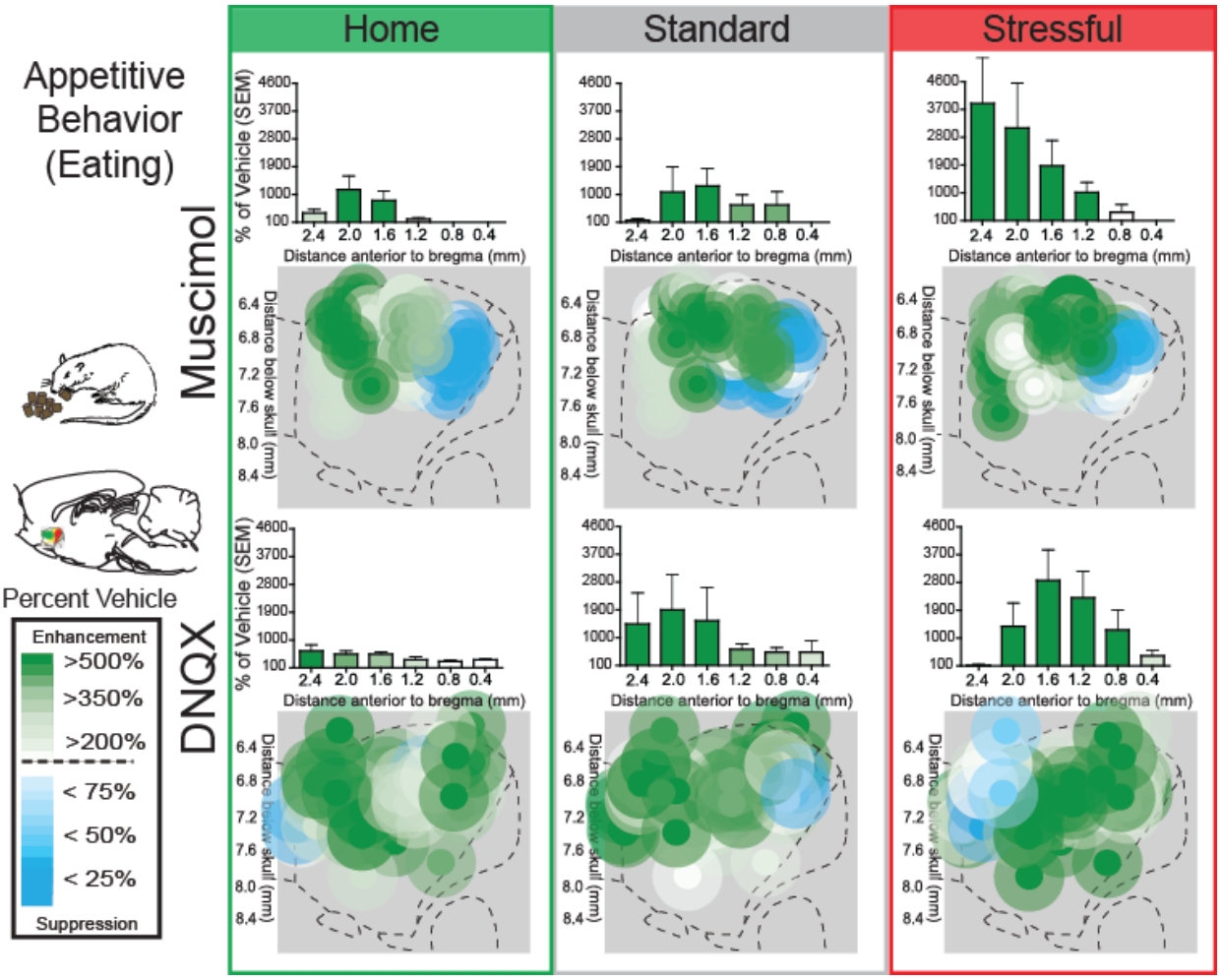


Figure 2.7. Effect of changing environmental ambience on eating produced by DNQX or muscimol. Fos plume maps (n=59) of the generation of eating by muscimol (top) or DNQX (bottom) in the Home (left), Standard (middle), or Stressful (right) environments. Changing environmental ambience had inconsistent effects on eating produced by DNQX or muscimol. Histogram bars show treading as percent of vehicle at each rostrocaudal level as marked along the medial shell (error bars = SEM).

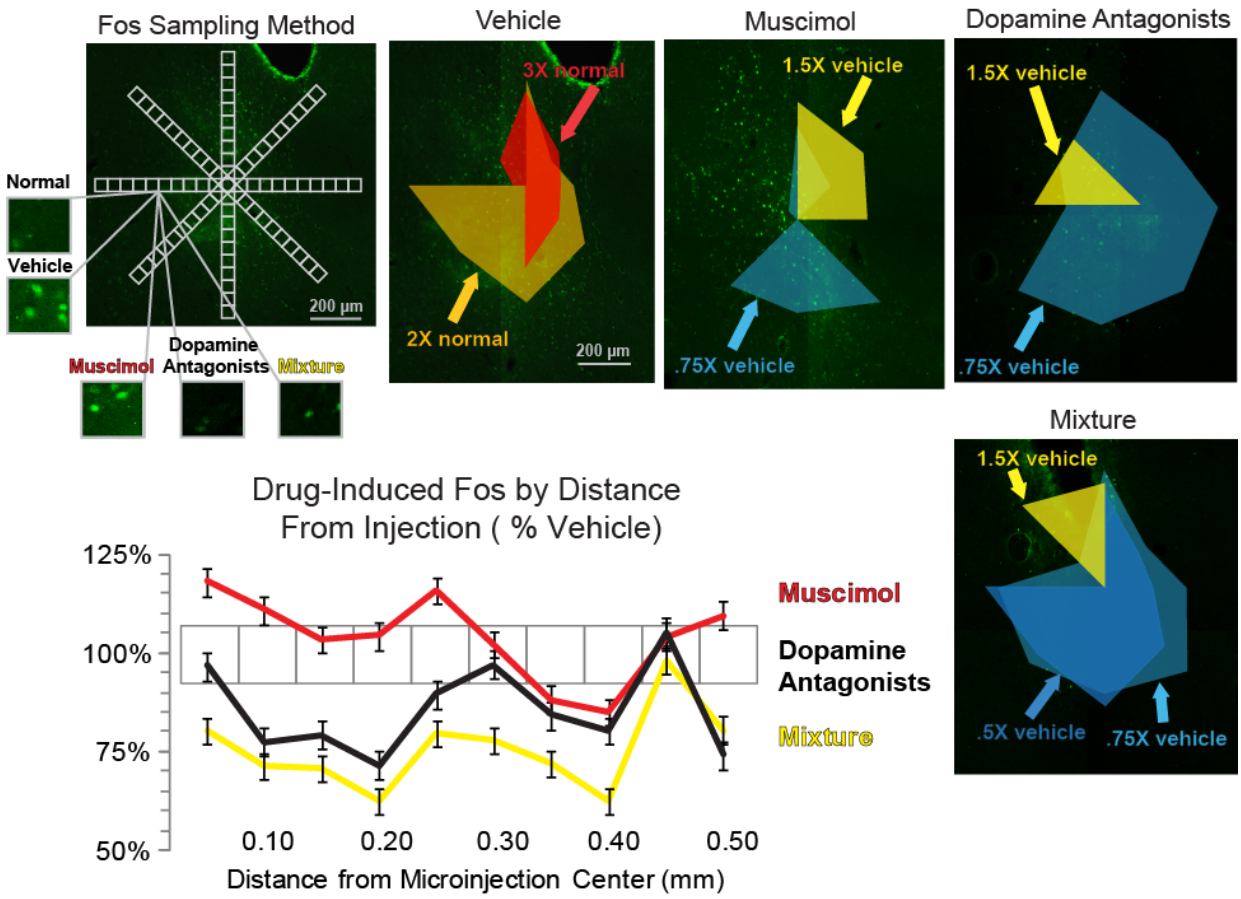


Figure 2.8. Fos plume analysis. Fos expression was assessed following microinjections of vehicle, muscimol, dopamine antagonists alone, or mixture of muscimol and dopamine antagonists. Fos labeled cells were individually counted within successive blocks (50 µm x 50 µm), along 8 radial arms emanating from the center of the site, with 10x magnification. For vehicle microinjections colors indicate levels of Fos expression of 3x (red), and 2x (orange) levels of Fos expression found in normal (uninjected) tissue. For drug microinjections, colors indicate levels of Fos expression of 1.5x (yellow), .75x (light blue), and .50x (darker blue) vehicle level Fos expression. Line graphs show that muscimol (red) reduced Fos expression approximately ~.35 mm away from the microinjection center, and that dopamine antagonists (black) and mixture (yellow) reduced Fos expression from .15 mm to .40 mm away from the microinjection center.

Chapter 3

Nucleus Accumbens Dopamine/Glutamate Interaction Switches Mode to Generate Desire versus Dread: D1 Alone for Appetitive Eating but D1 and D2 Together for Fear

Introduction

Intense aberrant motivation is an important feature of psychopathological disorders, ranging from intense appetitive motivation in addiction and binge eating to more fearful paranoia in schizophrenia and anxiety disorders (Barch, 2005; Kalivas and Volkow, 2005; Howes and Kapur, 2009; Woodward et al., 2011). Both appetitive and fearful motivations involve interactions between dopamine and glutamate in overlapping mesocorticolimbic circuits that converge on nucleus accumbens (NAc) (Kelley et al., 2005b; Faure et al., 2008; Meredith et al., 2008; Carlezon and Thomas, 2009; Kalivas et al., 2009; Humphries and Prescott, 2010).

NAc and dopamine-related circuits are best known for roles in appetitive motivation (Schultz, 2007; Wise, 2008), but are also implicated in some forms of aversive motivation related to fear, stress, disgust and pain (Levita et al., 2002; Salamone et al., 2005; Ventura et al., 2007; Matsumoto and Hikosaka, 2009; Zubieta and Stohler, 2009; Cabib and Puglisi-Allegra, 2012). Within medial shell of NAc, neuroanatomical coding plays an important role in determining appetitive versus fearful valence of intense motivations generated by glutamate disruptions. Local AMPA blockade (e.g., by DNQX microinjection) produces intense eating and/or fearful reactions in an anatomical keyboard pattern along a rostrocaudal gradient (Reynolds and Berridge, 2001, 2003; Faure et al., 2008; Reynolds and Berridge, 2008). At rostral

sites in medial shell, purely positive/appetitive behavior, such as intense eating, is produced by local glutamate disruptions (Maldonado-Irizarry et al., 1995; Kelley and Swanson, 1997). By contrast, as locations move caudally, disruptions generate progressively more fearful behaviors, including reactive distress vocalizations and escape dashes in response to touch, and spontaneous actively fearful behaviors such as an anti-predator response of defensive treading/burying, in which rodents use rapid forepaw movements to toss sand or bedding at a threatening stimulus (e.g., rattlesnake) (Coss and Owings, 1978; Treit et al., 1981; Reynolds and Berridge, 2001, 2003; Faure et al., 2008; Reynolds and Berridge, 2008). At intermediate sites in NAc shell, glutamate disruptions generate a mixture of both behaviors, and the dominant valence can be flexibly flipped between positive and negative by changing environmental ambience between familiar and stressful (Reynolds and Berridge, 2008).

We previously reported that endogenous dopamine activity was required locally for glutamate disruptions in NAc shell to generate feeding or fear (Faure et al., 2008). What remains unknown are the relative roles of D1-like versus D2-like dopamine receptors and their associated direct versus indirect output circuits in DNQX-generated motivations. Here we addressed these roles, and found that only D1 receptor stimulation, potentially involving the direct pathway to ventral tegmentum, was needed for glutamatergic disruptions to generate appetitive eating at rostral sites. In contrast, endogenous activity at both D1 and D2 receptors, potentially recruiting a stronger role of the indirect pathway to ventral pallidum and lateral hypothalamus, was needed for DNQX to generate fearful behavior at caudal sites. Further, we found that motivational valence trumped rostrocaudal location at flexible intermediate sites, which switched reversibly between an appetitive mode that required only D1 neurotransmission and a fearful mode that required simultaneous D1 and D2 neurotransmission.

Methods

Subjects

Male Sprague-Dawley rats (total n = 87; feeding and fear test groups, n = 51; Fos plume groups, n = 36), weighing 300 – 400 grams at surgery, were housed at ~21°C on a reverse 12:12 light:dark cycle. All rats had *ad libitum* access to both food and water. All of the following experimental procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Cranial cannulation surgery

Rats were anesthetized with intraperitoneal injections of ketamine hydrochloride (80 mg/kg) and xylazine (5 mg/kg), and treated with atropine (0.05 mg/kg) to prevent respiratory distress, and then placed in a stereotaxic apparatus (David Kopf Instruments). The incisor bar was set at 5.0 mm above intra-aural zero, angling cannula trajectory so as to avoid penetrating the lateral ventricles. Under surgical anesthesia, rats (n=87) received bilateral implantation of permanent cranial cannulae (14 mm, 23 gauge stainless-steel) aimed at staggered points throughout the rostrocaudal extent of medial shell of NAc. Cannulae were bilaterally inserted at coordinates between anteroposterior (AP) +2.4 to +3.1, mediolateral (ML) +/- .9 to 1.0 mm, and dorsoventral (DV) -5.6 to 5.7 mm from bregma. Cannulae were anchored to the skull using surgical screws and dental acrylic. Stainless steel obturators (28 gauge) were inserted in cannulae to avoid occlusion. After surgery, each rat received subcutaneous injection of chloramphenical sodium succinate (60 mg/kg) to prevent infection and carprofen (5 mg/kg) for pain relief. Rats received carprofen again 24 hrs later, and were allowed to recover for at least 7 days before testing began.

Drugs and intracerebral microinjections

Localized glutamate disruptions in medial shell were induced before behavioral tests by bilateral microinjections of DNQX, an AMPA/kainate receptor glutamate antagonist (6,7-dinotroquinoxaline-2,3(1H,4H)-dione; Sigma, St. Louis, MO) at a dose of 450 ng/ 0.5 µl per side. Either DNQX or vehicle (0.5 µl per side) was microinjected alone, or in combination with a) the selective D1 antagonist SCH23390 (*R*(+)-7-chloro-8-hydroxy-3-methyl1-phenyl-2,3,4,5,-tetrahydro-1H-3-benzazepine, Sigma) at a dose of 3 µg/ 0.5 µl per side; or b) the selective D2 antagonist raclopride (3,5-dichloro-N-{[(2S)-1-ethylpyrrolidin-2-yl]methyl}-2-hydroxy-6-methoxybenzamide) at a dose of 5 µg/ 0.5 µl per side, or c) both SCH23390 and raclopride. Drug doses were chosen based on Faure et al. (2008) and Reynolds and Berridge (2003). All drugs were dissolved in a vehicle of 50% DMSO mixed with 50% 0.15 M saline, and microinjected at a volume of 0.5 µl per side. The pH was normalized to 7.0 to 7.4 using HCl for both drug and vehicle microinjections. On test days, solutions were brought to room temperature (~21°C), inspected to confirm the absence of precipitation, and bilaterally infused at a speed of 0.3 µl/minute by syringe pump via PE-20 tubing through stainless-steel injectors (16 mm, 29 gauge) extending 2 mm beyond the guide cannulae to reach NAc targets. Injectors were left in place for 1 minute following microinjection to allow drug diffusion, after which obturators were replaced and rats were immediately placed in the testing chamber.

Glutamate/dopamine interaction group: Each rat tested for motivated behavior (n=23) received the following 5 drug microinjections on different days, spaced 48 hours apart, in counter-balanced order: 1) vehicle alone, 2) DNQX alone (in order to elicit motivated behavior), 3) a mixture of DNQX plus SCH23390 (D1 blockade), 4) DNQX plus raclopride (D2 blockade),

and 5) DNQX plus both SCH23390 and raclopride (combined dopamine blockade) (Faure et al., 2008).

Independent dopamine blockade group: A separate group of rats (n=18) was tested for motivated behavior after receiving microinjections of dopamine antagonists alone (without DNQX), or DNQX alone, or vehicle to ensure that dopamine antagonists in NAc shell did not prevent DNQX from generating motivations by simply eliminating motoric capacity or normal motivated behavior. Use of different groups ensured that the number of microinjections any rat received was limited to 5 or 6. This dopamine antagonist group received the following 5 drug conditions: 1) vehicle, 2) SCH23390 alone, 3) raclopride alone, 4) SCH23390 plus raclopride, and 5) DNQX alone (as a positive contrast to confirm that motivated behaviors could be generated at high intensities in these rats). All drug conditions were administered in counterbalanced order within each group and tests were spaced at least 48 hours apart.

Environmental shift group: A separate environment-shift group (n=10) was used to assess whether changing the environmental ambience flexibly altered the mode of dopamine-glutamate interactions at a particular site within the intermediate two-thirds of medial shell that is capable of generating both appetitive and fearful motivations (Reynolds and Berridge, 2008). Rats in this group had microinjection cannulae aimed at intermediate rostral-caudal sites. Each rat was tested on different days in two environments: comfortable and familiar "Home" versus overstimulating and "Stressful" (described below) in counterbalanced order. Rats were tested in each environment three times, also in counterbalanced in order, after microinjections of either: 1) vehicle, 2) DNQX, or 3) DNQX plus raclopride. Thus each rat received 6 test conditions; all separated at least 48 hrs apart in balanced order.

Behavioral tests of spontaneous motivated behaviors

Following 3 days of handling, all rats tested for motivated behavior (n = 51) were habituated to the testing procedure and apparatus on 4 days for 1 hour each. On the 4th day of habituation, rats received mock microinjections of vehicle prior to entering the test chamber, in order to habituate them to the microinjection procedure. On each test day, rats received one of the drug conditions described previously and were placed immediately in the transparent testing chamber (23 x 20 x 45 cm) which contained pre-weighed food (~20g rat chow) and *ad libitum* water, to allow the expression of appetitive behavior. The chamber also contained granular cob bedding spread on the floor ~3 cm deep to allow the expression of defensive treading behavior. Behavior in the chamber was videorecorded for 60 minutes, to be scored later offline for analysis. At the end of each session, rats were removed by the experimenter's gloved hand using a standardized slow-approach hand motion in order to quantify any fearful distress calls, escape attempts or defensive bites elicited by human touch. Following a ~5 second approach towards the testing cage, the experimenter slowly reached towards the rat, taking ~2 seconds. Upon contact, the experimenter lightly brushed the side of the rat with gloved fingertips, taking ~1 sec, before lifting the rat from the chamber in a gentle movement that lasted ~2 sec. The observer recorded any attempts by the rat to escape when touched, as well as bites and audible distress vocalizations.

All behavioral tests for the above groups (n = 41) were conducted in a "Standard" lab environment (Reynolds and Berridge, 2008), following a brief transport from the Home room. The Standard environment was intended to be similar to most behavioral neuroscience laboratories in lighting, sounds, and odors, and to be of relatively neutral ambience (in between positive Home and negative Stressful of the next experiment). This Standard environment

consisted of a conventional laboratory testing room (daylight illumination conditions of white fluorescent light intensity 550-650 lux, ambient noise sound intensity 65 – 70 decibels) as described previously (Reynolds and Berridge, 2008).

Rats in the environmental shift group were tested in 2 environments of opposite extreme valence: 1) the "Home" environment, which consisted of normal dim red lighting (5-10 lux) and quiet levels of ambient noise (65-70 decibels, primarily rat noise and static noise from ventilation systems), as well as familiar odors and sights of the rat's own home-room; versus 2) the "Stressful" high-intensity sensory-stimulation environment, which was conducted in the standard laboratory except that additional incandescent lamps were directed at the test chamber (1000-1300 lux within the cage) and loud, unpredictable sound was presented continuously throughout the test (raucous rock music from the continuous full-album soundtrack of "Raw Power" by Iggy & The Stooges [1973; Iggy Pop reissue 1997]; 80-86 decibels). In preference tests, rats have been shown to prefer the Home environment over the Standard and to prefer the Standard lab environment over the Stressful (Reynolds and Berridge, 2008).

Behavioral coding

The incidence of elicited fearful distress vocalizations, escape dashes, and bite attempts directed at the experimenter's hand were scored when the rat was gently picked up at end of the test session (Reynolds and Berridge, 2003), after which total grams of chow pellets consumed were recorded. Behaviors emitted spontaneously and videotaped during the 1-hr test were subsequently scored by experimenters blind to treatment for the total cumulative duration (seconds) for each of the following: eating behavior (involving both appetitive approach and voluntary initiation of ingestion plus consummatory chewing and swallowing of food), drinking behaviors (licking from water spout), and fearful defensive treading/burying behavior (defined as

active spraying or pushing of bedding with rapid alternating thrusts of the forepaws, spatially directed generally towards the brightly lit front or corners of the cage). Additionally, the number of bouts of appetitive behaviors such as food carrying and food sniffs, as well as less-valenced behaviors such as rearing, cage crosses, and grooming behavior were also recorded.

Histology

Following behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital. Rats in which Fos plumes were measured were perfused and brains treated as described previously (Reynolds and Berridge, 2008). These included rats behaviorally tested in the environmental shift group (n=10; which therefore received a 7^{th} final drug or vehicle microinjection and behavioral test 90 minutes prior to perfusion) and a separate dedicated Fos group (n = 36; which were histologically assessed after just a single drug or vehicle microinjection into locations staggered throughout medial shell, administered under conditions identical to the first day of testing for behavioral rats). The purpose of the dedicated Fos group was to assess maximal local impact radius, and avoid danger of under-estimating plume size due to progressive necrosis/gliosis over a series of microinjections that might shrink a final plume. If shrinkage occurred in the behaviorally tested group, that in turn could give rise to overly precise estimates of localization of function in brain maps. This potential distortion of impact estimates by plume shrinkage was prevented in the dedicated group that received only one microinjection.

All rats used for Fos analysis were anesthetized and transcardially perfused 90 minutes after their final or sole bilateral microinjection of vehicle (n=10), DNQX alone (n=13), DNQX plus SCH23390 (n=6), DNQX plus raclopride (n=10), DNQX plus raclopride and SCH23390 (n=3) or no solution (normal, n=3). Brain slices were processed for Fos-like immunoreactivity using NDS, goat anti-cfos (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat

Alexa Fluor 488 (Invitrogen, Carlsbad, CA) (Faure et al., 2008; Reynolds and Berridge, 2008). Sections were mounted, air-dried and coverslipped with ProLong Gold antifade reagent (Invitrogen). Zones where the expression of fluorescent Fos was elevated in neurons surrounding microinjection sites ("Fos plumes") were assessed via microscope as described previously (Reynolds and Berridge, 2008).

Other brains were removed and fixed in 10% paraformaldehyde for 1-2 days and in 25% sucrose solution (0.1 M NaPB) for 3 days. For assessment of microinjection site locations in behaviorally tested rats, brains were sliced at 60 microns on a freezing microtome, mounted, air-dried and stained with cresyl violet for verification of microinjection sites. Bilateral microinjection sites for each rats were placed on coronal slices from a rat brain atlas (Paxinos and Watson, 2007), which were used to extrapolate the position of each site on one sagittal slice. Mapping in the sagittal view allows for the presentation on the same map of the entire rostrocaudal and dorsoventral extents of NAc medial shell. Functional effects on appetitive and fearful behaviors were mapped using color-coding to express the intensity of changes in motivated behaviors for individual behaviorally-tested rats. Symbols were sized to match the maximal diameter of Fos plumes measured as described below. Sites were classified as rostral shell if their NAc placements were located +1.4 to +2.6 mm ahead of bregma, and as caudal shell if their placements were located +0.4 to +1.4 mm ahead of bregma.

Statistical analysis

The effects of DNQX on parametric behaviors were assessed using a three-factor mixed within- and between-subject ANOVA (drug x group [glutamate/dopamine interaction versus independent dopamine blockade] x anatomical level [rostral versus caudal]) to verify elicitation of eating and defensive behavior along a rostrocaudal gradient. The effects of antagonism at D1-

and D2-like receptors on DNQX-induced behavior was assessed using an additional two-factor mixed within- and between subject ANOVA to compare with behavior on DNQX-alone (D1 antagonism x D2 antagonism). The effects of environmental modulation were assessed using a two-factor within-subject ANOVA (environment x drug). When significant effects were found, rats were split by anatomical location and additional analysis was done using a one-way ANOVA and pairwise comparisons using Sidak corrections for multiple comparisons. For nominal data, differences between drug conditions were assessed using McNemar's repeated-measures test.

Results

Local AMPA receptor blockade in medial shell elicits eating and defensive treading behavior in a rostrocaudal gradient

Localized glutamate disruptions in medial shell induced by microinjections of DNQX, an AMPA/kainate receptor glutamate antagonist, stimulated intense appetitive and/or fearful behaviors depending on placement along a rostrocaudal gradient as expected (Figure 3.1a). At rostral sites in medial shell, NAc glutamate disruptions generated robust elevations nearly 5-times over vehicle levels in amounts of eating behavior and food consumed during the 1-hr test (cumulative duration of eating: drug x site interaction, $F(1,32) = 10.0$, $p = .003$; food intake measured in grams consumed: drug x site interaction, $F(1,32) = 14.5$, $p = .001$, Figures 3.2a-b, 3.3a). Conversely, at caudal sites in medial shell, DNQX microinjections did not elevate food intake (and in some caudal rats actually suppressed eating and food intake below control vehicle levels; Figure 3.2a-b), but instead generated profound elevations in the incidence of fearful distress vocalizations (Figures 3.2d and 3.3c; 73% of rats after DNQX microinjection vs 0% after

vehicle, McNemar's test, p = .001) and of fearful escape attempts to human touch (Figures 3.2e, and 3.3c; 40% of rats after DNQX vs 0% after vehicle, McNemar's test, p = .031). Likewise, caudal DNQX microinjections generated nearly 10-fold increases in the spontaneous emission of defensive treading-burying behavior over vehicle control levels (Figures 3.2c and 3.3b; drug x site interaction in cumulative duration of treading, $F(1,32) = 6.9$, $p = .013$). Defensive treading typically was not diffuse or random, but rather was directionally focused on a particular target: usually towards the transparent front of the cage (beyond which objects and people in the room could be seen) and towards light-reflecting front corners of the transparent plastic chamber.

D1 dopamine receptor transmission alone needed for DNQX to generate appetitive behaviors at rostral sites

A novel finding here was that endogenous local dopamine stimulation was needed only at D1-like (D1, D5) receptors around the microinjection site in rostral shell for the generation of intense appetitive behavior by DNQX microinjections. Rostral D2-like receptors (D2, D3, D4) appeared essentially irrelevant to glutamate-related amplification of eating behavior and food intake (Figures 3.1-3.3). That is, when the dopamine D1-antagonist, SCH23390, was added to the rostral DNQX microinjection, the D1 blockade abolished the ability of DNQX to increase time spent eating or food intake, leaving eating behavior and intake at control levels seen after vehicle microinjections (Figures 3.2a-b and 3.3a, eating: SCH23390, $F(1,7) = 13.3$, $p = .008$; Figure 2b, grams intake: SCH23390, $F(1,7) = 11.1$, $p = .010$).

By contrast, combining the D2-like antagonist raclopride with DNQX microinjection for rostral sites failed to prevent or even impair the DNQX-enhancement of eating (cumulative duration; Figures 3.2a-b and 3.3a, raclopride, $F(1,8) < 1$, $p = .743$) or food intake (grams consumed; Figure 3.2b, raclopride, $F(1,8) < 1$, $p = .517$). Quite the opposite, at least at caudal

shell sites, adding the D2 antagonist allowed caudal DNQX to further increase time spent eating to even higher levels that were 245% above vehicle, or 156% above eating levels produced by DNQX alone (Figures 3.2a and 3.3a; DNQX stimulation of eating at caudal sites was usually low due to the rostrocaudal gradient: average of 566 sec +/- 101 sec on DNQX plus raclopride versus 362 sec on DNQX alone and 230 sec on vehicle; raclopride x DNQX, $F(1,10) = 6.0$, $p = 0.035$). A slight caveat to this additional enhancement is that adding the D2 antagonist did not actually boost the physical amount of food consumed for this group, even though it nearly doubled the proportion of time during the trial in which rats ate (Figure 3.2b, raclopride, $F(1,11) < 1$, $p = .930$). However, we note that raclopride did boost stimulation of food consumption as well as of eating behavior for caudal DNQX microinjections in a separate experiment tested below (in tests conducted in a more stressful environment).

As expected, combining both the D1 antagonist and the D2 antagonist together with DNQX completely prevented DNQX from enhancing eating (similar to D1 antagonist above), and kept levels of intake equivalent to vehicle baseline levels (Figure 3.2a-b; versus vehicle: grams intake, $F(1,7) < 1$, $p = .973$; eating, $F(1,7) = 1.1$, $p = .322$). However, the D1-D2 mixture of antagonists was no more effective than adding just the D1 antagonist alone to DNQX, which also completely prevented appetitive increases (Figure 3.2a; eating, SCH23390 plus raclopride versus SCH23390 alone, $F<1$, $p = 1.000$). In short, we conclude that only local endogenous D1 receptor neurotransmission is needed to enable glutamate disruptions in rostral sites of medial shell to stimulate appetitive behavior and food intake. By contrast, local D2 receptor neurotransmission is essentially irrelevant to rostral eating stimulation, being neither necessary nor even contributing additively in any detectable way (and possibly even inhibiting the

stimulation of eating at caudal sites, perhaps via generation of fearful reactions as described below that could compete with or suppress appetitive eating).

Ruling out general suppression of appetitive/fearful behavior by dopamine antagonists

Finally, the prevention of DNQX-induced increases in food intake or eating by D1 receptor blockade appeared to reflect a specific interaction of dopamine receptors with glutamate disruptions rather than a general independent suppression of eating motivation or capacity induced by dopamine blockade. Neither microinjections of the D1 antagonist by itself (without DNQX) nor of the D2 antagonist by itself (without DNQX) suppressed baseline levels of eating below control vehicle levels of about 1 gram of chow per session (eating: SCH23390, $F(1,14) = 1.9$, $p = .194$, 149 sec +/- 52 SEM on SCH23390 versus 166 sec +/- 54 SEM on vehicle; raclopride: $F(1,14) < 1$, $p = .389$, 227 sec +/- 56 SEM; grams intake: SCH23390, $F(1,14) < 1$, $p = .514$, 1.15 grams +/- .36 SEM on SCH23390 versus .94 grams +/- .23 SEM on vehicle; raclopride, $F(1,14) = 3.9$, $p = .068$, 1.82 grams +/- .42 SEM). Thus local dopamine blockade in NAc at these doses did not impair either normal levels of motivation to eat or the motor capacity for ingestive movements. Instead our results seem to reflect a specific role of D1 receptor dopamine signals in enabling local AMPA receptor glutamate disruptions in rostral shell to stimulate eating behavior to high levels.

Fearful behaviors elicited by local glutamate disruption depend on concurrent local D1 and D2 receptor stimulation from endogenous dopamine

By contrast, simultaneous endogenous signaling at both D1 and D2 receptors in caudal sites of medial shell appeared necessary for DNQX microinjection to generate intense fearful behaviors (Figures 3.1-3.3). Mixing either the D1 antagonist or the D2 antagonist with DNQX effectively prevented the production of any defensive treading at caudal sites, as well as the

generation of any distress calls or escape reactions to human touch that otherwise were potentiated by DNQX microinjections (Figures 3.2c-e, 3.3b-c; defensive treading: SCH23390, $F(1,10) = 7.1$, $p = 0.024$, raclopride, $F(1,10) = 5.4$, $p = 0.043$; escape attempts & jumps: DNQX alone: 40% of rats, DNQX plus SCH23390: 0%, $p = 0.031$ [compared to DNQX, McNemar's test], DNQX plus raclopride: 13%, $p = .219$; distress calls: DNQX alone: 73% of rats, DNQX plus SCH23390: 13% of rats, $p = .012$, DNQX plus raclopride: 20% of rats, $p = .008$). In short, all fearful behaviors remained at near-zero control levels when either dopamine antagonist was mixed with DNQX.

Ruling out general suppression by dopamine antagonist microinjections

Again, D1 and D2 receptor contributions to DNQX fear induction appeared to reflect a specific interaction of these dopamine receptors with the glutamate disruption in caudal shell, because giving microinjections of either or both dopamine antagonists in the absence of DNQX did not change defensive treading from vehicle baseline levels (treading: SCH23390, $F(1,14) < 1$, $p = .913$; raclopride, $F(1,14) < 1$, $p = .476$). However, it must be noted that vehicle levels of fearful behaviors were near zero already, raising the possibility that a floor effect could have obscured a general suppression of fearful behavior by dopamine blockade. Therefore we turn to other evidence, which also suggests that dopamine antagonist microinjections, either with DNQX or by themselves, did not generally prevent most behaviors. For example, grooming, a nonvalenced behavior that was emitted at substantial rates after vehicle, remained unsuppressed by local blockade of D1 or D2 receptors. Dopamine antagonists alone did not suppress spontaneous grooming (average of 9.33 +/- 1.35 bouts on vehicle versus 8.09 +/- 1.13 on SCH23390 and 8.40 +/- 1.22 on raclopride; $F<1$). Likewise, adding dopamine antagonists to DNQX did not suppress grooming behavior ($F<1$). Microinjections of the dopamine antagonists

alone did moderately suppress locomotion expressed as rears and cage crosses by about 50% from vehicle levels, though this suppression was nowhere near as strong as the abolition of DNQX-induced elevations of eating or fearful defensive treading described above (rears: SCH23390, $F(1,13) = 17.6$, $p = .001$, raclopride, $F(1,13) = 9.8$, $p = .008$; cage crosses: SCH23390, $F(1,13) = 19.3$, $p < .001$, raclopride, $F(1,13) = 13.1$, $p = .002$). Further, DNQX microinjections stimulated locomotion to double or triple vehicle levels, and adding SCH23390 or raclopride to the DNQX microinjection did not prevent that rise in cage crosses and rears (main effect of DNQX: cage crosses, $F(1,33) = 12.0$, $p = .002$; rears, $F(1,33) = 6.8$, $p = .014$; SCH23390: $F<1$ for rears and cage crosses; raclopride: cage crosses, $F(1,19) = 2.2$, $p = .154$; rears, $F(1,19) = 3.2$, $p = .091$). Thus general suppression effects of dopamine antagonists were either missing or minimal, and did not appear sufficient to account for the abolition of DNQX-stimulated motivated behaviors described above.

Local mode of dopamine-glutamate interaction switches flexibly as ambience reverses motivation valence

Environmental ambience flips motivational valence. As expected, for most sites in the intermediate two-thirds of medial shell (i.e., all sites between far rostral 20% and far caudal 20%), changing environmental ambience from dark, quiet and familiar (similar to rats' home-room) to stressfully bright and noisy (extra light and raucous music) reversed the valence of motivated behavior generated by DNQX microinjections (Reynolds and Berridge, 2008) (Figure 3.4). Rats emitted almost exclusively appetitive behavior in the Home environment after DNQX microinjections, but emitted substantial amounts of fearful behaviors as well when tested in the Stressful environment after DNQX at the same NAc sites. The familiar, low-stimulation and presumably comfortable conditions of the Home environment (which rats have been shown to

prefer to standard lab illumination condition; Reynolds and Berridge, 2008) caused the appetitive-stimulating zone within NAc to expand from rostral sites and invade caudal sites of medial shell as well, so that 90% of all medial shell locations generated intense eating behavior and food intake (greater than 200% of vehicle; Figure 3.4a). Concomitantly, the Home environment virtually eliminated DNQX-induction of fearful behaviors, such as distress vocalizations, escape attempts or defensive treading (Figure 3.4a-b; treading, DNQX, $F(1,7) = 3.5$, $p = .102$; drug x site interaction, $F(1,7) < 1$, $p = .476$). Consequently, the size of the fear-inducing zone severely shrank in the Home environment, leaving most mid-caudal sites unable to generate fearful reactions. Thus only one rat (which had the farthest caudal shell site) displayed more than 20 seconds of defensive treading in the Home environment, or emitted a distress vocalization when touched after the test (Figure 3.4b).

In contrast, the loud and bright Stressful environment (which rats avoid over lab conditions and quickly learn to turn off when given the opportunity; Reynolds and Berridge, 2008) expanded the caudal fear-inducing zone to include substantial mid-rostral areas of medial shell, and increased the levels of defensive treading stimulated by DNQX to over 600% the corresponding levels induced in the Home environment (Figure 3.4b; DNQX, $F(1,7) = 23.8$, $p = .002$; site x drug interaction, $F(1,7) < 1$, $p = .429$). Similarly, the Stressful environment increased the incidence of distress vocalizations generated after DNQX when the rats were touched by the experimenter at the end of the session by five-fold compared to the Home environment (Figure 3.4d; 50% of rats versus 10% at Home; McNemar's test, $p = .063$). Conversely, the Stressful environment eliminated pure appetitive sites in the mid rostrocaudal zone, converting them into either mixed valence or purely fearful sites (Figure 3.4c). The Stressful environment also reduced the intensity of appetitive behaviors induced by DNQX at

midrostral sites to approximately 50% of Home levels, even for sites that still generated any eating (average of 507 sec +/- 142 SEM in the Stressful Environment versus 879 sec +/- 87 SEM in the Home Environment; drug x environment interaction, eating, $F(1,7) = 6.0$, $p = .044$; food intake, $F(1,7) = 2.9$, $p = .013$).

Fearful mode requires D2 receptor involvement, but appetitive mode does not. The most important novel finding here was that D1/D2 receptor requirements for endogenous dopamine stimulation at a given site dynamically changed with environmental ambience shifts in a manner tied to motivational valence generated by DNQX at the moment rather than to rostrocaudal location per se. Each DNQX site had two modes: appetitive and fearful, depending on external ambience of the moment. The appetitive mode (i.e. DNQX-stimulation of eating induced by the dark, quiet and familiar Home environment) did not require D2 receptor activation to enhance eating, whereas the fearful mode (i.e. DNQX-stimulation of defensive treading behavior and distress vocalizations induced by the loud and bright Stressful environment) always required D2 receptor activation for every site to stimulate fear, regardless of rostrocaudal location (just as caudal sites had required D2 for DNQX generation of fear in the previous experiment) (Figure 3.4). Flips in valence mode, between appetitive and defensive, occurred for 90% of sites tested, which comprised nearly all possible intermediate rostrocaudal locations in medial shell. For the remaining 10% of sites ($n = 1$), DNQX microinjected into far caudal shell always generated fearful behaviors in both environments (and fearful behaviors were always eliminated by D2 blockade).

More specifically, adding the D2 antagonist to DNQX microinjection completely blocked distress calls and defensive treading behavior at all sites that otherwise generated fear after DNQX in the Stressful environment (Figure 3.4; rostral sites, raclopride, $F(1,4) = 19.9$, $p = .021$,

all rats, raclopride, $F(1,7) = 10.7$, $p = .022$, site x drug interaction, $F(1,7) < 1$, $p = .730$). However, the D2 antagonist never blocked or suppressed eating behavior (i.e., appetitive motivation) generated at the same sites by DNQX in the Home environment; in fact, adding the D2 antagonist actually enhanced the levels of eating behavior generated by DNQX in the Stressful environment to 463% of vehicle levels and 140% of levels on DNQX alone for the same sites (Figure 3.4c; average of 712 sec +/- 178 SEM on DNQX plus raclopride versus 507 sec on DNQX alone and 153 sec on vehicle). In the Stressful environment, D2 blockade magnified DNQX-stimulation of eating and increased grams of food consumed, regardless of rostrocaudal location (within the intermediate zone), confirming that local D2 neurotransmission is not only unnecessary for eating enhancement but actually can oppose the generation of intense eating by local AMPA receptor blockade in medial shell (eating, raclopride, $F(1,7) = 18.5$, $p = .008$; site x drug interaction, $F(1,7) < 1$, $p = .651$; food intake, raclopride, $F(1,7) = 5.6$, $p = .064$, site x drug interaction, $F(1,6) = 2.5$, $p = .163$). While in the Standard environment D2 blockade disinhibited DNQX-eating only in caudal shell (Figure 3.2a), the Stressful environment expanded the fear generating zone and likewise expanded the zone in which D2-blockade disinhibits DNQX-eating to include mid-rostral zones of medial shell (Figure 3.4c; eating, raclopride x environment x site interaction, $F(1,25) = 6.2$, $p = .020$).

Dopamine receptor roles flip reversibly between multiple transitions. In rats that displayed ambivalent (both) motivations in the Stressful environment (60% of rats), DNQX-induced eating peaked in the first 15 minutes, while defensive treading peaked later in the trial (30 – 45 minutes after the microinjection, Figure 3.5a). During the 20 minutes period of maximal overlap between appetitive and defensive behavior (minutes 10 – 30), most rats transitioned from appetitive to defensive only once (16%) or 2 to 6 times (50%). With relatively few transitions

during the hour, any single minute was likely to consist of pure rather than mixed motivated behaviors (Figure 3.5b), consistent with previous reports (Reynolds and Berridge, 2008). Dopamine D2 receptor blockade did not block eating behavior (which dominated in the first 20 minutes of the session), but effectively blocked defensive treading behavior (which dominated in the final 20 minutes).

However, two rats stood out as especially ambivalent, transitioning between appetitive and defensive behavior more than 25 times each within the hour after pure DNQX microinjections in the Stressful environment. This represented the closest approach to simultaneous display of opposite motivations that we observed. Even in these rats, however, D2 receptor blockade consistently blocked only defensive behavior emitted under the loud and bright conditions, and never appetitive behavior (in either Stressful or Home environments) (example rat, Figure 3.5c) which continued to occur at similar levels and time points after DNQX plus D2 antagonist microinjection as after pure DNQX in the corresponding environment. Thus motivated behavior produced by dopamine-glutamate interactions appeared to be able shift rapidly and repeatedly between appetitive and fearful modes. When environmental conditions fostered ambivalence in a susceptible individual, a site could flip valence modes more than 20 times in a single hour.

Fos plume analysis: defining size of microinjection local impact

Localization of function was aided by assessing the extent of local impact of drug microinjections on nearby tissue, as reflected in Fos plumes around the microinjection center (Figure 3.1b). Rats used previously for behavioral testing in the environmental shift group were assessed for Fos plumes after the end of the experiment. However, as anticipated, we confirmed that rats that had already completed behavioral testing had shrunken Fos plumes compared to the

dedicated Fos group that received only a single microinjection, indicating that DNQX-induced plumes from rats that received 6 previous microinjections no longer represent the maximal impact radius of drug spread. DNQX produced plumes in the dedicated Fos group that were nearly 4 times larger in volume (nearly 2 times larger in radius) than in the previously behaviorally-tested group ($F(9,90) = 3.3$, $p < .002$). Therefore, when mapping functional drug spread in all figures, we relied on plume radius data from the dedicated Fos group (matched to initial behavioral test conditions) to avoid underestimation when assessing the maximal spread of local impact for microinjections, and to construct plume maps for localization of function. However, all other data besides plume radii shown in maps were obtained exclusively from the behaviorally-tested group (i.e., colors and bar graphs reflecting intensities of eating and fearful behaviors induced at particular sites).

Pure DNQX microinjections produced plume centers of double the intensity of vehicle-level Fos expression, in a small volume of 0.02 mm^3 for the dedicated Fos group (Figure 3.1b, top middle; radius = 0.18 +/- 0.04 mm SEM). Rats that had received 6 previous microinjections had an even smaller volume center of 0.004 mm^3 (radius = 0.1 mm). Surrounding plume centers, Fos expression in the maximal group had a larger halo of 0.23 mm^3 volume of milder elevation >1.5 times vehicle levels (radius = 0.38 +/- 0.05 mm SEM; rats previously tested 6 times had smaller outer halos of 0.05 mm^3 volume, radius = .23 mm). Addition of the D1 antagonist (SCH23390) shrank plumes and *attenuated* the intensity of DNQX-induced elevations in local Fos expression (Figure 3.1b, bottom middle; DNQX versus DNQX plus SCH23390, Post hoc pairwise comparison with Sidak corrections, $p < 0.01$). SCH23390 shrank the total volume of DNQX Fos plumes to less than 0.18mm^3 (outer halo radius = 0.35 +/- 0.05 mm SEM). By contrast, addition of the D2 antagonist (raclopride) expanded intense centers of Fos expression

and *enhanced* DNQX-induced elevation in local Fos expression (Figure 3.1b, bottom left; DNQX versus DNQX plus raclopride, Post hoc pairwise comparisons with Sidak corrections, $p < 0.05$). Raclopride expanded the inner center of doubled Fos expression produced by DNQX to a volume of 0.15 mm^3 (radius = .33 +/- 0.042 mm SEM), and left unchanged the radius and intensity of the outer plume halo (of 1.5x expression). We note that the D1 antagonist apparently predominates over the D2 antagonist in effects on local Fos when both are microinjected jointly with DNQX, as DNQX Fos plumes shrink following the addition of combined D1 and D2 antagonists (Faure et al., 2008).

Discussion

In rostral shell, only endogenous dopamine signaling at D1-like receptors was needed for DNQX microinjections to stimulate 5-fold increases in eating. By contrast, in caudal shell, simultaneous signaling at D1- and D2-like receptors was needed for DNQX to generate 10-times increases in fearful reactions (distress calls, escape attempts and active defensive treading directed at objects in cage or beyond). Yet, rostral sites in medial shell were not simply D1 dominant nor were caudal sites D1-D2 co-dominant for generation of motivations by glutamate disruptions. Most intermediate sites in shell switched flexibly between generating appetitive and fearful motivations when environmental ambience changed. For those sites, D2 activity was always required for fear generation by DNQX microinjection (in the stressful environment) but never required for appetitive generation of eating (in the familiar home environment). Not only was D2 signaling unnecessary, D2 receptor blockade actually disinhibited DNQX-stimulation of eating at sites when placement/environment combination otherwise facilitated fear. In short, rostrocaudal placement strongly biases the valence of motivational salience produced by

glutamatergic disruptions, but dopamine interaction modes are more closely tied to appetitive/fearful valence generated at a given moment than to location per se (Reynolds and Berridge, 2008).

Mechanism of interaction between dopamine and glutamate blockade

The precise mechanism of NAc dopamine-glutamate interaction in generating intense incentive salience versus fearful salience remains a puzzle. Purely speculatively, we offer several possibilities. In the absence of glutamatergic input during AMPA blockade, NAc neurons reduce already low rates of firing, become hyperpolarized, and possibly disinhibit downstream targets in ventral pallidum (VP), lateral hypothalamus (LH) and ventral tegmentum (VTA) to stimulate motivated behaviors (Taber and Fibiger, 1997; Kelley, 1999; Meredith et al., 2008; Roitman et al., 2008; Krause et al., 2010). However, if dopamine primarily modulates glutamatergic depolarizations (Calabresi et al., 1997) then dopamine might be viewed as largely irrelevant to such hyperpolarizations.

Still, one possibility is that D2 receptor activation attenuates remaining excitatory AMPA postsynaptic impact (Cepeda et al., 1993), and so D2 blockade might prevent AMPA attenuation, disrupting local hyperpolarizations. Alternatively, D1-receptor activation may facilitate hyperpolarization in relatively inhibited neurons (Higashi et al., 1989; Pennartz et al., 1992; Moyer et al., 2007; Surmeier et al., 2007), and so D1 blockade might likewise disrupt those hyperpolarizations. Presynaptic mechanisms might also contribute, based on potential suppression of glutamate release by NAc D1 receptor activation on hippocampal or amygdala terminals, and similar presynaptic D2 suppression at prefrontal terminals (Pennartz et al., 1992; Nicola et al., 1996; Charara and Grace, 2003; Bamford et al., 2004). Presynaptic dopamine

blockade might disrupt such suppressions, and consequently increase glutamate release, potentially overcoming DNQX effects.

A remaining class of explanation could involve more subtle dopamine/glutamate interaction. For example, DNQX microinjections might shift AMPA/NMDA activation ratios towards NMDA, potentially relevant if NMDA receptors provide current contributions in the absence of AMPA currents (Cull-Candy and Leszkiewicz, 2004; Hull et al., 2009). Additionally, DNQX-induced local hyperpolarization may, via GABAergic connections between neighbors, laterally disinhibit surrounding neurons (Mao and Massaquoi, 2007; Faure et al., 2008 ; Tepper et al., 2008). Dopamine blockade could counteract both of these effects by disrupting both NMDA-mediated currents (Cepeda et al., 1993; Surmeier et al., 2007; Sun et al., 2008) and lateral inhibition (Taverna et al., 2005; Grace et al., 2007; Moyer et al., 2007; Nicola, 2007). The actual roles of these or other mechanisms in generating these phenomena will need future clarification**.**

Direct and indirect output pathways in D1 and D2 dependent motivation

Direct and indirect pathways from shell may differentially contribute to incentive versus aversive motivation (Hikida et al., 2010). In general for striatum, D2-expressing outputs travel chiefly via the indirect pathway, and D1-expressing outputs travel via the direct pathway (Gerfen and Young, 1988; Gerfen et al., 1990; Bertran-Gonzalez et al., 2008; Matamales et al., 2009). For NAc medial shell in particular, D1-expressing neurons similarly constitute the direct output pathway to VTA, whereas equal populations of D1 and D2-dominant neurons project along the indirect pathway to VP and LH (Figure 3.6) (Haber et al., 1985; Heimer et al., 1991; Lu et al., 1998; Zhou et al., 2003; Humphries and Prescott, 2010). Additionally, 15% - 30% of shell neurons, likely projecting along the indirect pathway, co-express both D1 and D2 receptors,

which sometimes form a conjoined heteromer (Humphries and Prescott, 2010; Perreault et al., 2010; Perreault et al., 2011). Speculatively, the importance of D1 receptors in enabling glutamate disruptions to generate appetitive behavior might reflect a primacy of the direct pathway from NAc to VTA. In contrast, the need for D1 and D2 co-activation for DNQX-fear generation might highlight a greater contribution of the indirect pathway.

Valence mode shifts and rostrocaudal biases: Mesocorticolimbic circuits

Shifts between familiar and stressful environmental ambience modulate mesocorticolimbic circuits, likely altering glutamatergic inputs to NAc from prefrontal cortex, basolateral amygdala (BLA), hippocampus and thalamus (Swanson, 2005; Zahm, 2006; Belujon and Grace, 2008), which may interact with D1/D2 dopamine signals. For example, after theta burst firing from the BLA, rostral shell neurons can show decreased responsiveness to subsequent BLA stimulations, whereas neurons in caudal shell are more likely to increase subsequent firing to the same BLA stimulations, a difference which requires D2 receptors and which might modulate the size of appetitive vs. fear-generation zones within medial shell (Gill and Grace, 2011). Particular features of mesocorticolimbic inputs may also be important for the shell's intrinsic rostrocaudal gradient. For instance, norepinephrine from hindbrain is released chiefly in caudal regions of shell, facilitated by dopamine D1 stimulation but inhibited by D2, and might help modulate motivation valence (Berridge et al., 1997; Delfs et al., 1998; Vanderschuren et al., 1999; Schroeter et al., 2000; Park et al., 2010). Finally, point-to-point corticolimbic targeting from prefrontal cortex zones to subregions of medial shell, VP/LH and their downstream targets, permit multiple *segregated* loops to travel through mesocorticolimbic circuits (Thompson and Swanson, 2010), which could further contribute to localization of desire and dread generators.

Caveats regarding D1 and D2 receptors in motivated behavior

We believe our findings do not necessarily conflict with others' reports of D2/D3 involvement in incentive motivation (Bachtell et al., 2005; Bari and Pierce, 2005; Xi et al., 2006; Heidbreder et al., 2007; Gardner, 2008; Khaled et al., 2010; Song et al., 2012). As caveat, we note our findings are strictly limited to mechanisms that simultaneously involve: a) glutamate-dopamine interactions, b) within NAc medial shell, that c) generate intense elevation of appetitive/fearful motivations. Although our conclusions are consistent with reports that D1 (but not D2) blockade in NAc shell prevents appetitive VTA-stimulated eating (MacDonald et al., 2004) and prevents appetitive self-stimulation via optogenetic activation of glutamatergic amygdala-NAc projections (Stuber et al., 2011), as well as reports that D2 signaling contributes to active defensive behaviors (Filibeck et al., 1988; Puglisi-Allegra and Cabib, 1988), our results do not preclude other roles for D2/D3 receptors in generating appetitive motivation in different situations. In particular, we do not contradict appetitive roles produced in different brain structures, involving different reactions (e.g., learned rather than unconditioned) or that involve deficits below normal levels of motivation. Understanding dopamine receptor roles in generating motivations will eventually require integration of all relevant facts.

GABA and metabotropic glutamate generation of motivated behavior

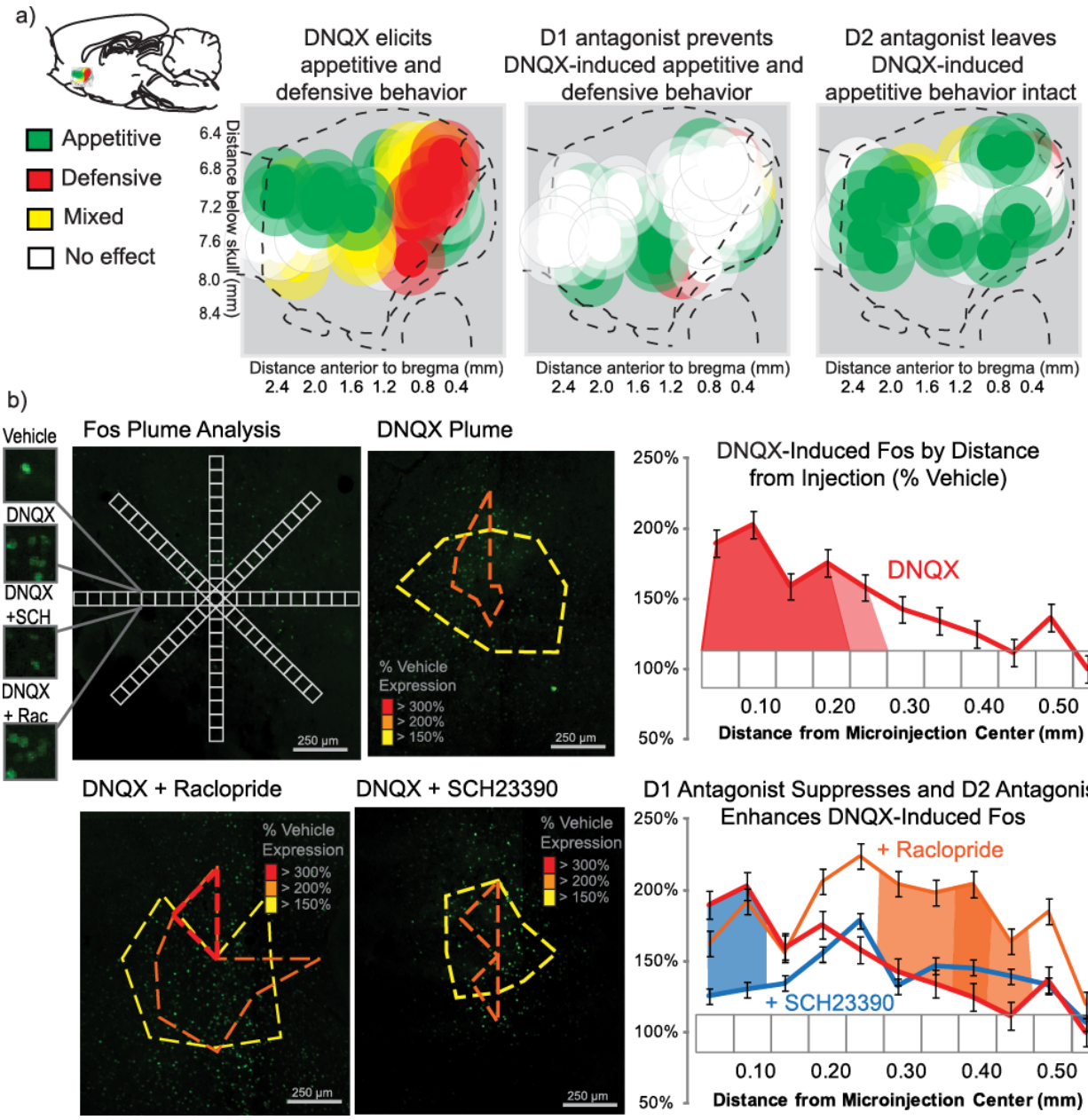
We suggest that rostral dopamine/glutamate interactions here generated positive incentive salience, making food perceived as more attractive to eat. By contrast, caudal or negatively-valenced interactions generated fearful salience, making objects and experimenter perceived as threatening. We previously reported metabotropic glutamate blockade at sites throughout medial shell to generate fear and disgust (Richard and Berridge, 2011a), and reported local GABAergic hyperpolarizations to generate rostrocaudal gradients of feeding and fear, similar to the keyboard

pattern described here (Reynolds and Berridge, 2001; Faure et al., 2010). However, we do not suggest that the dopamine interactions with ionotropic glutamatergic disruptions identified here necessarily apply to metabotropic or to GABAergic NAc mechanisms of motivation. There are several neuronal differences (e.g., direct GABAergic hyperpolarizations of neurons versus glutamate blockade-mediated hyperpolarization) and functional differences (e.g., shifts in hedonic impact versus induction of motivated behavior) that could prove important.

Implications for psychopathology

Corticolimbic dopamine-glutamate interactions have been linked to both intense incentive salience and fearful salience, contributing to appetitive motivation in addiction and to intense fearful motivation in psychotic paranoia (Wang and McGinty, 1999; Barch, 2005; Taylor et al., 2005; Lapish et al., 2006; Faure et al., 2008; Jensen et al., 2008; Kalivas et al., 2009). Flips in the valence of pathologically intense motivational salience also can occur (Morrow et al., 2011). Amphetamine addicts can experience fearful "amphetamine psychosis" similar to paranoia, which may involve pathological exaggerations of fearful salience (Featherstone et al., 2007; Jensen et al., 2008; Howes and Kapur, 2009). Conversely, some schizophrenic patients exhibit higher brain activations that encode appetitive *incentive* salience (Elman et al., 2006; Diaconescu et al., 2011). Overall, understanding how glutamate-dopamine interactions within NAc shell create intense appetitive and/or fearful motivations may illuminate the mechanisms underlying such intense but opposite disorders of motivation.

Figure 3.1. Summary maps of behavior and Fos plume analysis. Summary maps (A) show in sagittal section the motivated behaviors generated by microinjections in NAc medial shell of DNQX alone (left), DNQX plus SCH23390 (D1 antagonist, middle), or DNQX plus raclopride (D2 antagonist, right) in the Standard laboratory environment. Each subject (n=23) was designated as producing primarily appetitive (green symbols), fearful (red symbols) or mixed appetitive and fearful (yellow symbols) motivated behavior following DNQX microinjection. Purely appetitive eating behavior and food intake (criteria for including a site was a >200% increase in eating) was primarily stimulated in rostral shell by DNQX. Fearful distress calls, escape attempts and spontaneous emission of defensive treading-burying behavior directed toward specific targets (criteria for including a site was a >500% increase over vehicle levels) were primarily stimulated in caudal shell by DNQX. Intermediate sites in medial shell often met both appetitive and defensive criteria and were designated as generating 'mixed valence'. Combining the D1 antagonist in the DNQX microinjection prevented the elicitation of all appetitive (rostral) and fearful (caudal) behaviors. By contrast, combining the D2 antagonist in the DNQX microinjection only prevented the elicitation of defensive behavior, but left appetitive behavior intact. Maximal Fos plumes (B) were analyzed for each drug microinjection condition. Fos labeled cells were individually counted within successive blocks (50 µm x 50 µm), along 8 radial arms emanating from the center of the site, with 10x magnification. Colors indicate levels of Fos expression of 3x (red), 2x (orange) and 1.5x (yellow) vehicle level Fos expression. Line graphs show that DNQX (red) produced elevated Fos expression starting at the center of the microinjection to zones ~0.3mm away. DNQX-induced increases in Fos expression were suppressed by adding D1 antagonist (SCH23390, blue) but were enhanced by adding D2 antagonist (raclopride, orange) to the DNQX microinjection.


a)
Appetitive
Defensive
Mixed
No effect
DNQX elicits appetitive and defensive behavior
D1 antagonist prevents DNQX-induced appetitive and defensive behavior
D2 antagonist leaves DNQX-induced appetitive behavior intact
Distance below skull (mm)
6.4
6.8
7.2
7.6
8.0
8.4
Distance anterior to bregma (mm)
2.4 2.0 1.6 1.2 0.8 0.4
b)
Vehicle
DNQX
DNQX +SCH
DNQX + Rac
Fos Plume Analysis
DNQX Plume
% Vehicle Expression
> 300%
> 200%
> 150%
250 µm
DNQX-Induced Fos by Distance from Injection (% Vehicle)
250%
200%
150%
100%
50%
DNQX
0.10 0.20 0.30 0.40 0.50
Distance from Microinjection Center (mm)
DNQX + Raclopride
DNQX + SCH23390
D1 Antagonist Suppresses and D2 Antagonist Enhances DNQX-Induced Fos
+ Raclopride
+ SCH23390

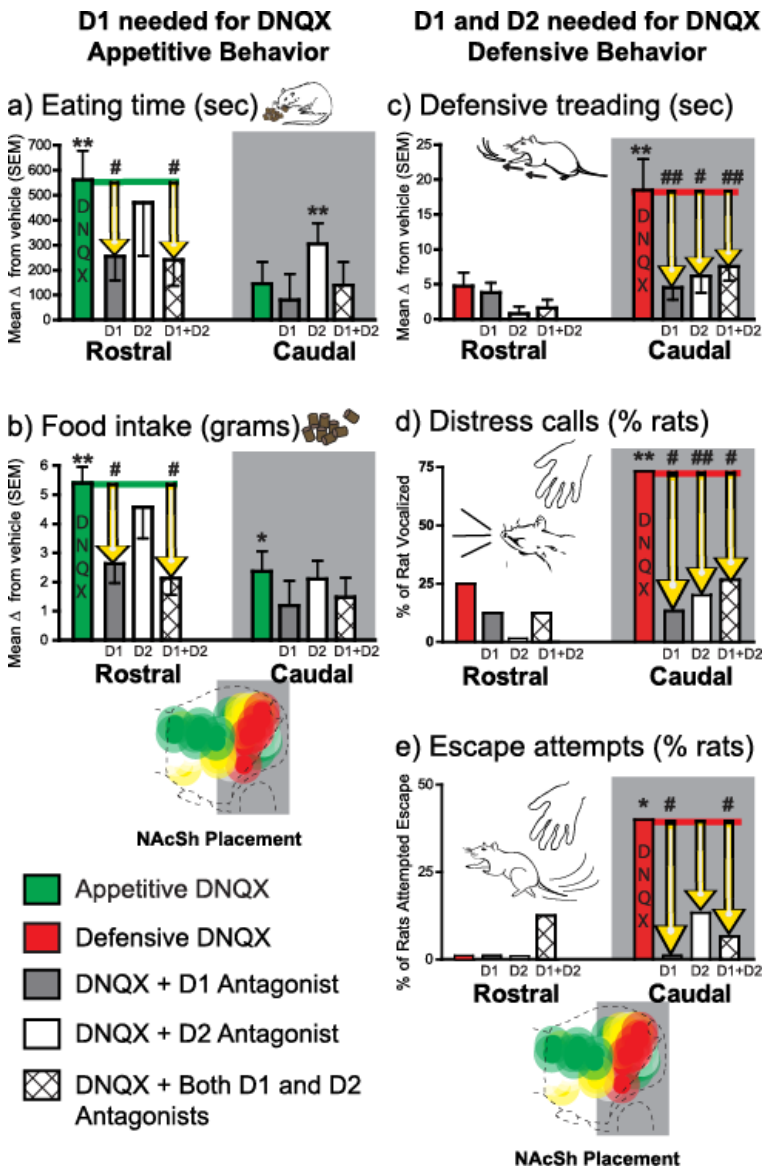


Figure 3.2. Motivated behavior summary graphs. Generation of increases in eating behavior (A), food intake (B), spontaneous defensive treading/burying behavior (C), incidence of distress vocalizations in response to human touch after the test (D), and of escape attempts in response to human touch (E). Effects are shown for microinjections of DNQX alone, DNQX plus SCH23390 (D1 antagonist), DNQX plus raclopride (D2 antagonist) and DNQX plus both D1 and D2 antagonists in rostral (n=9) and caudal (n=14) regions of medial NAc shell (relative to vehicle microinjections in the same rats). Data are presented as change from vehicle, errors bars indicate SEM, * $p < 0.05$, ** $p < 0.01$ change from vehicle, # $p < 0.05$, ## $p < 0.01$ change from DNQX, pairwise comparisons using Sidak corrections for multiple comparisons (eating, food intake and defensive treading) or McNemar's test (distress vocalizations and escape attempts).

Figure 3.3. Effects of D1 and D2 antagonism on DNQX-induced eating and defensive fearful behaviors. Fos plume maps (n=23) in sagittal plane of the generation in medial shell of eating (A), defensive treading (B), and fearful vocalizations and escape attempts (C) by DNQX (left), DNQX plus SCH23390 (D1 antagonist, middle), and DNQX plus raclopride (D2 antagonist, right). The D1 antagonist prevented DNQX from generating either eating or fear, while the D2 antagonist left DNQX-induced eating intact, but prevented DNQX-induced generation of fear. Histograms bars below the maps show behavior as percent of vehicle (eating, A; treading, B) or percent of subjects (vocalizations and escape attempts, C) for each behavior at rostrocaudal level as marked along the medial shell (error bars = SEM).

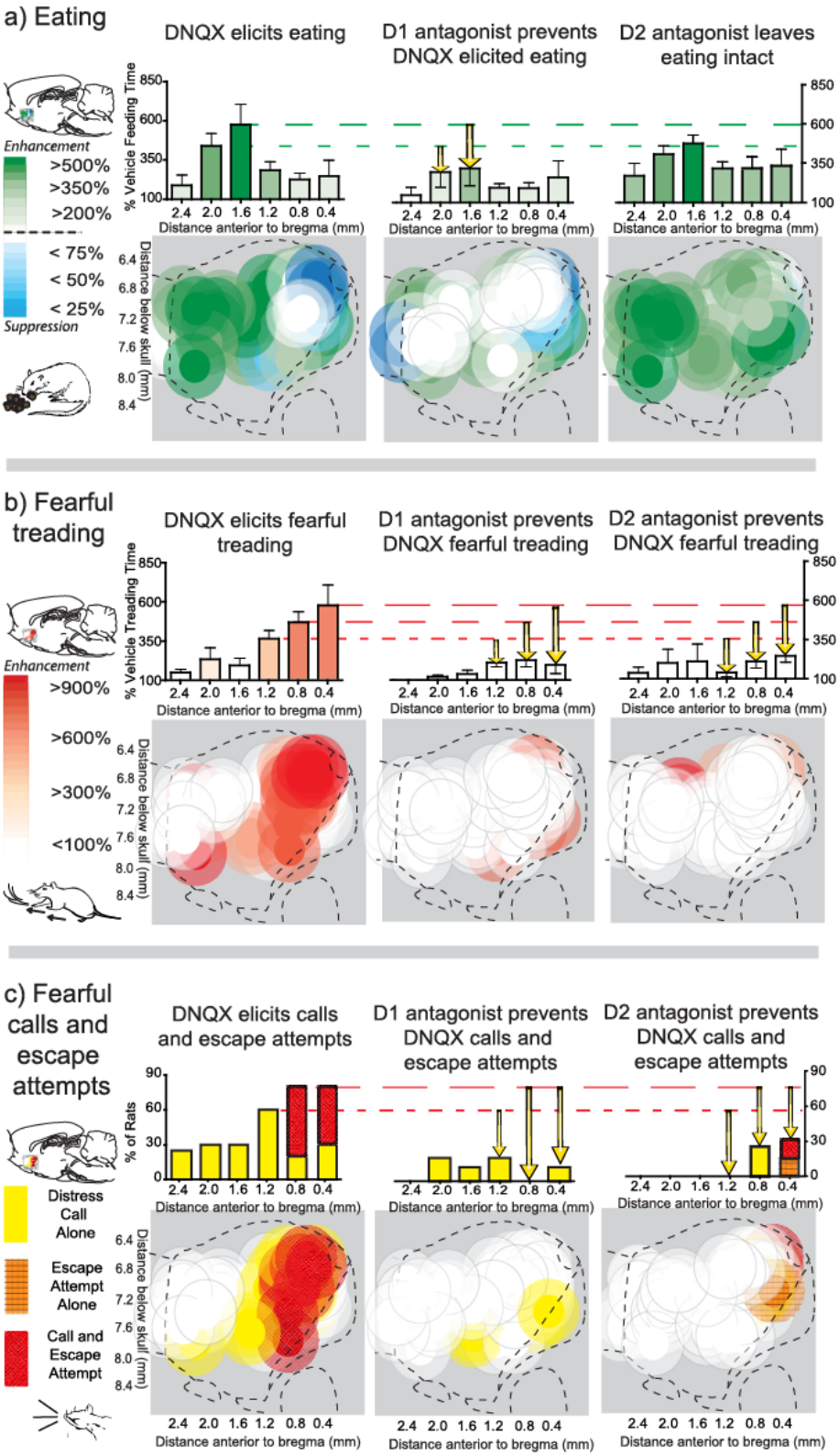
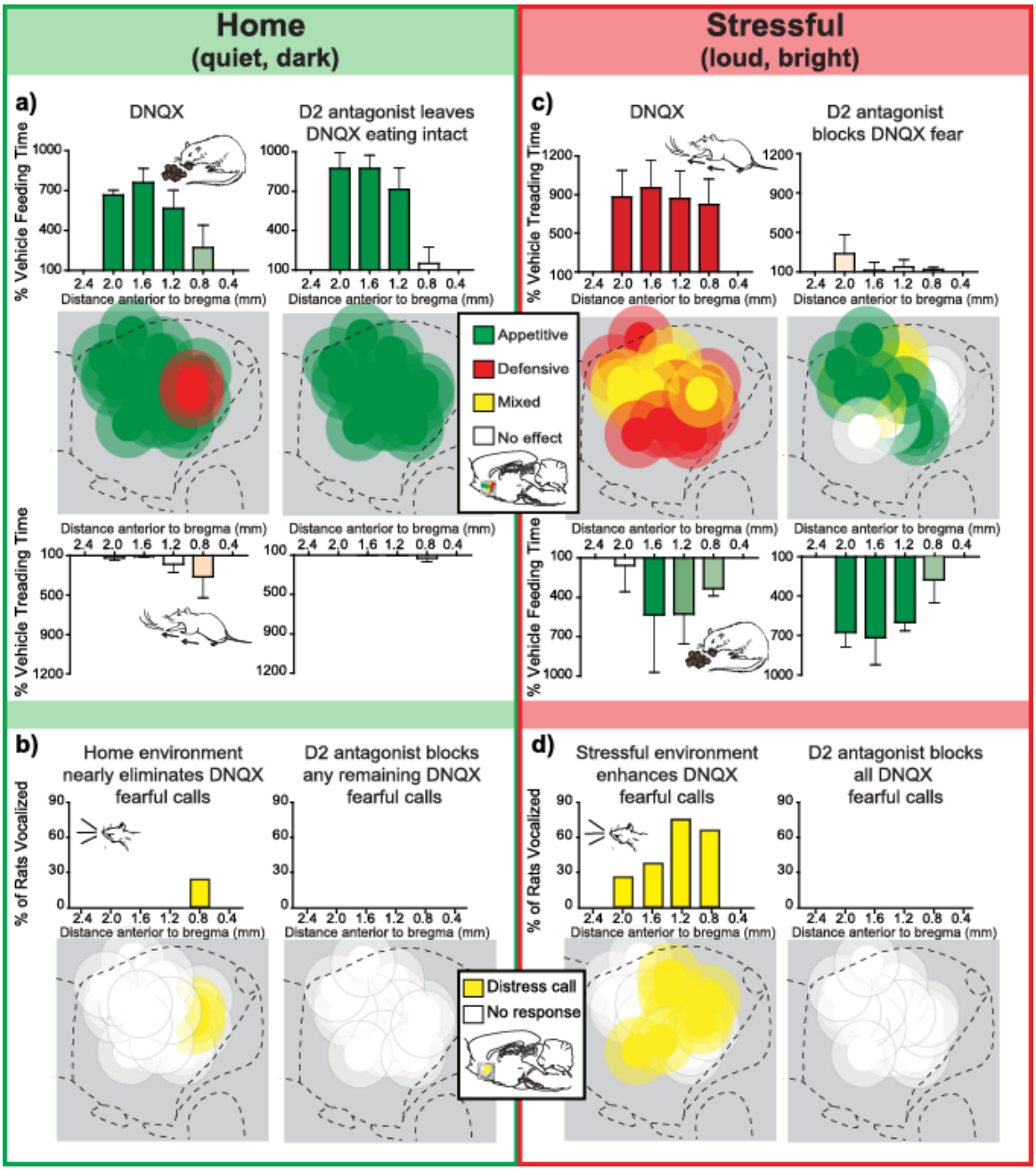

a) Eating
DNQX elicits eating
D1 antagonist prevents DNQX elicited eating
D2 antagonist leaves eating intact
Enhancement
>500%
>350%
>200%
< 75%
< 50%
< 25%
Suppression
% Vehicle Feeding Time
850
600
350
100
2.4 2.0 1.6 1.2 0.8 0.4
Distance anterior to bregma (mm)
Distance below skull (mm)
6.4
6.8
7.2
7.6
8.0
8.4
b) Fearful treading
DNQX elicits fearful treading
D1 antagonist prevents DNQX fearful treading
D2 antagonist prevents DNQX fearful treading
Enhancement
>900%
>600%
>300%
<100%
% Vehicle Treading Time
c) Fearful calls and escape attempts
DNQX elicits calls and escape attempts
D1 antagonist prevents DNQX calls and escape attempts
D2 antagonist prevents DNQX calls and escape attempts
% of Rats
90
60
30
0
Distress Call Alone
Escape Attempt Alone
Call and Escape Attempt

Figure 3.4. Environmental ambience shifts glutamate-dopamine interaction mode. Summary maps (n=10) in sagittal plane show behavior produced by microinjections in the same rats of either DNQX alone or DNQX plus a D2 antagonist (raclopride), each tested both in the Home environment (A) and in the Stressful (C) environment. Each site was designated in a particular environment as producing primarily appetitive (green symbols), fearful (red symbols) or mixed valence (yellow symbols) motivated behavior following DNQX microinjection. Testing in the Home environment caudally expanded the area which DNQX generated purely appetitive behavior (criteria for including a site was a 200% increase in feeding behavior), and nearly eliminated the ability of DNQX to generate defensive treading behavior, distress calls or escape attempts at any site. Conversely, testing in the Stressful environment rostrally expanded the area of medial shell capable of generating intense fearful behaviors (criteria for including a site was a 500% increase in treading over vehicle levels), so that nearly every rostrocaudal location became able to generate fearful reactions after DNQX microinjections (relative to vehicle). The addition of the D2 antagonist to the microinjection blocked DNQX generation of defensive treading regardless of site. In contrast, the D2 antagonist never blocked the ability of DNQX to stimulate eating behavior and increase food intake. Histograms bars below the maps show mean behavior as percent of vehicle for each behavior at rostrocaudal level as marked along the medial shell, with the dominant behavior stimulated in each environment appearing along the top (Home environment: eating; Stressful environment: treading; error bars = SEM). Maps of fearful vocalizations indicate which rats emitted audible distress calls in response to the experimenter's touch in the Home environment (B) and the Stressful environment (D) following DNQX alone or DNQX plus the D2 antagonist. Histograms bars above the maps show the percentage of subjects which vocalized at each rostrocaudal level.


Home
(quiet, dark)
Stressful
(loud, bright)
a)
DNQX
D2 antagonist leaves DNQX eating intact
% Vehicle Feeding Time
% Vehicle Treading Time
Distance anterior to bregma (mm)
c)
DNQX
D2 antagonist blocks DNQX fear
Appetitive
Defensive
Mixed
No effect
b)
Home environment nearly eliminates DNQX fearful calls
D2 antagonist blocks any remaining DNQX fearful calls
% of Rats Vocalized
d)
Stressful environment enhances DNQX fearful calls
D2 antagonist blocks all DNQX fearful calls
Distress call
No response

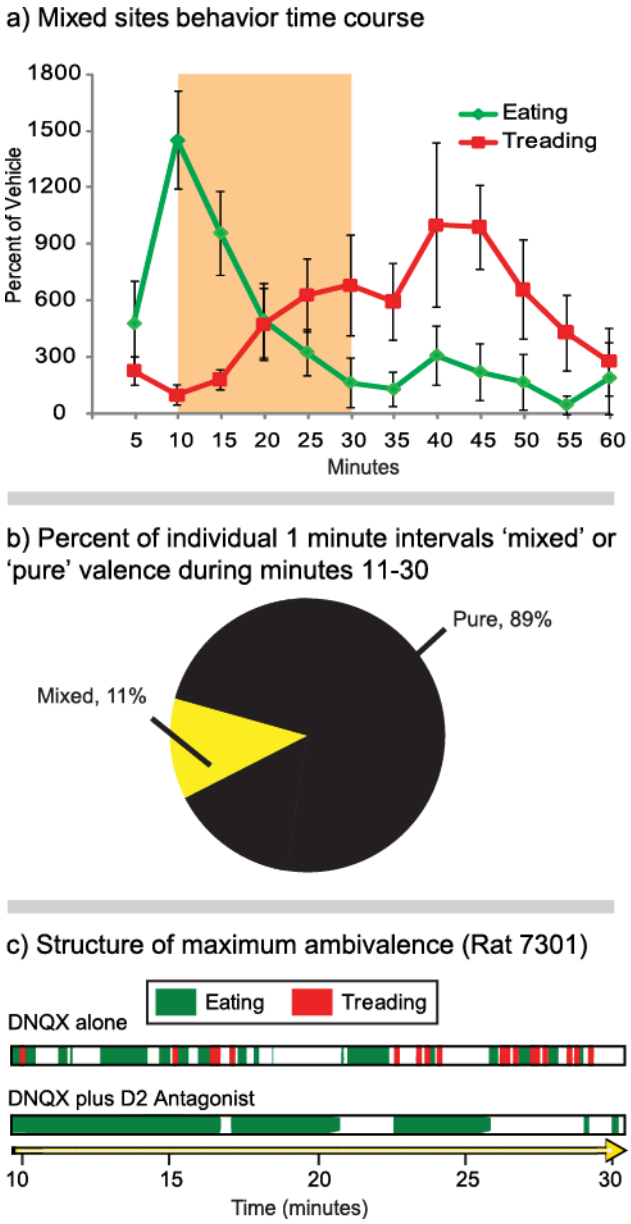


Figure 3.5. Appetitive and defensive behavior elicited from mixed valence sites in the Stressful environment. Time course (A) of eating and defensive treading over the 1-hour trial (n=6): eating behavior peaks early in the trial, while defensive treading emerges towards the mid-point of the trial (average % of vehicle, error bars = SEM). During the period of greatest overlap (minutes 11 – 30, highlighted in A), most individual minutes (B) for a given rat consisted of purely appetitive or purely aversive, rather than mixed, and transitions between appetitive and defensive valence tend to be limited (<10 for most rats). Yet, even for the rat that demonstrated the greatest ambivalence (shown in C, ~31 transitions between appetitive versus defensive behaviors in the 1-hour test), the addition of the D2 antagonist (raclopride) completely eliminated fearful behavior, and left DNQX-stimulation of eating intact.

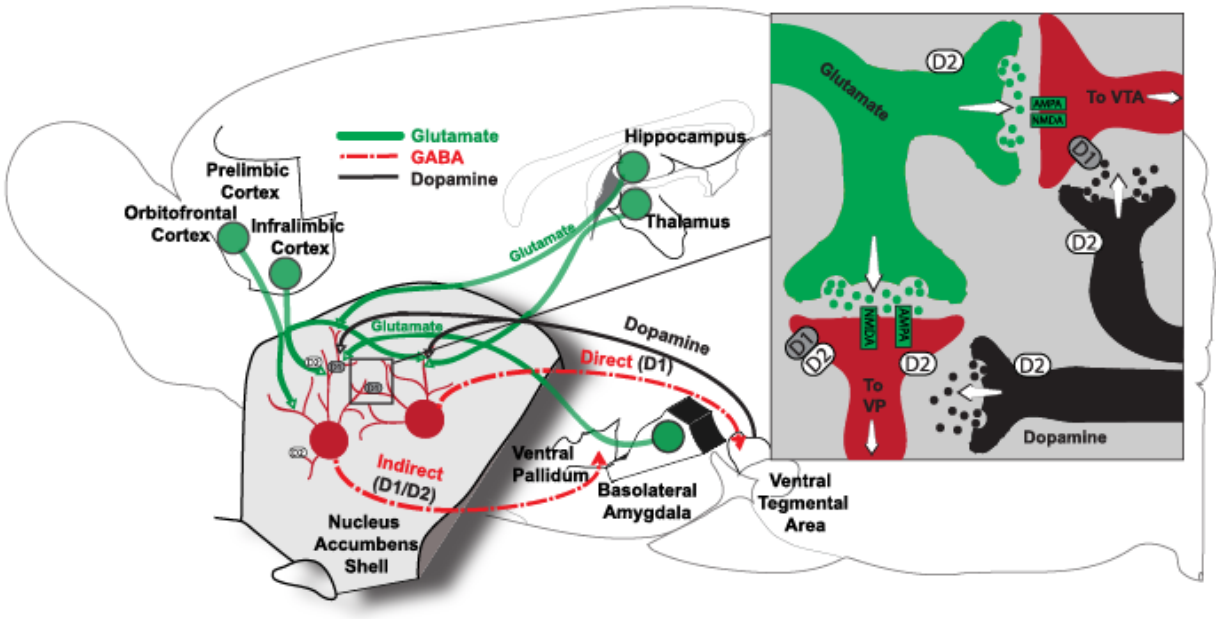


Figure 3.6. Mesocorticolimbic circuits impacted by glutamate-dopamine interactions. Close-up representation of synapses in NAc medial shell and position in larger circuits. D1 receptors are located postsynaptically on medium spiny neurons (red) that project via direct output path to ventral tegmentum (VTA), and via indirect output path to ventral pallidum (VP) and lateral hypothalamus (LH). D2 receptors are shown postsynaptically on medium spiny neurons that project via indirect output path to VP and LH (also D1-D2 co-expressing neurons). Dopamine receptors are also shown presynaptically in NAc on dopamine (black) and glutamate (green) neurons. Glutamatergic inputs (green) are shown from medial prefrontal cortex, orbitofrontal cortex, hippocampus, thalamus, and basolateral amygdala. Dopaminergic inputs (black) to NAc shell are shown from ventral tegmental area. GABAergic output pathways are shown to VP and LH (indirect path; D1, D2 and D1-D2 expressing neurons) and to VTA (direct path; D1).

Chapter 4

Prefrontal Cortex Modulates Desire and Dread Generated by Nucleus Accumbens Glutamate Disruption

Introduction

Motivations or emotions generated by subcortical circuits involving the nucleus accumbens (NAc) may be powerfully modulated by "top-down" corticolimbic controls from prefrontal cortex (Phillips et al., 2008; Kompus et al., 2009). In humans, successful voluntary suppression of subjective cravings or emotional responses is accompanied by activation of prefrontal cortical areas and simultaneous reduction of subcortical activity in NAc, amygdala and ventral tegmentum (McDonald, 1991b; Jongen-Relo et al., 1995; Hussain et al., 1996; Alheid et al., 1999; Kompus et al., 2009).

Here, we sought to probe corticolimbic modulation of intense unconditioned appetitive and defensive behaviors generated by disruptions in medial shell of NAc in the rat. The medial shell of NAc is an important node in the generation of both positive desire and aversive dread (Kerfoot et al., 2007; Nicola, 2007; Delgado et al., 2008; Humphries and Prescott, 2010; Sesack and Grace, 2010; Muschamp et al., 2011; Richard and Berridge, 2011a). Localized disruptions of glutamate transmission in medial shell, via microinjections of the AMPA antagonist DNQX, generate intense unconditioned appetitive and/or fearful behaviors organized along a rostrocaudal gradient (probably involving disinhibition of ventral pallidum, hypothalamus, and related targets from GABAergic suppression, thus releasing motivation-generating circuits)

(Taber and Fibiger, 1997; Kelley, 1999; Meredith et al., 2008; Roitman et al., 2008; Krause et al., 2010). Rostral disruptions in NAc shell evoke purely appetitive behaviors like voracious eating (Maldonado-Irizarry et al., 1995; Kelley and Swanson, 1997). Caudal disruptions instead evoke increasingly fearful behaviors including audible distress vocalizations, escape attempts and spontaneous defensive treading, an innate anti-predator reaction in which rodents toss debris at a threatening stimulus (e.g., rattlesnake) (Coss and Owings, 1978; Treit et al., 1981; Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2008). Intermediate disruptions produce ambivalent mixtures of appetitive and fearful behaviors (Reynolds and Berridge, 2008; Richard and Berridge, 2011b).

The valence of motivation produced by glutamate disruption at many shell sites can be retuned by changes in external ambience, which might reflect top-down influences from cortex and related structures that send glutamate inputs to NAc (Reynolds and Berridge, 2008; Richard and Berridge, 2011b). This study probed three regions of prefrontal cortex that send direct glutamate projections to the medial shell. First, the medial orbitofrontal cortex (Brodmann's area 10) (Van Eden and Uylings, 1985; Hoover and Vertes, 2011; but see Schilman et al., 2008), which is implicated in pleasure and emotion (Kringelbach, 2005, 2010). Second, infralimbic cortex (Sesack et al., 1989), which is homologous to human subgenual or deeply ventral anterior cingulate cortex (Brodmann's area 25) (Ongur and Price, 2000; Barbas et al., 2003; Uylings et al., 2003), and has been suggested to suppress motivated behaviors, such as reinstatement of cocaine and food seeking (Rhodes and Killcross, 2004; Peters et al., 2008) and conditioned fear (Quirk et al., 2000). Third, prelimbic cortex (potentially homologous to Brodmann's areas 24 and 32 of anterior cingulate cortex) (Ongur and Price, 2000; Barbas et al., 2003; Uylings et al., 2003) projects to NAc shell and core, and has been suggested to participate in appetitive and fearful

motivations (Capriles et al., 2003; Vidal-Gonzalez et al., 2006; Peters et al., 2009) and in some forms of inhibitory control (Hayton et al., 2010; MacLeod and Bucci, 2010). Here, we tested the effects of reversible activation versus inhibition of medial orbitofrontal, infralimbic, and prelimbic cortex on the ability of glutamate disruptions within NAc shell to produce unconditioned appetitive and fearful motivated behaviors.

Methods

Experimental design

To investigate whether activity in infralimbic, prelimbic, and orbitofrontal regions of medial prefrontal cortex modulates the generation of strong unconditioned motivations by NAc shell glutamate disruptions, we simultaneously generated eating or fearful defensive behaviors via microinjections in medial shell of a low dose of the AMPA antagonist DNQX (6,7-dinotroquinoxaline-2,3(1H,4H)-dione; 250 ng/ 0.2 µl per side in 50% saline/50% DMSO), while temporarily either activating or inhibiting each prefrontal region (Figure 4.1a). Temporary activation of prefrontal cortex was produced by microinjections of the GABA-A antagonist bicuculline (.1 µg / .2 µl side in ACSF), which by preventing local inhibition induces relative activation of neurons (Berretta et al., 2005). Using bicuculline allowed receptor-based comparison to the opposite polarity effects of GABA *agonist* microinjections at the same prefrontal sites (muscimol/baclofen microinjections). We conducted a careful slow motion video analysis of behavior to ensure that no seizure indicators were ever produced by bicuculline in prefrontal cortex, which is consistent with previous reports of no seizure manifestations after bicuculline injected into cortex at doses similar to ours or even higher (Qu et al., 2006; Dang et al., 2010; Enomoto et al., 2011; Paine et al., 2011; Markham et al., 2012; Murphy et al., 2012).

Temporary *inhibition* of cortex was produced by a mixture of $GABA_A$ and $GABA_B$ agonists, muscimol (5 ng per side) and baclofen (65 ng per side in .2 µl ACSF) (Peters et al., 2008). Microinjection spread was assessed by Fos plumes surrounding drug microinjections. We have previously found that the diameter of drug-induced Fos plumes shrinks after several microinjections (Richard and Berridge, 2011b), and therefore we used a dedicated Fos group measured after a single microinjection to capture maximal diameter.

Subjects

Male Sprague-Dawley rats [n = 124; Prefrontal Activation, n = 68, Prefrontal Inhibition, n = 30, Fos analysis, n = 26), 300 – 400 grams prior to surgery] were housed at ~21 Cº on a reverse 12:12 light:dark cycle, with *ad libitum* access to both food and water. All experiments were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Cranial cannulation surgery

Under surgical anesthesia, all rats received bilateral implantation of permanent cranial cannulae aimed at points throughout the rostrocaudal extent of medial shell of NAc (19 mm, 23 gauge) and either the infralimbic, prelimbic, or medial orbitofrontal cortex regions of prefrontal cortex (15 mm, 23 gauge). Coordinates for each rat were bilaterally symmetrical, and aimed 2 mm above target sites. Following induction of anesthesia with ketamine hydrochloride (80 mg/kg) and xylazine (5 mg/kg), and treatment with atropine (0.05 mg/kg) to prevent respiratory distress, rats were placed in a stereotaxic apparatus (David Kopf Instruments) with a flat-skull angle (incisor bar set at -3.3 mm). Cannulae aimed at prefrontal cortex were angled laterally 5° so that injection sites were sufficiently medial following implantation centered on the following coordinates: medial orbitofrontal cortex (n = 45): anteroposterior (AP) +4.5 mm, mediolateral

(ML) +/-.8 mm, dorsoventral (DV) -3.0 mm; infralimbic (n = 34): AP +3.0 mm, ML +/-.8 mm, DV -3.0 mm; prelimbic (n = 14): AP +3.4 mm, ML +/-.8 mm, and DV -2.0 mm. To avoid the lateral ventricles, and to allow sufficient space between cannulae aimed at NAc and prefrontal cortex, cannulae aimed at NAc were angled laterally 15º and aimed at coordinates between AP +.8 to +2.5 mm, ML +/- 3.0 to +/- 3.2 mm, and DV, -6.0 to - 6.5mm. Cannulae were anchored to the skull using four surgical screws and dental acrylic, and stainless steel obturators (28 gauge) were inserted to avoid cannulae occlusion. Post-surgery, rats received injections of chloramphenical sodium succinate (60 mg/kg) to prevent infection and carprofen (5 mg/kg) for pain relief, and were allowed to recovery for at least 7 days before testing began.

Drug microinjections

Following one week of recovery, each rat (n = 68; infralimbic, n =26; prelimbic, n=11; orbitofrontal, n=29) in the prefrontal activation group was tested after the following 4 drug conditions for spontaneous motivated behavior (see Supplement for more detail): 1) vehicle in prefrontal cortex and vehicle in NAc, 2) bicuculline in prefrontal cortex and vehicle in NAc, 3) vehicle in prefrontal cortex and DNQX in NAc, and 4) bicuculline in prefrontal cortex and DNQX in NAc. Each rat in the prefrontal inhibition group (n = 30; infralimbic, n=9; prelimbic, n=5; orbitofrontal, n=16) received the following 4 drug conditions: 1) vehicle in prefrontal cortex and vehicle in NAc, 2) baclofen plus muscimol in prefrontal cortex and vehicle in NAc, 3) vehicle in prefrontal cortex and DNQX in NAc, and 4) baclofen plus muscimol in prefrontal cortex and DNQX in NAc (Figure 4.1a). Each rat in the Fos group received comparable single microinjections of one above condition. Fos plume groups received similar microinjections to the cortical excitation group in a between-subject comparison. All drug conditions were received in counterbalanced order, on testing days spaced at least 48 hours apart. Rats used for Fos analysis

received only one set of microinjections. On testing days, all solutions were brought to room temperature (~21ºC) and bilaterally infused, at a speed of 0.2 µl / minute, through stainless steel injectors (16 mm to prefrontal cortex, 21 mm to NAc, 29 gauge), which extended 2 mm beyond the tip of the cannulae into the target region. Following infusion, injectors were left in place for 1 minute to allow drug diffusion, after which obturators were replaced and rats were immediately placed in the testing chamber.

Behavioral tests of spontaneous motivated behaviors

Following 3 days of handling, all behavioral testing rats (n = 98) were habituated to the testing procedure and apparatus on 4 days for 1 hour each. On each testing day, rats received one of the drug conditions described previously, and were placed immediately in the transparent testing chamber (23 x 20 x 45 cm) which contained preweighed food (~20g of rat chow) and *ad libitum* water, to allow the expression of appetitive behavior, and ~3 cm deep of granular corn cob bedding, to allow the expression of defensive treading behavior. Behavior in the chamber was taped for 60 minutes and scored later offline for analysis. Rats were removed by the experimenter's gloved hand at the end of the session using a standardized slow-approach hand motion. Following a 5s approach toward the testing cage, the experimenter slowly reached toward the rat, taking 2s. Upon contact, the experimenter lightly brushed the side of the rat with gloved fingertips, before lifting the rat from the chamber in a gentle movement that lasted 2s. The observer recorded whether rats made any audible distress vocalizations, attempts to escape, or attempts to bite the experimenter.

Behavioral coding

Observers blind to drug treatment scored each 60 minute session for the total time (seconds) spent in each of the following behaviors: appetitive behaviors such as eating (mouth on

the food or engaged in chewing action) and drinking (licking the spout of the water bottle), fearful behavior consisting of defensive treading (spraying or pushing of bedding by rapid alternating thrusts of the forepaws), and grooming (a stereotyped sequence described in (Aldridge et al., 1993)). Observers scored the total number for behaviors which tended to occur as discrete events, including appetitive behaviors such as food carrying (transportation of food pellets in the mouth) and food sniffs (sniffing near the food for at least 1 second), and two general motor activities: rearing (forepaws at least one inch off the floor) and cage crosses (forepaws and head cross the halfway point of the cage). Observers also looked for any indicators of seizure including: behavioral arrest or akinesia (freezing), stereotyped behaviors including repetitive blinking or rhythmic jaw-opening, head nodding, head shaking, wet dog shakes (repetitive shaking of the entire trunk), tonic seizures (sudden-onset tonic extension or flexion of the head, trunk and/or extremities for several seconds), sudden loss of posture (falling over), and myoclonic or clonic twitches (brief arrhythmic or rhythmic jerking of a muscle group).

Histology

Following all testing, behavioral testing rats were deeply anesthetized with an overdose of sodium pentobarbital. Brains were removed and fixed in 10% paraformaldehyde for 2 days and in 25% sucrose solution for 3 days. Brains were sliced at 60 microns on a freezing microtome, and stained with cresyl violet for verification of microinjection sites. Maps of bilateral Fos plume-sized placements in the sagittal plane were then color-coded to express changes in behavior for individual rats in the figures. NAc placements were classified as rostral if their placements were located +1.4 to +2.4 mm ahead of bregma, and caudal if their placements were located +0.4 to +1.4 mm ahead of bregma.

Fos-like protein immunohistochemistry

Brains were processed and Fos plumes were analyzed as described previously (Faure et al., 2008; Reynolds and Berridge, 2008; Richard and Berridge, 2011b). Rats used for Fos analysis (n=26) were anesthetized with an overdose of sodium pentobarbital and transcardially perfused 90 minutes after bilateral microinjection of 1) vehicle in prefrontal cortex and vehicle in NAc (n = 6), 2) bicuculline in prefrontal cortex and vehicle in NAc (n = 2), 3) vehicle in prefrontal cortex and DNQX in NAc (n = 8), or 4) bicuculline in prefrontal cortex and DNQX in NAc (n = 4) for Fos plumes analysis, and 5) vehicle in prefrontal cortex and no injection in NAc (n = 3), or 6) bicuculline in prefrontal cortex and no injection in NAc (n = 3), for analysis of the effect of prefrontal activation on baseline NAc Fos. Following transcardial perfusions, brains used for Fos analysis were removed and placed in 4% paraformaldehyde for 4 – 24 hours, and then transferred to 25% sucrose (in 0.1 M NaPB) for at least 3 days. Brains were sliced at 40 microns on a freezing microtome, and processed for Fos-like immunoreactivity using NDS, goat anti-cfos (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat Alexa Fluor 488 (Invitrogen, Carlsbad, CA) as described previously (Faure et al., 2008; Reynolds and Berridge, 2008; Faure et al., 2010). Sections were mounted, air-dried and coverslipped with ProLong Gold antifade reagent (Invitrogen).

Fos identification and assessment: Local Fos plumes and PFC-NAC interactions

Immunoreactivity for Fos-like proteins was visualized using a Leica microscope equipped for fluorescent microscopy, using a filter with an excitation band at 480-505 nm for Fos-positive cells and images were taken using MCID Core software. For analysis of drug spread, Fos plumes images were taken in the areas surrounding the microinjection with the most intense areas of Fos expression, just medial to the end of the injector tip, surrounding a small focal point

of necrosis. Fos labeled cells were individually counted within successive blocks (50 µm x 50 µm), along 8 radial arms emanating from the center of the necrosis, with 10x magnification (Figure 4.1). Zones of Fos elevation (or "plumes") were assessed as described previously (Reynolds and Berridge, 2008). Additionally, to assess whether prefrontal cortex microinjections had direct neurobiological effects on NAc shell, we assessed immunoreactivity for Fos-like protein in NAc shell of rats who received no microinjections in NAc, following either vehicle or bicuculline prefrontal cortex microinjections. We counted Fos densities in NAc shell at three coronal sections (rostral, middle and caudal) in boxes (200 µm x 200 µm) centered over ventral and dorsal regions, spaced 200 µm apart dorsoventrally.

Statistical analysis

The effects of DNQX on each continuous behavior were assessed using a three-factor mixed within- and between-subject ANOVA (drug [DNQX versus vehicle] x group [activation versus inactivation] x NAc placement [rostral versus caudal]) to verify elicitation of eating and defensive behavior along a rostrocaudal gradient, equally within both groups. The effects of prefrontal activation and inactivation on DNQX-induced behavior were assessed using an additional four-factor mixed within- and between-subject ANOVA (Prefrontal Drug x DNQX x Prefrontal Placement x NAc Placement). When significant interactions were found, rats were split by anatomical location and additional analysis was done using a two-way ANOVA (Prefrontal drug x NAc drug) and additional one-way ANOVAs with pairwise comparisons using Sidak corrections for multiple comparisons. For the purposes of visualizing data, NAc placements were further divided into six rostrocaudal bins and dorsal and ventral prefrontal placements were divided into eight rostrocaudal bins. Bar graphs of these bins were placed next to maps of behavioral effects produced at individual NAc or prefrontal sites.

Results

Local glutamate disruptions in medial shell of NAc induce appetitive and defensive behavior organized along a rostrocaudal gradient

When prefrontal cortex received no drug manipulation (vehicle microinjection), localized glutamate disruptions in medial shell generated intense appetitive eating and/or fearful behaviors as expected, organized by valence along the usual rostrocaudal gradient (Figure 4.1b). Medial shell microinjections of a moderate dose of DNQX produced robust stimulation of eating and food consumption to above five-times vehicle control levels, with most intense eating (of up to 10 grams) occurring from the most *rostral* sites (Figure 4.1b; average of 611 seconds +/- 65 SEM eating after rostral DNQX versus 154 seconds on vehicle control; eating time: drug x placement, $F(1,74) = 5.415$, $p = .006$; average of 5.5 grams +/- .4 SEM grams consumed after rostral DNQX versus 1.1 grams on vehicle control; food intake: drug x placement, $F(1,74) = 7.557$, $p = .001$). At *caudal* sites the same microinjections produced active fearful behaviors, including audible distress vocalizations and escape attempts to touch (vocalizations: 59% of rats on DNQX vs. 0% on vehicle, McNemar's test, $p < .001$; escape: 27% of rats on DNQX vs. 0% on vehicle, $p = .031$) and spontaneous defensive treading at 60-times vehicle control levels (Figure 4.1c; average of 31.3 seconds +/- 6.13 SEM after caudal DNQX versus .49 seconds on vehicle control; drug x placement, $F(1,74) = 9.550$, $p = < .001$).

Medial orbitofrontal activation specifically enhances appetitive motivation produced by NAc shell

Co-activation of medial orbitofrontal cortex (immediately rostral to infralimbic cortex) selectively *enhanced* eating induced by NAc DNQX at middle and caudal sites that otherwise produced only fear (Figures 4.2a and 4.3a). Orbitofrontal activation made caudal DNQX

generate levels of eating that were 250% of levels without orbitofrontal activation, and as high as any eating generated at rostral DNQX sites (up to 9.5 grams; eating time: DNQX x bicuculline, $F(1,20) = 4.483$, $p = .047$; DNQX x bicuculline, $F(1,21) = 4.376$, $p = .049$; Figure 4.3a). At more rostral NAc sites for DNQX, which generated high levels of food intake to begin with (>5-times control), robust eating remained unchanged by orbitofrontal co-activation (Figure 4.3a). Orbitofrontal activation never altered DNQX generation of fearful behaviors (Figures 4.2b and 4.3b; medial orbitofrontal cortex: defensive treading, DNQX x bicuculline, $F<1$; DNQX x bicuculline x NAc placement, $F(2,19) = 1.764$, $p = .198$; vocalizations and escape attempts, $p = .375$ to 1.00). Likewise medial orbitofrontal activation had no effect on nonvalenced motor behaviors such as grooming ($Fs<1$), cage crosses ($F(1,14) = 2.788$, $p = .117$) or rears ($F(1,14) = 1.424$, $p = .253$).

Infralimbic activation suppressed eating and fear generated by NAc shell glutamate disruption

Infralimbic activation nonspecifically suppressed all intense motivated behaviors generated by NAc disruptions, appetitive and fearful. Bicuculline-induced activation roughly cut in half the high level of eating behavior and food intake otherwise induced by DNQX in rostral sites of NAc shell (Figures 4.2a and 4.3c; eating time, DNQX x bicuculline, $F(1,20) = 4.563$, $p = .045$; food intake, DNQX x bicuculline, $F(1,23) = 10.903$, $p = .003$; DNQX x bicuculline x NAc placement, $F(2,23) = 3.522$, $p = .046$). Activation of infralimbic cortex similarly nearly abolished fearful distress vocalizations, escape attempts, and defensive treading behavior otherwise produced by DNQX at caudal shell sites (at least 96% reduction; Figure 4.2b and 4.3d; defensive treading, DNQX x bicuculline, $F(1,23) = 37.906$, $p < .001$; DNQX x bicuculline x NAc placement, $F(2,23) = 31.177$, $p < .001$; vocalizations, McNemar's test, DNQX alone versus DNQX plus bicuculline, $p = .021$).

Infralimbic suppression is specific to DNQX-induced levels of motivation

Infralimbic cortex activation did not interfere with baseline levels of spontaneous appetitive and defensive behaviors after vehicle microinjection in NAc. Infralimbic microinjections of bicuculline did not suppress moderate baseline levels of eating or drinking (Fs<1, Figure 4.3c), nor change baseline defensive behaviors, which remained near zero (Figure 4.3d; defensive treading, $F<1$, vocalizations and escape attempts, McNemar's tests, $p = 1.00$). It also did not prevent NAc DNQX microinjections from stimulating nonvalenced activities such as grooming ($F<1$), and even slightly enhanced locomotion (cage crosses, main effect of bicuculline, $F(1,16) = 5.125$, $p = .038$; rears, main effect of bicuculline, $F(1,16) = 7.981$, $p = .012$).

Prelimbic activation has no effect on motivated behaviors generated by NAc shell DNQX

By contrast to co-activation of *infralimbic* cortex, which suppressed DNQX appetitive and fearful behaviors, co-activation of the immediately dorsal region of *prelimbic* cortex failed to alter NAc DNQX-induced eating or food intake (Figure 4.2a; eating time, DNQX x bicuculline, $F<1$; DNQX x bicuculline x NAc placement, $F<1$; food intake, DNQX x bicuculline, $F(1,5) = 2.330$, $p = .187$; DNQX x bicuculline x NAc placement, $F<1$). Prelimbic cortex co-activation also failed to alter defensive treading behavior, distress calls or escape attempts induced by glutamate disruptions in NAc caudal shell (Figure 4.2b; prelimbic cortex: defensive treading, Fs<1). Finally, co-activation of prefrontal cortex had no impact on baseline defensive behaviors in the absence of NAc DNQX, which remained near zero (defensive treading, $F<1$, McNemar's Test, vocalizations and escape attempts, $p = 1.00$).

No behavioral indicators of seizure were observed after microinjections of bicuculline in either prelimbic, infralimbic or medial orbitofrontal regions. Thus we conclude that seizures were not induced by prefrontal activations.

Inhibition of all prefrontal cortex regions leaves unchanged levels of unconditioned appetitive or defensive behaviors produced by NAc shell glutamate disruption

Inhibition of infralimbic, prelimbic or medial orbitofrontal regions of prefrontal cortex, via combined microinjection of GABA agonists baclofen and muscimol, all failed to alter the DNQX NAc shell generation of intense levels of appetitive or defensive behaviors, regardless of prefrontal subregion (Figure 4.4a and b; eating time: Fs<1; food intake: DNQX x baclofen-muscimol, $F(1,14) = 2.580$, $p = .131$; DNQX x baclofen-muscimol x prefrontal placement, F<1; defensive treading, DNQX x baclofen-muscimol, F<1; DNQX x baclofen-muscimol x prefrontal placement, $F(2,14) = 2.536$, $p = .115$; vocalizations and escape attempts, $p = 1.00$; Figures 4.2 and 4.3). Inhibition of prefrontal cortex also had no impact on baseline levels of eating or defensive behaviors (food intake, Fs<1; eating, baclofen-muscimol, $F(1,14) = 1.833$, $p = .197$; baclofen plus muscimol x prefrontal placement, $F(2,14) = 1.241$, $p = .319$; defensive treading, Fs<1).

Fos plume analysis: determining functional microinjection drug spread

We assessed Fos plumes produced by DNQX microinjections in NAc and bicuculline microinjections in orbitofrontal and infralimbic prefrontal cortex, and also assessed whether prefrontal bicuculline modulated distant Fos plumes in NAc induced by DNQX in medial shell (Figure 4.5). DNQX microinjections in NAc shell produced plumes containing a small .008 mm^3 volume center where Fos expression was more than doubled (radius = .125 mm), surrounded by a larger .056 mm^3 sphere of mildly elevated Fos expression between 1.5 to 2 times vehicle levels (radius = .24 mm; Figure 4.5b). Prefrontal bicuculline microinjections similarly produced enhancement of Fos expression surrounding the microinjection. Infralimbic and orbitofrontal plumes contained a small excitatory .00075mm^3 center of tripled Fos expression (radius = .056

mm), surrounded by a larger .03 mm^3 middle zone of more than doubled Fos expression (radius = .19 mm) and an outer .15 mm^3 halo of mildly elevated Fos expression between 1.5 to 2 times vehicle (radius = .33 mm) (Figure 4.5a). In addition, bicuculline activation of either infralimbic or medial orbitofrontal cortex amplified distant Fos expression in medial shell (main effect of bicuculline, $F(1,8) = 7.737$, $p = .024$; bicuculline x prefrontal placement, $F(1,8) = 1.484$, $p = .258$; Figure 4.5c). Fos expression surrounding NAc shell DNQX microinjections was elevated to 3-times DNQX alone levels in a radius of .15 mm from microinjection center (volume = .0014 mm^3) and 2-times DNQX levels up to .26 mm away (volume = .073 mm^3) following bicuculline microinjections. Prefrontal bicuculline enhanced distant Fos in uninjected NAc shell to between 550 and 1150% over normal levels (main effect of drug, $F(33,2) = 15.895$, $p < .001$), with greatest effects in dorsal shell and in particular in the most rostral and dorsal portion of medial shell (bicuculline x dorsoventral location, $F(1,10) = 8.566$, $p = .015$; bicuculline x rostrocaudal location x dorsoventral location, $F(2,30 = 4.914$, $p = .014$; Fig. 4.5c).

Discussion

Medial orbitofrontal amplification of subcortically generated eating

We found that activation of medial orbitofrontal cortex biased the valence of motivation generated by NAc DNQX in an appetitive direction. Activation of medial orbitofrontal neurons by bicuculline microinjection specifically enhanced the eating generated by microinjections in caudal NAc shell, which otherwise produced mostly fearful behaviors. The intensity of eating generated at caudal NAc sites rose to levels equaling those normally produced by DNQX microinjections at more rostral sites, which produced almost purely appetitive valence. However, orbitofrontal activation never generated behavior on its own, nor further enhanced the already

intense eating generated by DNQX in rostral NAc. That pattern suggests that orbitofrontal cortex specifically modulates intense eating generated by NAc, up to the level of a response ceiling. Alternatively, it is possible that orbitofrontal co-activation can enhance positive valence only in the presence of a pre-existing state of negatively-valenced fear or anxiety.

In human studies of sensory rewards, neuroimaging activation of a specific mid-anterior region of orbitofrontal cortex (Kringelbach, 2005) is specifically associated with subjective pleasure for food, as well as drugs, music, etc. (Blood and Zatorre, 2001; de Araujo et al., 2003; de Araujo and Rolls, 2004; Vollm et al., 2004; Kringelbach, 2005; Grabenhorst and Rolls, 2009). A special role for orbitofrontal cortex in coding human pleasure seems consistent with our finding that orbitofrontal activation specifically enhanced positive incentive motivation generated by some sites of NAc (Kringelbach and Rolls, 2004; Kringelbach, 2005).

Infralimbic cortex suppresses subcortically generated fear and eating

We found that activation of infralimbic cortex (corresponding in humans to deeply ventral or subgenual anterior cingulate cortex; area 25) generally inhibited the intensity of both positive and negative motivations produced by glutamate disruptions in NAc shell. Thus, infralimbic activation acted primarily as a nonspecific brake: suppressing appetitive behavior elicited by disruption at rostral shell sites, and suppressing fearful behaviors elicited at caudal shell sites. Our findings therefore support the hypothesis that infralimbic cortex activation might generally regulate or inhibit the subcortical generation of intense motivations of either positive or negative valence.

This hypothesis may fit with neuroimaging evidence from humans. For instance, anterior cingulate cortex is activated when people successfully engage in voluntary efforts to suppress their aversive emotional reactions to distressing photos or to suppress their appetitive cravings to

images of palatable foods, and those anterior cingulate activations are accompanied by reductions of activity in NAc, ventral tegmental area and extended amygdala otherwise triggered by viewing the same images (Hussain et al., 1996; Alheid et al., 1999). Infralimbic suppression of subcortically-generated motivation is also consistent with findings in rodent studies that infralimbic cortex suppresses reinstated seeking of cocaine and food rewards (Rhodes and Killcross, 2004; Ishikawa et al., 2008a; Peters et al., 2008; Ghazizadeh et al., 2012; LaLumiere et al., 2012), and similarly suppresses reinstatement of conditioned fear responses (Milad et al., 2004; Vidal-Gonzalez et al., 2006; Peters et al., 2009). Our findings extend infralimbic suppression to include intense unconditioned appetitive and fearful behaviors, which do not depend on learning nor explicitly require top-down control to be generated.

Prefrontal cortical excitation modulates but is not necessary for enhanced motivation

Our results also showed an asymmetrical role of cortical excitation versus inhibition in modulating motivations generated by inhibition of NAc shell. *Excitation* of medial orbitofrontal and infralimbic cortex modulated motivations released by NAc shell DNQX microinjections as described above, but *inhibition* of the same orbitofrontal or infralimbic cortex regions failed to alter NAc shell desire or dread in any detectable way. This asymmetrical pattern suggests that unconditioned motivations elicited by NAc shell disruptions may not need input from prefrontal cortex, but that *supra-normal* levels of prefrontal activation are nonetheless able to modulate these motivations. Additionally, our finding that neither prefrontal excitation nor inhibition affected normal levels of baseline eating and fearful behavior may indicate that normal levels of unconditioned eating and fear do not require prefrontal control.

Overall, our results are consistent with the notion that infralimbic cortex and orbitofrontal cortex hierarchically control NAc production of intense desire and dread (Gallistel, 1980).

Prefrontal cortex acted here as hierarchically superior in the functional sense of being able to suppress and/or modulate the valence of robust motivations triggered by disruptions of NAc. At the same time, while subordinate, the NAc still possessed a degree of autonomy that is characteristic of a hierarchical element. That is, only manipulations of NAc and not prefrontal cortex were capable of producing intense levels of motivated behaviors. Such features of suppression/modulation by a hierarchically superior unit, combined with semi-autonomy of a subordinate unit, have been suggested to characterize functional hierarchies (Gallistel, 1980).

Neurobiological bases of infralimbic versus orbitofrontal top-down regulation

Local AMPA blockade by DNQX likely produces relative hyperpolarizations in NAc neurons by reducing glutamatergic depolarizations (Hu and White, 1996). This likely reduces firing and GABA release by NAc projection neurons, and thus disinhibits downstream targets neurons in ventral pallidum, lateral hypothalamus and ventral tegmentum to generate intense levels of motivated behaviors (Taber and Fibiger, 1997; Kelley, 1999; Meredith et al., 2008; Roitman et al., 2008; Krause et al., 2010).

How does prefrontal cortex activation interact with NAc release of intense motivations? There may be a relatively straightforward explanation for the effects of infralimbic cortex activation, which directly opposed or suppressed NAc DNQX-induced motivation. If infralimbic activation increases glutamate release on NAc neurons that are hyperpolarized by DNQX, that elevated glutamate level may compete with and perhaps partially overcome the local glutamate disruption, thus reducing the intensity of DNQX-elicited motivations. The explanation of orbitofrontal modulation of NAc DNQX-generated eating is probably more complex. One possibility is that orbitofrontal activation may inhibit some NAc shell neurons by activation of inhibitory GABAergic interneurons. NAc shell neurons are reported to be excited by either

orbitofrontal activation or infralimbic activation, but not both (Asher and Lodge, 2011). That is, if a particular NAc neuron is excited by infralimbic cortex, it may be inhibited by medial orbitofrontal cortex (Asher and Lodge, 2011). Conversely, if a NAc neuron is excited by orbitofrontal cortex, it is inhibited by infralimbic cortex. This mutual exclusivity suggests two parallel corticolimbic channels, with mutual inhibition between them. Thus, orbitofrontal activation may inhibit some NAc shell neurons (specifically those excited by infralimbic activation). If inhibition of these NAc shell neurons contributes to intense appetitive behaviors, then orbitofrontal activation may increase the intensity of eating by augmenting their hyperpolarization (Figure 4.6a). If this hypothesis is correct, then future pharmacological or optogenetic inactivation of local interneurons might modulate orbitofrontal-NAc interactions in producing intense motivated behaviors.

A second category of explanation for infralimbic or medial orbitofrontal effects goes beyond direct projections to involve indirect modulations via wider mesocorticolimbic networks, whereby prefrontal activations could recruit third-party structures to modulate the valence or intensity of appetitive and defensive motivations produced by NAc inhibition (including brain structures such as basolateral amygdala, lateral and medial dorsal hypothalamus or brainstem) (Figure 4.6b) (Freedman et al., 2000; Gabbott et al., 2005; Marchant et al., 2010; Hoover and Vertes, 2011; Millan et al., 2011). Combined manipulations of these other subcortical structures could be used in future to test their roles in modulating prefrontal-NAc shell interactions.

Clinical implications

Improved top-down control could help deal with maladaptively intense emotions in a variety of psychopathologies involving corticolimbic circuitry, including addiction, schizophrenia and PTSD (Waltz and Gold, 2007; Everitt et al., 2008; Shin and Liberzon, 2010;

Volkow et al., 2011). Speculatively, treatments aimed at enhancing medial orbitofrontal function might conceivably help increase positively-valenced emotion or shift the balance away from negatively-valenced emotions. Reduced orbitofrontal volume is found in patients with schizophrenia, panic disorders, PTSD and obsessive compulsive disorder (Baare et al., 1999; Rauch et al., 2003; Asami et al., 2009; Roppongi et al., 2010; Sobanski et al., 2010). Extrapolation of our results to such conditions would suggest that enhancing orbitofrontal activity might help add to positive appetitive motivation in cases where intense but negative valenced emotion already exists, such as in pathological anxiety or fearful paranoia .

In contrast, reduced activity in subgenual anterior cingulate cortex (homologous to infralimbic cortex in our study) has been reported to leave some patients with reduced ability to regulate certain unwanted emotions, such as in post-traumatic stress disorder (Bremner et al., 1999). Additionally, abnormalities in area 25 in cocaine addicts are associated with reduced top-down control and poor decision making (Bechara, 2005). Again speculatively applying our findings, these results support the view that activation of deep anterior cingulate area 25, homologous to infralimbic cortex here, might suppress the levels of unwanted intense emotions, regardless of whether the valence of the pathological emotion was appetitive or fearful.

In conclusion, our results suggest orbitofrontal cortex may play an important role in enhancing the positive valence of intense emotional states that might otherwise be purely fearful or anxious. Additionally, deep anterior cingulate or infralimbic cortex may be important in suppressing intense emotional states involving either desire or dread. These demonstrations of top-down hierarchical control over intense motivations generated by subcortical neural events in nucleus accumbens illustrate corticolimbic mechanisms that may contribute to regulating normal emotional well-being.

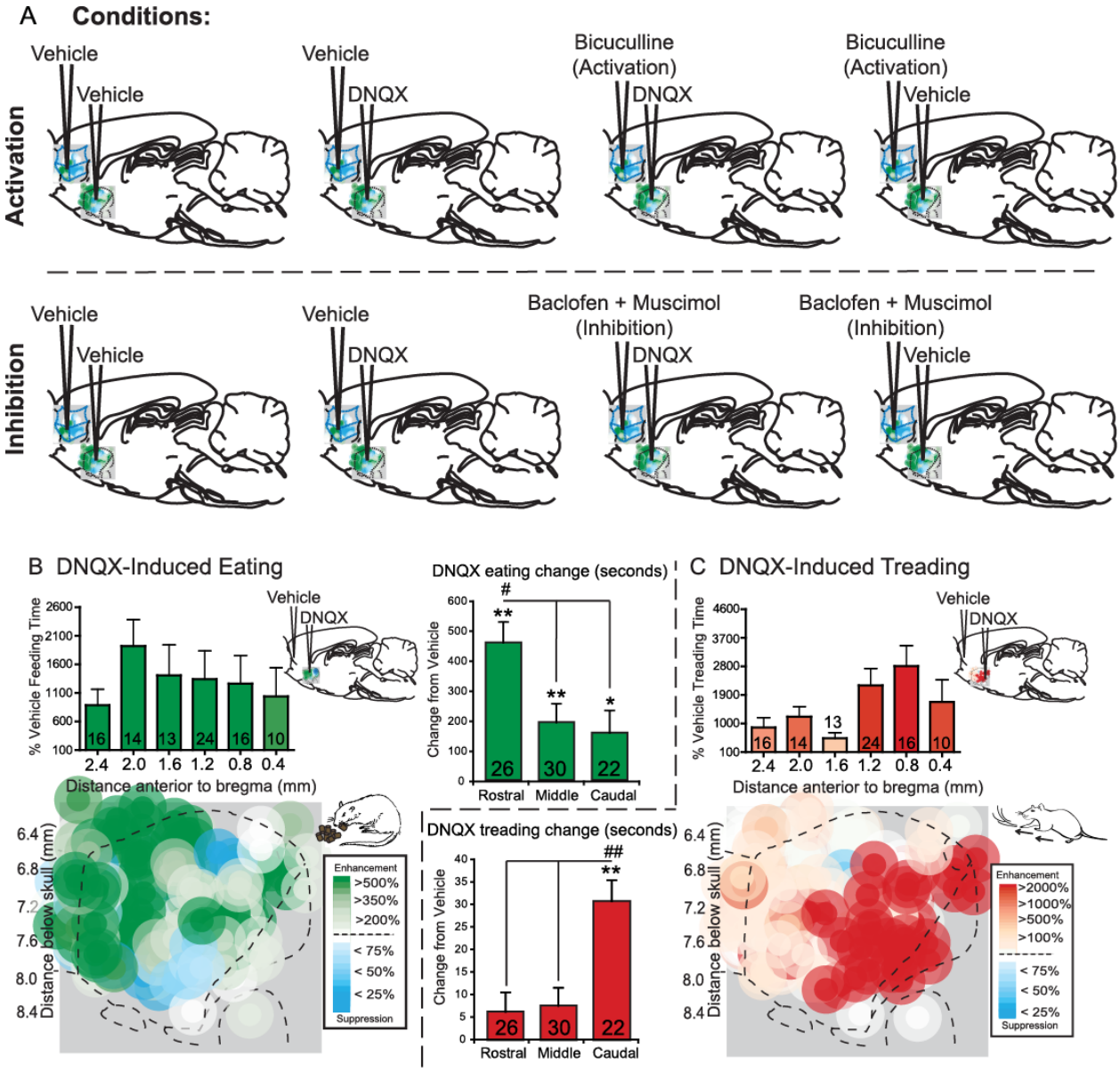


Figure 4.1. Microinjection conditions and DNQX effects. Rats received the following microinjections (A): rats in the activation group (top, n=68) received either DNQX or vehicle in NAc shell and bicuculline or vehicle into prefrontal cortex, and rats in the inactivation group (bottom, n=30) received either DNQX or vehicle in NAc shell and a baclofen plus muscimol combination or vehicle in prefrontal cortex. Fos plume maps show the effects of DNQX alone (vehicle in prefrontal cortex) on eating (B, green) or defensive treading behavior (C, red). Histogram bars above the maps show mean behaviors as a percent of vehicle at each rostrocaudal level (errors bars = SEM). Summary bar graphs show the DNQX induced eating (B) and treading (C) as change from vehicle at rostral (n=26), middle (n=30) and caudal (n=22) locations in NAc shell; data is given as seconds per hour, ** $p < .01$, * $p < .05$ versus vehicle, ## $p < .01$, # $p < .05$ subregion difference, with Sidak corrections for multiple comparisons.

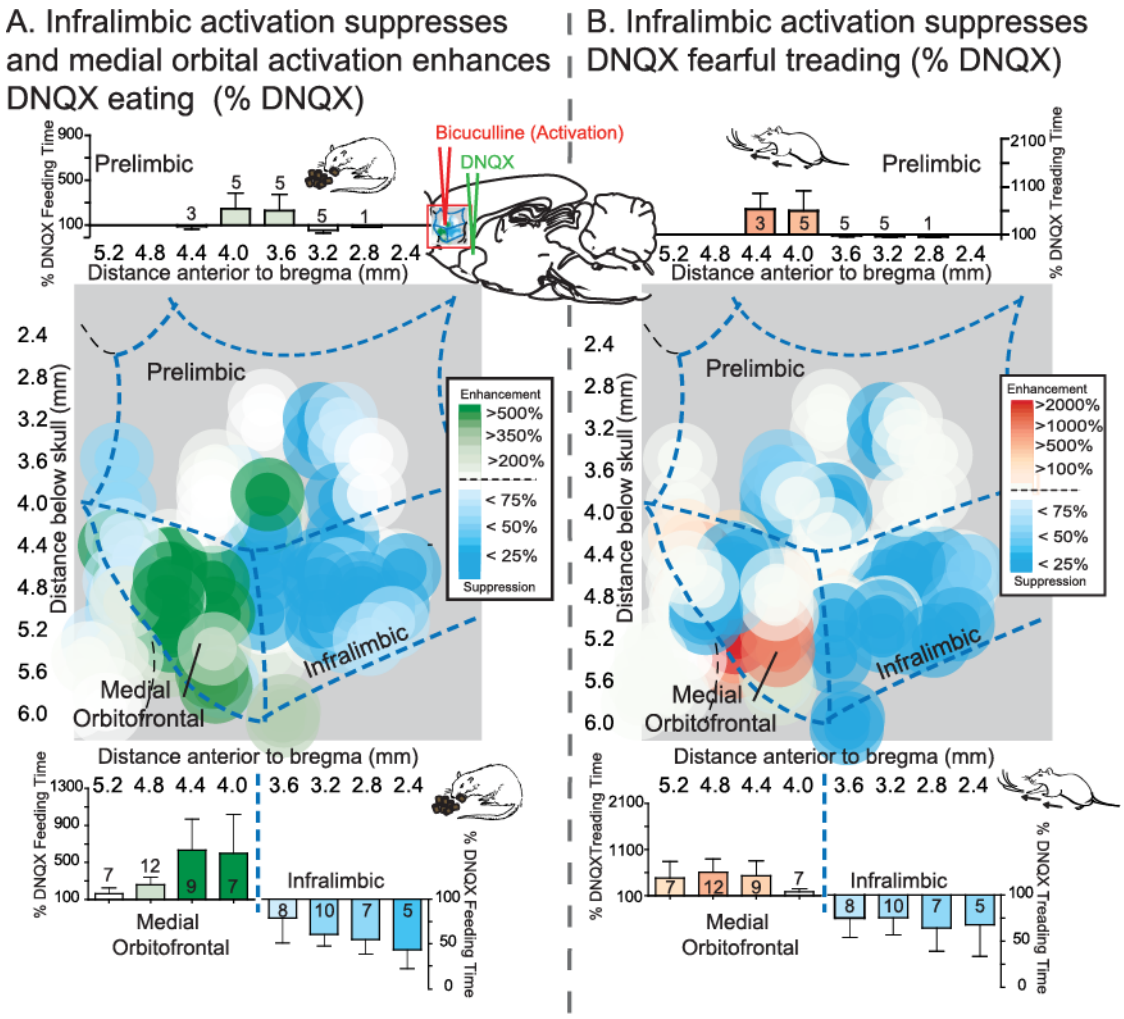


Figure 4.2. Maps of prefrontal activation effects on NAc shell DNQX generated eating and defensive treading. Maps show the effects of prefrontal activation (n=68) on DNQX-induced eating (A, left) or defensive treading (B, right) at sites mapped on the sagittal plane of prefrontal cortex, color-coded for changes in behavior as a percent of DNQX. Histograms bars show mean behavior as percent of DNQX at each rostrocaudal level, split by dorsal (prelimbic, top; n=11) and ventral (medial orbitofrontal, n=29, and infralimbic, n=26, bottom) areas of prefrontal cortex (error bars = SEM).

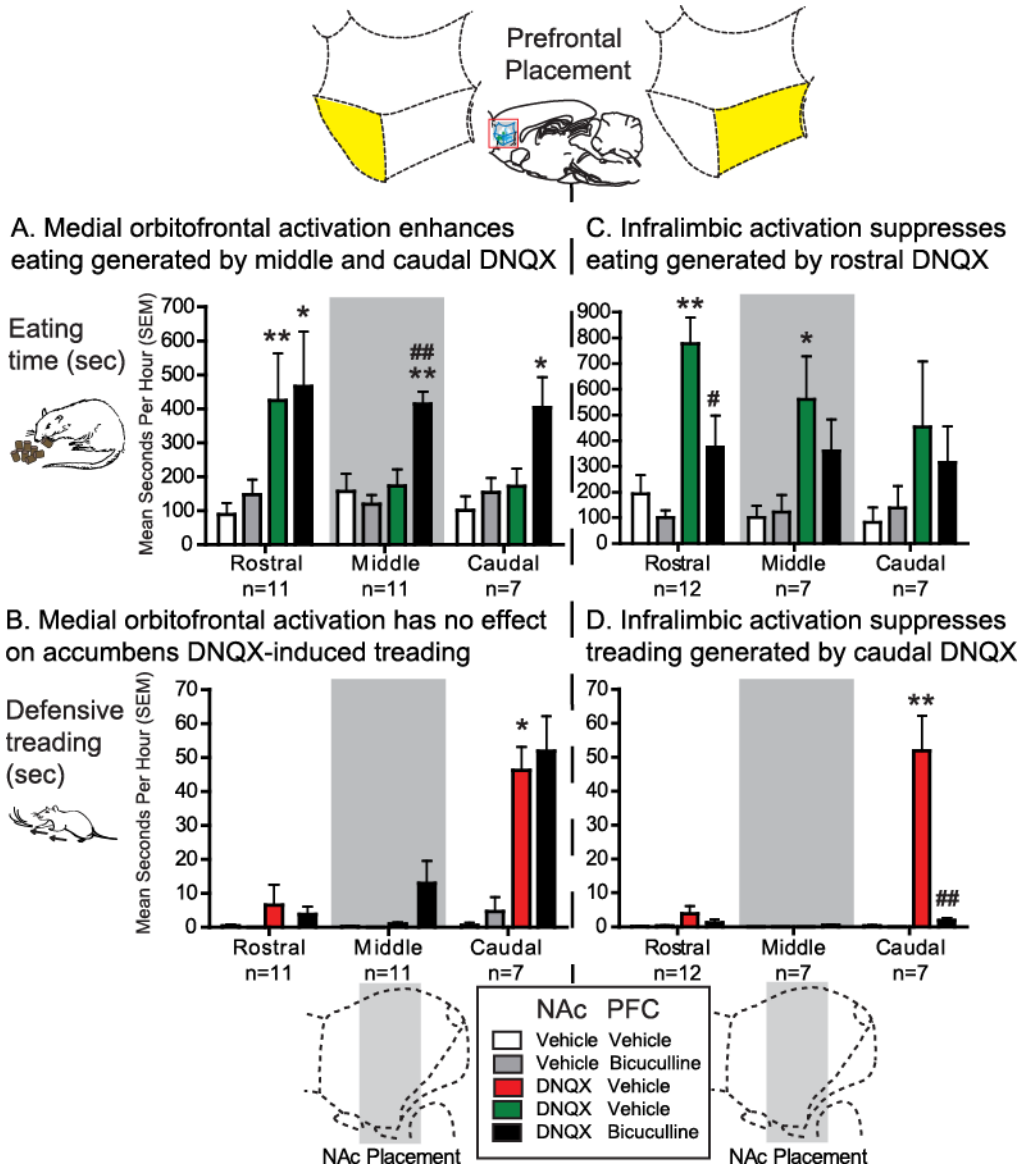


Figure 4.3. Motivated behavior graphs. Graphs demonstrating the specific effects of medial orbitofrontal activation (left) and infralimbic activation (right) on appetitive eating (top) and defensive treading (bottom), depending on particular rostrocaudal location (rostral, middle or caudal). Simultaneous microinjections of bicuculline in medial orbitofrontal (n=29) with DNQX in NAc shell (black, left) produced enhancement of DNQX induced eating (green, A), specifically at more middle (n=11) and caudal (n=7) locations but not rostral (n=11), and had no effect on DNQX induced treading (red, B). Microinjections of bicuculline in infralimbic cortex (n=26) with simultaneous DNQX in NAc shell (black, right) produced suppression of both DNQX-induced eating (green) from rostral sites (n=12) and treading (red) from caudal sites (n=7; middle sites, n=7). Data is given as seconds per hour, errors bars indicate SEM, * $p < .05$ versus vehicle, ** $p < .01$ versus vehicle, # $p < .05$ versus DNQX, ## $p < .01$ versus DNQX, pairwise comparisons using Sidak corrections.

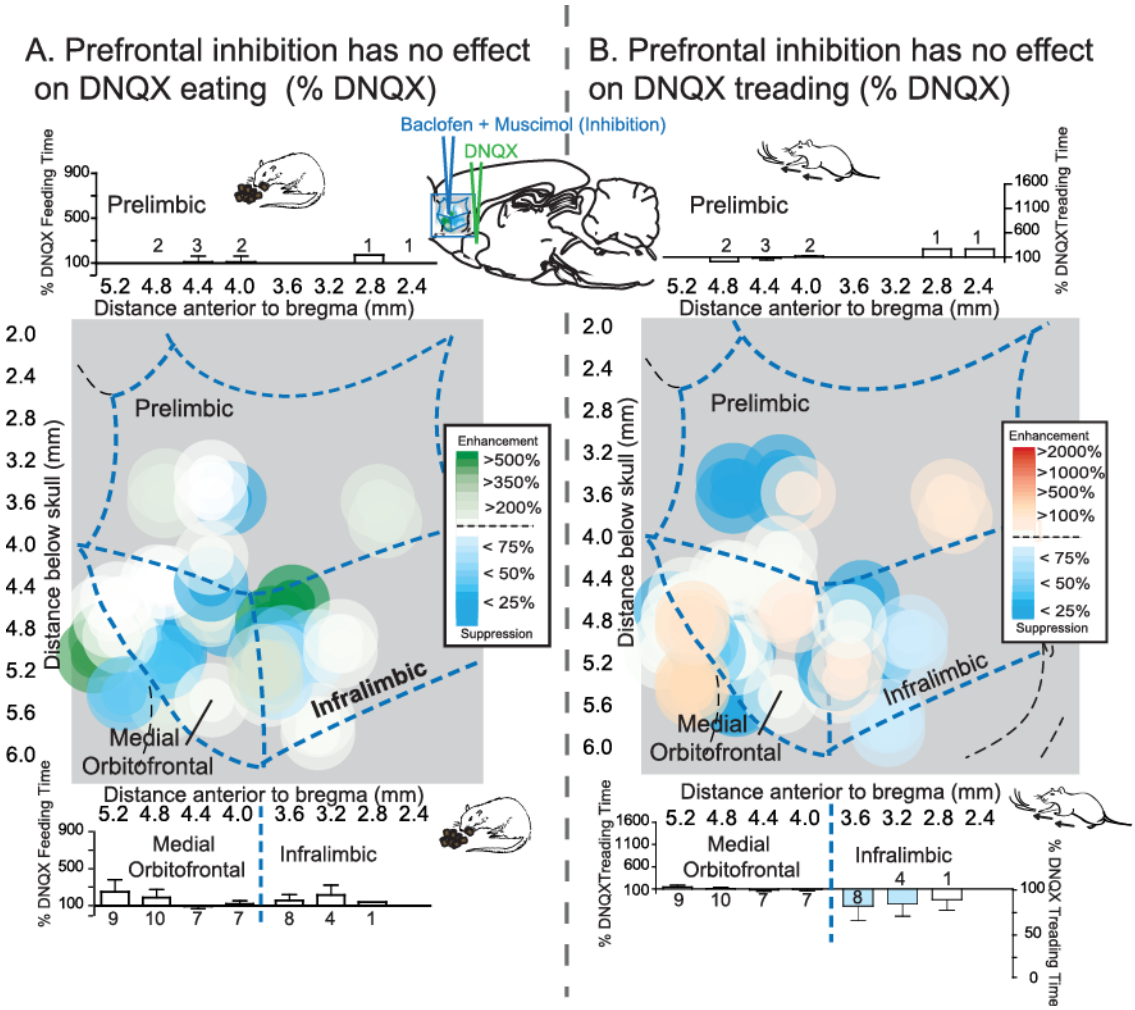
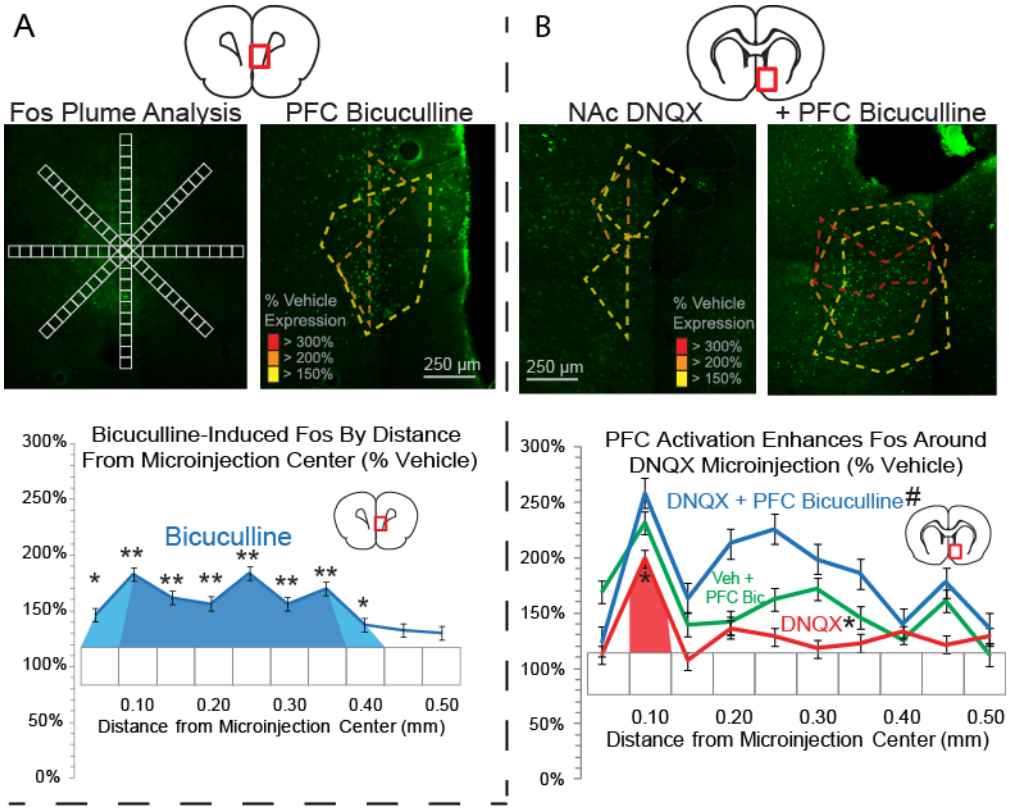


Figure 4.4. Maps of prefrontal inhibition effects on NAc shell DNQX generated eating and defensive treading. Maps show the effects of prefrontal inhibition (n=30) on DNQX-induced eating (A, left) or defensive treading (B, right) at sites mapped on the sagittal plane of prefrontal cortex, color-coded for changes in behavior as a percent of DNQX. Histograms bars show mean behavior as percent of DNQX at each rostrocaudal level, split by dorsal (prelimbic, top; n=5) and ventral (medial orbitofrontal, n=16, and infralimbic, n=9, bottom) areas of prefrontal cortex (error bars = SEM).

Figure 4.5. Fos plume analysis and NAc-prefrontal interactions. Fos plumes were analyzed for functional drug spread of bicuculline in prefrontal cortex (A) and DNQX in NAc shell (B). Fos labeled cells were individually counted within successive blocks (50 µm x 50 µm), along 8 radial arms emanating from the center of the site, with 10x magnification (A). Colors indicate levels of Fos expression of 3x (red), 2x (orange) and 1.5x (yellow) vehicle level Fos expression. Line graphs show levels of Fos expression following bicuculline (blue, A) and DNQX (red, B), as well as the impact of prefrontal bicuculline on levels of Fos expression in NAc shell following either DNQX (blue, B) or vehicle (green, B) microinjections in NAc shell. Analysis of Fos expression in uninjected NAc shell (C) showed that bicuculline elevated NAc shell Fos even in the absence of NAc shell microinjections at levels more than 500% (yellow), 650% (orange), and 800% (red) of vehicle. Bar graphs indicate levels of elevated Fos at three rostrocaudal and two dorsoventral levels. * $p < .05$, ** $p < .01$ versus vehicle, # $p < .05$, ## $p < .01$ versus DNQX.



C PFC Bicuculline Increases NAc Shell Fos in the absence of NAc microinjection

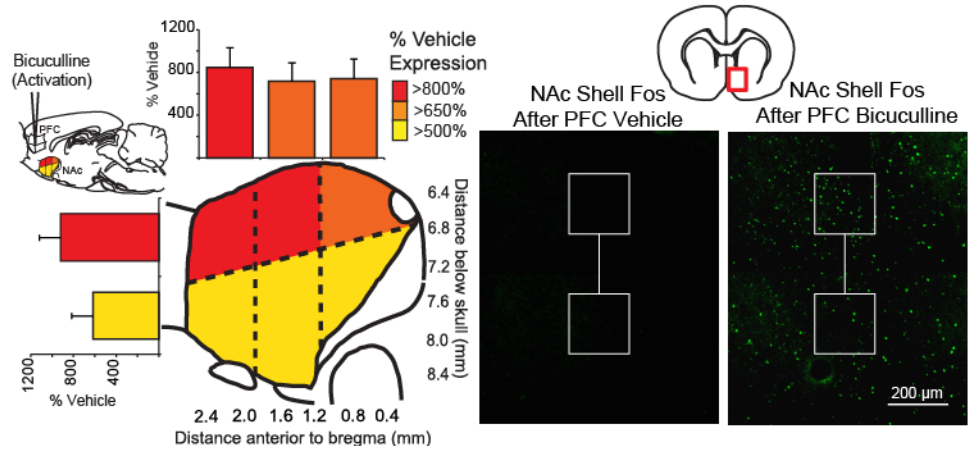
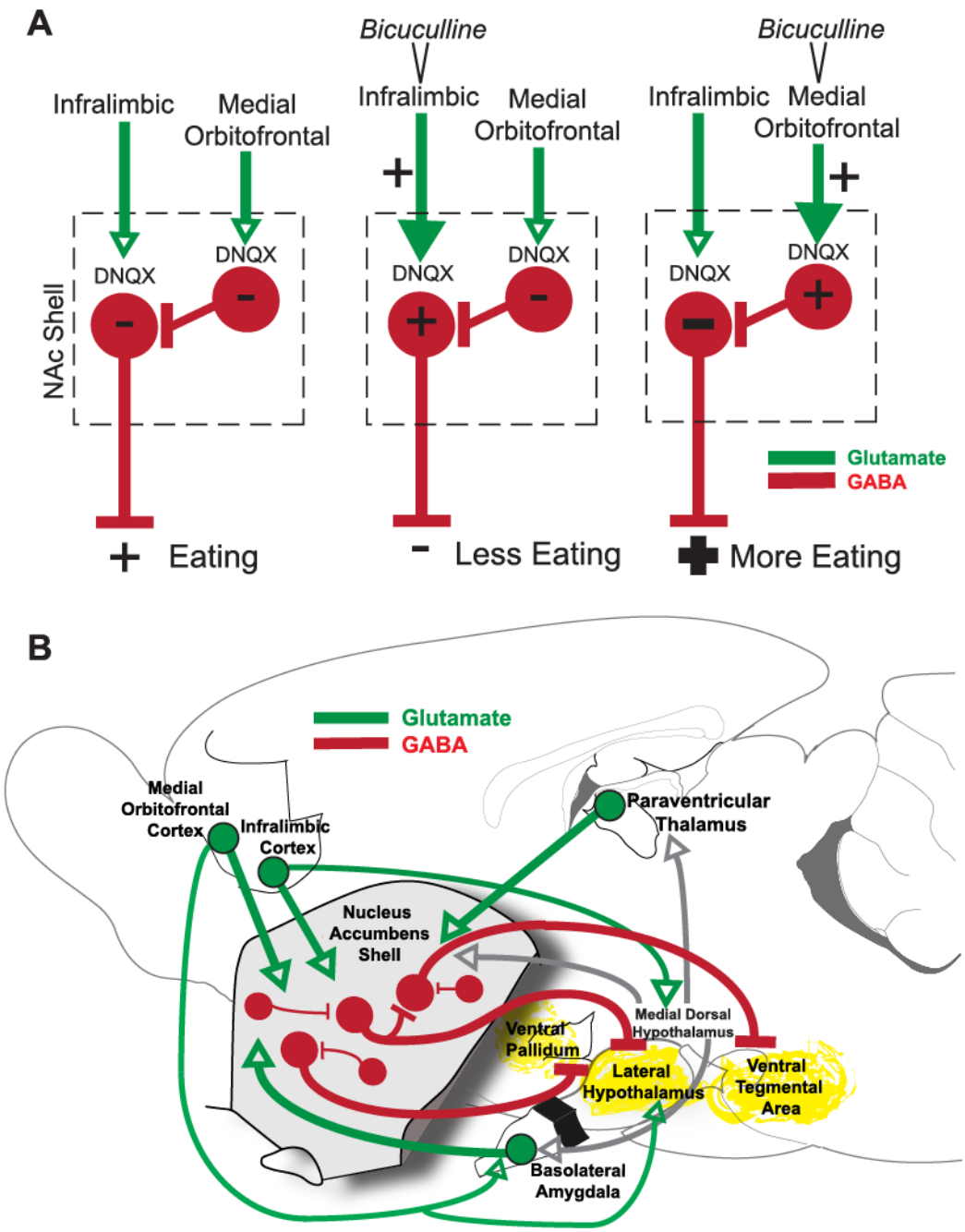


Figure 4.6. Potential mechanisms of prefrontal modulation of DNQX generated motivated behaviors. Proposed direct prefrontal to NAc shell mechanism (A) mediating opposite infralimbic versus orbitofrontal effects on DNQX-induced eating. Bicuculline infusions excite glutamate inputs (green) from infralimbic or medial orbitofrontal cortex. DNQX alone inhibits rostral NAc shell projection neurons (red), resulting in disinhibition of downstream targets and intense eating. Infralimbic activation may overcome DNQX inhibition of these same neurons, suppressing eating. Medial orbitofrontal activation may instead activate GABAergic interneurons, which further inhibit neurons already inhibited by DNQX, potentiating eating. A circuit diagram (B) shows prefrontal and NAc shell projections to relevant third-party structures that may mediate larger circuit interactions. DNQX microinjections likely inhibit neurons in NAc shell, disinhibiting downstream structures such as ventral pallidum, lateral hypothalamus, and ventral tegmental area (yellow) via GABAergic projections neurons (red). Medial orbitofrontal and infralimbic activation may act to modulate DNQX-induced behaviors via direct glutamate (green) projections to NAc shell or parallel projections to structures such as medial dorsal hypothalamus, lateral hypothalamus and basolateral amygdala.


A
Bicuculline
Bicuculline
Infralimbic
Medial Orbitofrontal
Infralimbic
Medial Orbitofrontal
Infralimbic
Medial Orbitofrontal
NAc Shell
DNQX
DNQX
DNQX
DNQX
DNQX
DNQX
Glutamate
GABA
+ Eating
- Less Eating
More Eating
B
Glutamate
GABA
Medial Orbitofrontal Cortex
Infralimbic Cortex
Paraventricular Thalamus
Nucleus Accumbens Shell
Medial Dorsal Hypothalamus
Ventral Pallidum
Lateral Hypothalamus
Ventral Tegmental Area
Basolateral Amygdala

Chapter 5

New and Future Directions

Introduction

Inhibitions of medial shell of nucleus accumbens (NAc), either via corticolimbic glutamate blockade or GABAergic inhibition, generate desire and dread along a rostrocaudal gradient: rostral inhibitions generate intense eating and increases in food intake, whereas caudal inhibitions generate increasingly fearful behaviors (Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2008; Faure et al., 2010). These medial shell inhibitions may exert their effects solely by disinhibiting downstream targets in ventral pallidum, lateral hypothalamus, ventral tegmental etc., releasing appetitive and defensive motivated behaviors (Stratford and Kelley, 1999; Krause et al., 2010; Stratford and Wirtshafter, 2012a). Alternatively, desire and dread generated by NAc inhibitions may also require potentiated excitations in a subset of NAc shell neurons, either via lateral disinhibitions (Nicola et al., 2004; Mao and Massaquoi, 2007; Moyer et al., 2007; Faure et al., 2008; Tepper et al., 2008) or potentially via shifts in AMPA/NMDA ratio towards NMDA (Cull-Candy and Leszkiewicz, 2004; Hull et al., 2009).

It is important to determine whether inhibition of NAc shell, or its output pathways, is sufficient to generate fear and eating. In order to more selectively target particular cell types and efferent pathways from NAc shell for direct inhibition, optogenetic and/or pharmacogenetic techniques can be an advantageous tool. Optogenetics utilizes viral-mediated expression of light-sensitive opsins, which when illuminated by particular wavelengths of light can either excite (i.e.

with channelrhodopsin) or inhibit (i.e. with halorhodopsin or archaerhodopsin) the cells in which they are expressed (Chow et al., 2010; Yizhar et al., 2011). In a similar vein, one type of pharmacogenetics utilizes viral-mediated expression of specially-designed G-protein coupled receptors (GPCRs), or designer receptors exclusively activated with designer drugs (DREADDs), which respond to a drug (clozapine-N-oxide; CNO) that has no known effects on endogenous receptors in the brain, but either excites (with the hM_3D receptor; G_s) or inhibits (with the hM_4D receptor; G_i) the cells in which these DREADDs are expressed (Dong et al., 2010; Ferguson et al., 2011; Ferguson and Neumaier, 2012). Viral promoters that are currently available can be used to selectively restrict the expression of opsins and/or DREADDs, either to neurons in general (using hSyn promoters, for example: Diester et al., 2011; Stefanik et al., 2012), to putative excitatory neurons (using a CamKIIα promoter: Stuber et al., 2011; Tye et al., 2011), to enkephalin or dynorphin-expressing neurons (Enk or Dyn promoters: Ferguson et al., 2011) or to any neuronal type in which a transgenic mouse or rat expresses cre-recombinase (Kravitz et al., 2010; Adamantidis et al., 2011; Witten et al., 2011; Kravitz et al., 2012). In dorsal striatum, selective manipulations of D1/dynorphin- and D2/enkephalin-containing neurons largely target the direct versus indirect pathways, but manipulations of these particular cell types within medial shell likely still recruit more than one output pathway. To target specific efferents, one can combine viral expression in one neuronal structure, with light or drug infusion targeted to a particular output structure (Stuber et al., 2011; Tye et al., 2011; Stefanik et al., 2012). This approach may be especially valuable given the number of NAc shell efferents and their potential heterogeneity in terms of neurochemical characteristics.

But first, it is critical to determine whether general NAc shell inhibitions by these methods generate eating or fear in a similar manner to pharmacological inhibitions. Therefore, I

tested whether medial NAc shell inhibitions mediated by DREADD receptors could generate either eating or defensive behaviors similarly to traditional pharmacological inhibitions.

DREADD-Mediated Inhibition of Medial Shell

To test whether DREADD-mediated inhibition of medial NAc shell would generate similar behaviors to inhibition via GABA agonism or glutamate blockade, I used adeno-associated viral vectors (AAV) with a general neuronal promoter (hSyn) to express an engineered GPCR (Gi-coupled human muscarinic M4, hM_4D) which is activated by the otherwise inert ligand, clozapine-*N*-oxide (CNO). Viral expression was targeted to points throughout the rostrocaudal extent of NAc shell. After an incubation period of 4 weeks rats received systemic injections of CNO and were tested for the generation of spontaneous motivated behaviors.

Methods

Subjects

Male Sprague-Dawley rats (n = 17), weighing 300 – 400 grams at surgery, were housed at ~21°C on a reverse 12:12 light:dark cycle. All rats had *ad libitum* access to both food and water. All of the following experimental procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Virus infusion surgeries

Rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (5 mg/kg), and treated with atropine (0.05 mg/kg) to prevent respiratory distress, and then placed in a stereotaxic apparatus. The incisor bar was set at 5.0 mm above intra-aural zero, angling the

microinjector trajectory so as to avoid penetrating the lateral ventricles. Rats (n=17) received bilateral infusions of 1.5 µl per side of hSyn-HA-hM$_4$D-mCitrine viral vector (AAV5, ~1012 infectious units/ml, UNC Vector Core) aimed at staggered points throughout the rostrocaudal extent of medial shell of NAc. Virus was infused at coordinates between anteroposterior (AP) +2.4 to +3.1 mm, mediolateral (ML) +/- .9 to 1.0 mm, and dorsoventral (DV) -7.6 to 7.7 mm from bregma, through 28-gauge stainless steel injectors over a 10 minute period. Injectors were left in place for 10 minutes post-infusion to allow diffusion from the injector tip. After surgery, each rat received subcutaneous injection of chloramphenicol sodium succinate (60 mg/kg) to prevent infection and carprofen (5 mg/kg) for pain relief. Rats received carprofen again 24 hrs later, and were allowed to recover for at least 4 weeks before testing began, to allow sufficient time for virus expression.

DREADD inhibition and behavioral tests of spontaneous motivated behaviors

DREADD-mediated inhibitions in medial shell were induced by systemic intraperitoneal injections of CNO (Santa Cruz Biotechnology) at doses of 0 (vehicle control), 1, 3, and 10 mg/kg in saline vehicle. Doses were given in counterbalanced order and testing days were spaced at least 48 hours apart. Rats in the DREADD group (n=17) were habituated to the testing procedure and apparatus on 3 days for 2 hours each before testing began. On each test day, rats received one of the drug conditions described previously and were placed immediately in the transparent testing chamber (23 x 20 x 45 cm) which contained pre-weighed food (~20g rat chow) and *ad libitum* water, to allow the expression of appetitive behavior. The chamber also contained granular cob bedding spread on the floor ~3 cm deep to allow the expression of defensive treading behavior. Behavior in the chamber was videorecorded for 120 minutes, to be scored later offline for analysis. Behavioral sessions were longer than described previously to allow

sufficient time for systemic injections of CNO to act at the NAc shell DREADD receptors (Faure et al., 2008; Faure et al., 2010; Richard and Berridge, 2011b, 2012). At the end of each session, rats were removed by the experimenter's gloved hand using a standardized slow-approach hand motion in order to quantify any fearful distress calls, escape attempts or defensive bites elicited by human touch. Following a ~5 second approach towards the testing cage, the experimenter slowly reached towards the rat, taking ~2 seconds. Upon contact, the experimenter lightly brushed the side of the rat with gloved fingertips, taking ~1 sec, before lifting the rat from the chamber in a gentle movement that lasted ~2 sec. The observer recorded any attempts by the rat to escape when touched, as well as bites and audible distress vocalizations.

Behavioral coding: DREADD Inhibition

The incidence of elicited fearful distress vocalizations, escape dashes, and bite attempts directed at the experimenter's hand were scored when the rat was gently picked up at end of the test session (Reynolds and Berridge, 2003), after which total grams of chow pellets consumed were recorded. Behaviors emitted spontaneously and videotaped during the 2-hr test were subsequently scored by experimenters blind to treatment for the total cumulative duration (seconds) for each of the following: eating behavior (involving both appetitive approach and voluntary initiation of ingestion plus consummatory chewing and swallowing of food), drinking behaviors (licking from water spout), and fearful defensive treading/burying behavior (defined as active spraying or pushing of bedding with rapid alternating thrusts of the forepaws, spatially directed generally towards the brightly lit front or corners of the cage). Additionally, the number of bouts of appetitive behaviors such as food carrying and food sniffs, as well as less-valenced behaviors such as rearing, cage crosses, and grooming behavior were also recorded.

Histology

Following behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused. Brains were removed and fixed in 4% paraformaldehyde for 4-24 hours and in 25% sucrose solution (0.1 M NaPB) for 3 days. Brains were sliced at 40 microns on a freezing microtome, mounted, air-dried and coverslipped with ProLong Gold antifade reagent (Invitrogen). Zones of viral expression were assessed via visualization of mCitrine fluorescence using a Leica DM6000 microscope (10x to 20x magnification). Bilateral virus expression sites for each rat were placed on coronal slices from a rat brain atlas (Paxinos and Watson, 2007), which were used to extrapolate the position of each site on one sagittal slice. Mapping in the sagittal view allows for the presentation on the same map of the entire rostrocaudal and dorsoventral extents of NAc medial shell. Functional eating effects were mapped using color-coding to express the intensity of changes in motivated behaviors for individual behaviorally-tested rats. Symbols were sized to match the maximal diameter of virus expression. The extent of virus expression was assessed by identifying fluorescently labeled cells bodies within successive blocks (50 µm x 50 µm) along 8 radial arms emanating from the center of virus expression with 10x magnificent (Figure 5.1). The average radius for each area of virus expression was computed based on the furthest distance of labeled cell bodies along each radial arm. Sites were classified as rostral shell if virus expression occurred +1.4 to +2.6 mm ahead of bregma, and as caudal shell it occurred +0.4 to +1.4 mm ahead of bregma.

Statistical analysis

The effects of CNO were assessed using linear mixed-models (LMM) analysis, based on the best-fitting model of repeated measures covariance. Depending on the model, some degrees

of freedom were adjusted to non-integer values. Pairwise comparisons for each dose were conducted using Sidak corrections for multiple comparisons.

Results and Discussion

DREADD-Mediated Inhibition Generates Intense Eating

In order determine whether DREADD-mediated inhibition could be used to determine the relative roles of inhibition of specific NAc shell output pathways, I tested whether DREADD-mediated inhibition of medial shell would generate similar behaviors to inhibition via GABA agonism or glutamate blockade. I used adeno-associated viral vectors (AAV) with a general neuronal promoter (hSyn) to express an engineered GPCR (Gi-coupled human muscarinic M4, hM_4D) in rostral or caudal medial shell of NAc. Four weeks after viral infusions, DREADD-mediated inhibitions in medial shell were induced by systemic injections of CNO. Of the 17 rats tested, 8 rats had visible viral expression restricted to rostral shell, 4 rats had viral expression in caudal shell, and 5 rats had no visible viral expression or expression outside of medial shell. Virus expression was induced in NAc shell cell bodies in a zone with an average radius of .33 +/- .025 mm, approximately .15 mm^3 in volume (Figure 5.1).

CNO injections generated increases in time spent eating and food intake to approximately double vehicle levels in rats with DREADD expression in rostral, but not caudal locations in NAc shell (Figures 5.1 and 5.2; eating: main effect of CNO in rostral rats, $F(3, 6.827) = 5.154$, $p = .035$; caudal rats, $F(3, 2.974) = 1.001$, $p = .500$; food intake: rostral rats, $F(3,6.819) = 4.161$, $p = .056$; caudal rats, $F(3, 3.025) = 2.680$, $p = .219$). No significant effects on defensive treading, defensive reactions to the experimenter, drinking, or motor behaviors including cage crosses, rearing and grooming were induced by systemic CNO in rats that had either rostral or

caudal shell expression (Figure 5.3; drinking, $F<1$; defensive treading, $F(3,10.344) = 1.070$, $p = .404$; grooming, $F<1$; cage crosses, $F(3,13.385) = 1.427$, $p = .279$; rears, $F<1$). The lack of significant fearful behavior may be due in part to a low number of subjects with caudal DREADD expression (n=4) (Figure 5.1). One caudal rat tread for more than 200 seconds after receiving 1 mg/kg CNO, and attempted to escape after receiving 3 mg/kg CNO, which may indicate that more thorough mapping is necessary to identify DREADD-mediated generation of defensive treading or other defensive behaviors.

Alternatively, DREADD-mediated inhibition may be more similar to other inhibitions mediated by the Gi-coupled GPCRs, such as mu-opioid stimulation, which generates eating, but not fear, throughout NAc shell (Peciña and Berridge, 2005). Further testing will be necessary to fully determine whether caudal DREADD-mediated inhibition is capable of generating eating or fear. If Gi-DREADD-mediated inhibition fails to generate fearful behaviors in further tests, then while DREADD-mediated inhibition could be used to investigate the importance of inhibition of particular NAc shell neuronal types or efferent pathways in the elicitation of intense eating, it may be less efficacious in investigations of defensive behavior. If DREADD-mediated inhibition cannot generate defensive behaviors, optogenetic methods that exert more direct inhibitions, perhaps more similarly to $GABA_A$ agonism or AMPA blockade, may be more useful in investigating the precise neural mechanisms of NAc shell dread.

Optogenetic Inhibition of Medial Shell: Pilot Experiment

I have conducted pilot testing to begin to ask whether optical inhibition of NAc shell (via ArchT) will generate appetitive and fearful motivated behaviors. Rats received NAc shell infusions of AAV vectors with a general promoter (CAG) to express ArchT along with fiber

optic implants aimed at NAc shell. After an incubation period of 3 weeks rats received illumination of NAc shell to test for generation of spontaneous motivated behaviors. The following experiment is still ongoing.

Methods

Subjects

Male Sprague-Dawley rats (n = 5), weighing 300 – 400 grams at surgery, were housed at ~21°C on a reverse 12:12 light:dark cycle. All rats had *ad libitum* access to both food and water. All of the following experimental procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Optical fiber implants and patch cables

Chronic optical fiber implants and optical patch cables were constructed in-house based on published protocols (Sparta et al., 2012). For construction of fiber implants, 200 µm core fibers (0.37 NA; Thor Labs) were epoxied into 1.25 mm zirconia ferrules (230 µm inner diameter; Precision Fiber Products) with 7 mm of fiber extending beyond the bottom of the ferrule. Fibers were cut at the top of the ferrule, polished, and calibrated to determine the percentage of light transmission at the fiber tip based on output from the patch cables. For construction of patch cables, 200 µm core fibers were epoxied on one end into 1.25 mm zirconia ferrules, and on the other end into FC/PC steel ferrule connectors (230 µm inner diameter; Thor Labs). Fibers were cut, polished and tested for acceptable levels of maximum light transmission.

Virus infusion and fiber implantation surgeries

Rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (5 mg/kg), and treated with atropine (0.05 mg/kg) to prevent respiratory distress, and then placed in a

stereotaxic apparatus. The incisor bar was set at 5.0 mm above intra-aural zero, angling the microinjector trajectory so as to avoid penetrating the lateral ventricles. Rats (n=5) received bilateral infusions of 1.5 µl per side of CAG-ArchT-GFP viral vector (AAV5, ~1012 infectious units/ml, UNC Vector Core) aimed at staggered points throughout the rostrocaudal extent of medial shell of NAc. Virus was infused at coordinates between anteroposterior (AP) +2.4 to +3.1 mm, mediolateral (ML) +/- .9 to 1.0 mm, and dorsoventral (DV) -7.6 to 7.7 mm from bregma, through 28-gauge stainless steel injectors over a 10 minute period. Injectors were left in place for 10 minutes post-infusion to allow diffusion from the injector tip. After virus infusion, rats in the optogenetic group received implantation of bilateral chronic optical fibers targeted directly about NAc shell, which were secured with surgical screws and dental acrylic; a stainless steel wound clip was fixed into the dental acrylic to allow the rats to be attached to a tethering system. After surgery, each rat received subcutaneous injection of chloramphenicol sodium succinate (60 mg/kg) to prevent infection and carprofen (5 mg/kg) for pain relief. Rats received carprofen again 24 hrs later, and were allowed to recover for at least 3 weeks before testing began, to allow time for virus expression.

Optical inhibition and behavioral testing

Rats were habituated to the testing apparatus on 3 days for 1 hour each; rats were connected to a stainless steel tether which was attached to a fiberoptic rotary joint, which also acted as a fiber splitter (50:50 split ratio; Doric Lenses). The rotary joint was attached to a weighted arm intended to reduce slack in the tethering system. The rotary joint allowed free movement of the rats throughout the 38 x 38 x 30 cm chamber, which contained ~3 cm corn cob bedding, rat chow (~20 g), and *ad libitum* water. On each test day, patch cables were bilaterally attached to the rat's fiber implants via zirconia sleeves; patch cables were run through the tether

and connected to the rotary joint, which interfaced with a fiber-coupled 623 nm diode laser (100mW red laser; OEM Laser Systems). Based on the calibration of each rat's fiber implants, rats received optical illumination of NAc shell at powers ranging from 1-10 mW on test days spaced 48 hours, starting with lower power levels and increasing across test days. Rats received light stimulation at these powers according to some set of the following parameters (each on separate test days): 1) 10 minutes light off baseline, 10 minutes light on, 10 minutes light off, 2) 20 minutes light off baseline, 20 minutes light on, 20 minutes light off, 3) 30 minutes light on, 4) 30 minutes no light baseline, and 5) 10 minutes baseline, 10 minutes alternating 15 seconds light on and 20 seconds off, and 10 minutes off. Rats received a total of up 13 testing sessions. Behavior was visibly observed by the experimenter during testing, and videorecorded for later offline analysis.

Histology

Following behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused. Brains were removed and fixed in 4% paraformaldehyde for 4-24 hours and in 25% sucrose solution (0.1 M NaPB) for 3 days. Brains were sliced at 40 microns on a freezing microtome, mounted, air-dried and coverslipped with ProLong Gold antifade reagent (Invitrogen). Zones of viral expression were assessed via visualization of GFP fluorescence using a Leica DM6000 microscope (10x to 20x magnification).

Results and Discussion

Of the 5 rats initially tested for the effects of optogenetic inhibition of NAc shell, 3 showed robust ArchT virus expression in NAc shell, based on GFP fluorescence (Figure 5.4). Initial online observations revealed no elicitation of eating or defensive behaviors by red laser

(635nm) illumination of NAc shell in rats expressing ArchT. In general, levels of eating and defensive behaviors were near zero levels during both light-on and baseline conditions, potentially indicating that the optogenetic testing apparatus and condition were not conducive to the production of normal motivated behaviors, indicating the perhaps additional habituation or modification of the apparatus is needed. Future detailed analysis of videorecordings may reveal subtle changes in behavior that could not be detected during online observations. Illumination of ArchT in NAc shell neurons may have failed to generate behaviors in this initial test group in part because the wavelength used (635 nm) suboptimally (~30% of maximum) generates photocurrents at this opsin (Han et al., 2011). Future experiments will utilize a 532 nm laser (green; 100mW; OEM Laser Systems), which should generate photocurrents closer to 60% of maximum levels (Han et al., 2011). Additional incubation time may also be necessary for allow for better ArchT expression at the initial testing point; while ArchT expression was robust, brains were removed and assessed at least 2 weeks after the initial behavioral testing session.

Future Directions

Targeted inhibition of accumbens shell pathways with DREADDs or opsins

In dorsal striatum, selective manipulations of D1/dynorphin- and D2/enkephalin-containing neurons largely target the direct versus indirect pathways, as most neurons there express either D1 or D2 receptors, but not both (Gerfen et al., 1990; Surmeier et al., 1996; Humphries and Prescott, 2010). While the differing dopamine receptor requirements for eating and fear (Chapter 3) may differentially implicate specific NAc shell pathways, the relationship between specific motivational valences and particular output pathways is unlikely to be one-to-one, given the unique organization of D1 and D2 receptors on GABAergic medium spiny

neurons (Bertran-Gonzalez et al., 2008; Perreault et al., 2010). The NAc shell indirect pathway, which consists of medium spiny neurons projecting to the ventral pallidum, is composed of equal populations of predominantly D1 and D2 receptor-containing neurons (Lu et al., 1998; Zhou et al., 2003) as well as, likely, many of the neurons which show D1/D2 co-localization and heteromerization (Humphries and Prescott, 2010). Because both classes of dopamine receptors are present in this population, neurons projecting to ventral pallidum may be important for inhibition-elicited eating (which is in some cases D1-dependent), as well as defensive treading (which in some cases is D1 and D2 dependent) (Chapter 3; Richard and Berridge, 2011b). Ventral pallidum is capable of generating either valence of behavior: ventral pallidum DAMGO microinjections produce both eating and defensive treading depending on the particular anteroposterior location (Smith and Berridge, 2005). One might think the evidence is perhaps stronger for the potential involvement of this projection in fearful behaviors, given that both D1 and D2 receptors are required for DNQX-induced defensive behaviors. Yet, while eating produced by NAc shell mu-opioid stimulation, via DAMGO microinjection, is not blocked by ventral pallidal lesions or opioid blockade (Smith and Berridge, 2007; Taha et al., 2009), NAc shell muscimol-induced eating *is* prevented by ipsilateral lesion of the ventral pallidum (Stratford and Wirtshafter, 2012a). The NAc shell direct pathway, which consists of medium spiny neurons projecting to the ventral tegmentum, is composed of primarily D1-dominant neurons (Lu et al., 1998; Zhou et al., 2003), and may be critical for both eating and fear generated by NAc shell inhibition.

The dopamine receptor makeup of the other NAc shell projections, described previously, remain unknown. This information may be critical for understanding the mechanism underlying the different D1 versus D2 receptor requirements for eating and fear generated by glutamate

disruption. For instance, the lateral hypothalamus is critical for eating induced by either muscimol or DNQX in NAc shell (Maldonado-Irizarry et al., 1995; Stratford and Kelley, 1999; Stratford and Wirtshafter, 2012a). Additionally, caudal shell specific projections, including those to areas of the extended amygdala and the parabrachial nucleus, could be critical to the preferential generation of defensive behaviors by caudal shell inhibitions.

Because more than cell-type specificity is needed to target particular NAc shell efferents, one can combine viral expression in one neuronal structure, with light or drug infusion targeted to a particular output structure (Stuber et al., 2011; Tye et al., 2011; Stefanik et al., 2012). This approach could be used to target GABAergic projections to ventral pallidum, ventral tegmentum and lateral hypothalamus that originate throughout the rostrocaudal extent of medial shell (Mogenson et al., 1983; Zahm and Heimer, 1990; Heimer et al., 1991), but also caudal shell specific projections to areas of the extended amygdala, including the sublenticular gray, the central and medial amygdala and the ventrolateral bed nucleus of the stria terminalis, as well as potentially to the retrorubral area, the substantia nigra, and the parabrachial nucleus (Heimer et al., 1991; Zahm and Heimer, 1993; Usuda et al., 1998), and rostral shell specific projections to the lateral preoptic area (Usuda et al., 1998).

How else can we target these different output pathways?

Several other approaches can also be utilized to test the importance of individual output pathways from NAc shell in the generation of eating versus fear. To test for recruitment of particular brain structures by eating- versus fear-generating microinjections, distant Fos analysis can be conducted to determine which brain regions are selectively activated by microinjections that generate particular behaviors (based on rostral versus caudal microinjection locations or changes in the emotional environment) (such as in Stratford and Kelley, 1999; Will et al., 2003).

To measure whether these activated output structures *encode* particular aspects of behavior (i.e. eating or defensive treading), electrophysiology recording can be utilized to determine whether particular patterns of neural activity in these brain structures are associated with the generation of desire versus dread (such as in Smith et al., 2011).

It is also important to test the necessity or sufficiency of these output structures in the effects of NAc shell inhibition. Unilateral microinjections of either DNQX or muscimol into NAc shell can be paired with lesions or temporary inactivation of specific output structures ipsilateral to the injection sites (with contralateral lesions/inactivations as controls), to test whether particular output structures are *necessary* for DNQX or muscimol induced behaviors (such as in Taha et al., 2009; Stratford and Wirtshafter, 2012a). This can alleviate problems caused by the more general necessity of specific structures for baseline levels of eating and fear and help to isolate their necessity for NAc-inhibition elicitations of intense eating and fear. For instance, bilateral lesions of ventral pallidum cause severe aphasia (loss of eating) and aversive 'disliking' reactions to tastes that were previously liked (Cromwell and Berridge, 1993); unilateral lesions have more limited effects and leave baseline eating relatively intact, but still prevent the elicitation of eating by ipsilateral NAc shell muscimol (Stratford and Wirtshafter, 2012a). Yet, this approach cannot inform us as to the relative importance or *sufficiency* of specific, direct projections from NAc shell, such as can be investigated using optogenetics or pharmacogenetics.

Other future directions: Clarifying the role of dopamine receptor subtypes

In Chapter 3 of the dissertation I examined the relative roles of D1-like versus D2-like dopamine receptors, but many questions remain regarding the role of different dopamine receptors subtypes, particularly within the D2-like family (D2, D3 and D4 receptors). D3

dopamine receptors in particular have been implicated in behaviors related to appetitive motivation including alcohol self-administration and reinstatement (Heidbreder et al., 2007) and cocaine self-administration (Song et al., 2012). While general D2 antagonism failed to block DNQX-induced eating in my experiment, D3 receptors may still play an important role in incentive motivation, especially reward seeking that relies heavily on learned cues (Beninger and Banasikowski, 2008). More selective manipulation of D3 receptors could be utilized to determine whether they are differently involved in the gradient effects reported here than D2-like receptors as a group.

Also deserving of further study are D1-D2 heteromer receptors, which are present on more than 30% of D1-expressing medium spiny neurons within medial shell (Perreault et al., 2010). These unique receptors are G_q coupled and therefore exert different cellular effects than either receptor type on its own (e.g. activation of phospholipase C) (Rashid et al., 2007). Within NAc, these receptors are located on neurons that co-express dynorphin and enkephalin (Perreault et al., 2010), likely projecting along the indirect pathway to ventral pallidum (Humphries and Prescott, 2010) or forming one of the NAc shell afferents that have not been characterized in terms of their dopamine receptor phenotype. These D1-D2 heteromers are upregulated by repeated amphetamine administration and in post-mortem brains of patients with schizophrenia (Perreault et al., 2010; Perreault et al., 2011). Based on importance of both D1 and D2 receptors for fearful behaviors reported here, as well as D1-D2 heteromer upregulation in people with schizophrenia, who may struggle with exaggerated fearful salience (Kapur, 2003), I hypothesize that D1-D2 heteromer receptors may be of particular importance for aversive motivation. While pilot testing indicates that a selective D1-D2 heteromer agonist may potentiate fearful behaviors generated by DNQX (unpublished observations), further investigation is needed, both into the

functional effects of these receptors on appetitive and aversive motivated behaviors, but also on their distribution along the rostrocaudal gradient in medial shell of NAc.

Examining changes in dopamine signaling

The importance of D1 in both appetitive and defensive motivation, and D2 in only fearful or defensive motivation may also indicate differing roles for phasic and tonic dopamine input to NAc shell in the generation of appetitive and defensive behavior, perhaps pointing to a role of phasic dopamine in eating, and both phasic and tonic for fearful motivation. D2-like receptors generally have a higher affinity for dopamine, and are thought to be constantly stimulated by basal, tonic release, whereas lower affinity D1-like receptors may require higher, phasic release in order reach sufficient levels of dopamine binding (Goto and Grace, 2005). This distinction, along with our current results, may indicate that eating induced by glutamate disruption may not rely on basal tonic dopamine, but instead might rely in particular on phasic (D1 receptor activating) dopamine inputs, whereas both phasic and tonic dopamine may be required to enable fearful motivation. We speculate that this could be a mechanism by which environmental ambience could alter the valence of motivated behavior produced by glutamate disruption: enhanced tonic dopamine release may, by preferentially enhancing D2-dopamine neurotransmission, biasing motivational valence toward fear in stressful environments, whereas in comfortable environments drops in tonic dopamine transmission might contribute to reduced defensive motivation and a bias towards appetitive eating behavior (Reynolds and Berridge, 2008).

To further investigate this hypothesis, it will be important to measure any changes in the nature of dopamine signaling in NAc shell in different emotional environments using a technique with sufficient temporal resolution to distinguish between tonic and phasic dopamine signals.

This can be accomplished using fast-scan cyclic voltammetry (FSCV), which has been used in NAc to measure real-time dopamine transmission during motivation-related behaviors (Robinson et al., 2003) The use of this technique in rostral versus caudal medial shell is somewhat limited, as norepinephrine, which is released mainly in caudal shell, cannot be distinguished from dopamine based on its cyclic voltammogram (Park et al., 2010). Therefore, reliable measurement of dopamine transmission can only occur in rostral shell. For this reason, it is important to keep in mind the heterogeneity of dopamine neurons, our understanding of which is still emerging. For instance, while dopamine neurons projecting to dorsolateral striatum and lateral shell of NAc tend to be "conventional" slow-firing dopamine neurons, dopamine neurons projecting to prefrontal cortex and to the core and medial shell of NAc tend to be less conventional, faster-firing neurons (Lammel et al., 2008). Additionally, while excitatory synapses onto dopamine neurons projecting to (rostral) medial shell are selectively modulated by cocaine administration (but not an aversive stimulus), synapses onto neurons projecting to lateral shell are modified by both cocaine administration and an aversive stimulus, formalin injection into the hindpaw (Lammel et al., 2011). While neither of these studies examined rostral versus caudal shell differences in terms of the characteristics of their dopamine afferents (they appear to have focused on projections to rostral medial shell), critical differences may exist, which should be kept in mind when interpreting any studies of dopamine transmission in rostral medial shell.

Neurobiological bases of infralimbic versus orbitofrontal top-down regulation

Further investigation is necessary to understand the mechanism by which prefrontal glutamate inputs to medial shell (as well as glutamate inputs from other sources) can modulate the valence or intensity of motivated behaviors generated by medial shell glutamate disruption. Regarding the infralimbic versus medial orbitofrontal effects reported in Chapter 4, two main

categories of explanation were hypothesized. Previous electrophysiological studies suggest that orbitofrontal and medial prefrontal glutamatergic inputs have nearly opposite effects on firing of individual neurons in medial NAc shell. As described in Chapter 4, NAc shell neurons tend to be excited by *either* orbitofrontal activation or infralimbic activation, but not both (Asher and Lodge, 2011). Instead, if a NAc neuron is excited by infralimbic cortex, it tends to be inhibited by orbitofrontal cortex. Conversely, if a NAc neuron is excited by orbitofrontal cortex, it tends to be inhibited by infralimbic cortex. This implies that neurons which receive monosynaptic excitatory inputs from one of these prefrontal inputs, only receive input from the other prefrontal structure via inhibitory interneurons (which reverse the polarity of the signal) (Asher and Lodge, 2011). This may provide one mechanism by which these two prefrontal regions can differently regulate intense motivations generated by medial NAc shell, via projections to the medial shell itself. Eating-related neurons in mid to rostral areas of NAc shell, which are hyperpolarized by rostral DNQX, may be excited by infralimbic activation, reversing DNQX-induced hyperpolarizations and suppressing eating. Yet these same neurons, perhaps only slightly hyperpolarized by middle or caudal DNQX, may be further hyperpolarized by orbitofrontal activations, acting via inhibitory interneurons, releasing eating which was not produced by DNQX alone.

In order to test the importance of inhibitory interneurons in the effects of medial orbitofrontal activation, one could selectively inactivate specific populations of interneurons using either pharmacogenetic or optogenetic approaches as new model organisms and viral promoters become available, or using selective pharmacologic approaches (Gittis et al., 2011). Electrophysiology recordings in NAc shell, following DNQX microinjection or DNQX plus prefrontal activations, would also shed critical light on potential mechanisms. To my knowledge, no recordings have been conducted in NAc shell following microinjections of DNQX or

muscimol into medial NAc shell (or any other region of NAc for that matter). While it is technically challenging to record from the site of a drug microinjection, similar experiments have been conducted by some laboratories and new technical advancements and methodological studies may make this more feasible in the future (Doherty and Gratton, 1999; Krupa et al., 1999; du Hoffmann et al., 2011).

Another important possibility is that infralimbic and medial orbitofrontal cortex exert their divergent effects on desire and dread generated by NAc shell glutamate disruption through wider networks, such as via parallel projections to third-party subcortical structures (including basolateral amygdala, lateral and medial dorsal hypothalamus or brainstem) (Freedman et al., 2000; Gabbott et al., 2005; Marchant et al., 2010; Hoover and Vertes, 2011; Millan et al., 2011). Medial orbitofrontal activation may potentiate eating via excitatory projections to basolateral amygdala or lateral hypothalamus (Hoover and Vertes, 2011), whereas infralimbic cortex may exert its motivation suppressing effects via projections to medial dorsal hypothalamus (Marchant et al., 2010; Millan et al., 2011). To test these possibilities, unilateral microinjections into NAc shell and prefrontal cortex could be utilized, with either ipsilateral (experimental condition) or contralateral (control) temporary inactivation of one of the structures of interest (basolateral amygdala, lateral hypothalamus medial dorsal hypothalamus), to test for the importance of these structures in the reported prefrontal-NAc interactions.

To selectively test for the importance of only those neurons in these third-party structures that are activated by infralimbic or medial orbitofrontal disinhibition, the Daun02 inactivation pharmacogenetic technique could be valuable (Koya et al., 2009; Bossert et al., 2011). This technique employs transgenic rats that express beta-galactosidase in activated neurons (Kasof et al., 1995): 90 minutes after the relevant behavioral event, Daun02 is injected into the relevant

area, which beta-galactosidase converts into daunorubicin, which disrupts normal activity in these select neurons for 3 days (Farquhar et al., 2002; Koya et al., 2009; Bossert et al., 2011).

One could potentially also take advantage of the finding that GABAergic generation of eating and fear is less susceptible to both changes in the emotional ambience of the environment and local dopamine blockade (Chapter 2), both of which may modulate glutamate inputs to NAc shell (Faure et al., 2008; Reynolds and Berridge, 2008; Richard and Berridge, 2011b). Based on these findings, one might expect that muscimol-elicited behaviors would be less susceptible to prefrontal modulation acting via direct serial mechanism (within NAc shell itself), but could be vulnerable if prefrontal control is acting via a parallel, convergent mechanism.

Ability of other glutamate inputs to modulate valence or intensity of DNQX desire and dread

Non-prefrontal glutamate inputs to NAc shell, including from basolateral amygdala, hippocampus and thalamus, likely play different roles in generating or modulating the various psychological functions of the medial shell. For instance, while medial prefrontal cortex is normally highlighted for its role in behavioral flexibility (Sesack and Grace, 2010) and top-down inhibitory control such as following extinction (Peters et al., 2009), the amygdala has commonly implicated in fear learning (Fanselow and LeDoux, 1999; Maren and Quirk, 2004; Paré et al., 2004), but also in linking sensory and emotional systems (LeDoux, 1993), as well as recognition and orienting to motivationally relevant (rewarding and fearful), novel, uncertain or unusual stimuli (Gallagher et al., 1990; Holland and Gallagher, 1999; Davis and Whalen, 2001; Holland et al., 2002; Everitt et al., 2003; Yun and Fields, 2003; Chang et al., 2012). Importantly, studies utilizing optogenetics have shown that mice will self-stimulate glutamate inputs from basolateral amygdala to the NAc, despite their unwillingness to stimulate glutamate inputs from prefrontal cortex (Stuber et al., 2011). Activation of glutamate inputs from the hippocampus, which may be

the largest source of glutamate to the medial shell, will also support self-stimulation, as well as "real time" place preference, in which entries into a particular chamber are instrumentally paired with activation of these glutamate inputs (Britt et al., 2012). Pharmacological, pharmacogenetic and optogenetic techniques should be brought to bear on the questions of 1) how these various glutamate inputs modulate eating and fear generated by NAc inhibition along the rostrocaudal gradient, and 2) what role they may play in retuning caused by changes in environmental ambience.

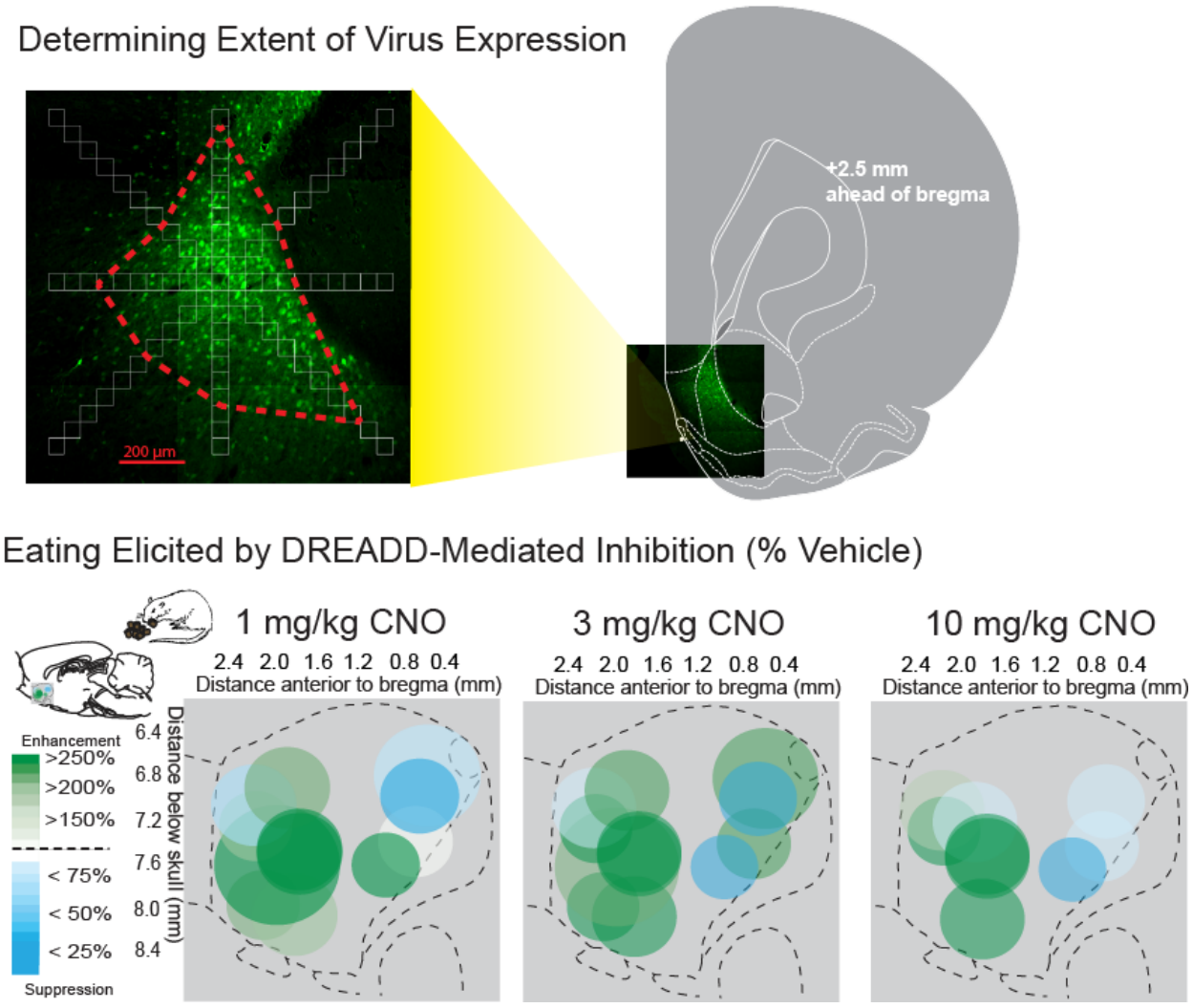


Figure 5.1. DREADD expression and maps of eating produced by DREADD-mediated inhibition. Image (top) shows example expression of DREADD virus in rostral medial shell, and the zones along each radial arm that were determined to have cell bodies positive for virus expression. Maps (bottom) show eating as a percent of vehicle levels after injections 1 mg/kg CNO (left), 3 mg/kg CNO (middle) and 10 mg/kg CNO (right). Injections of CNO generate increases in food intake and eating in rats expressing hM_4D DREADD receptors in rostral (n=8), but not caudal (n=4) medial shell.

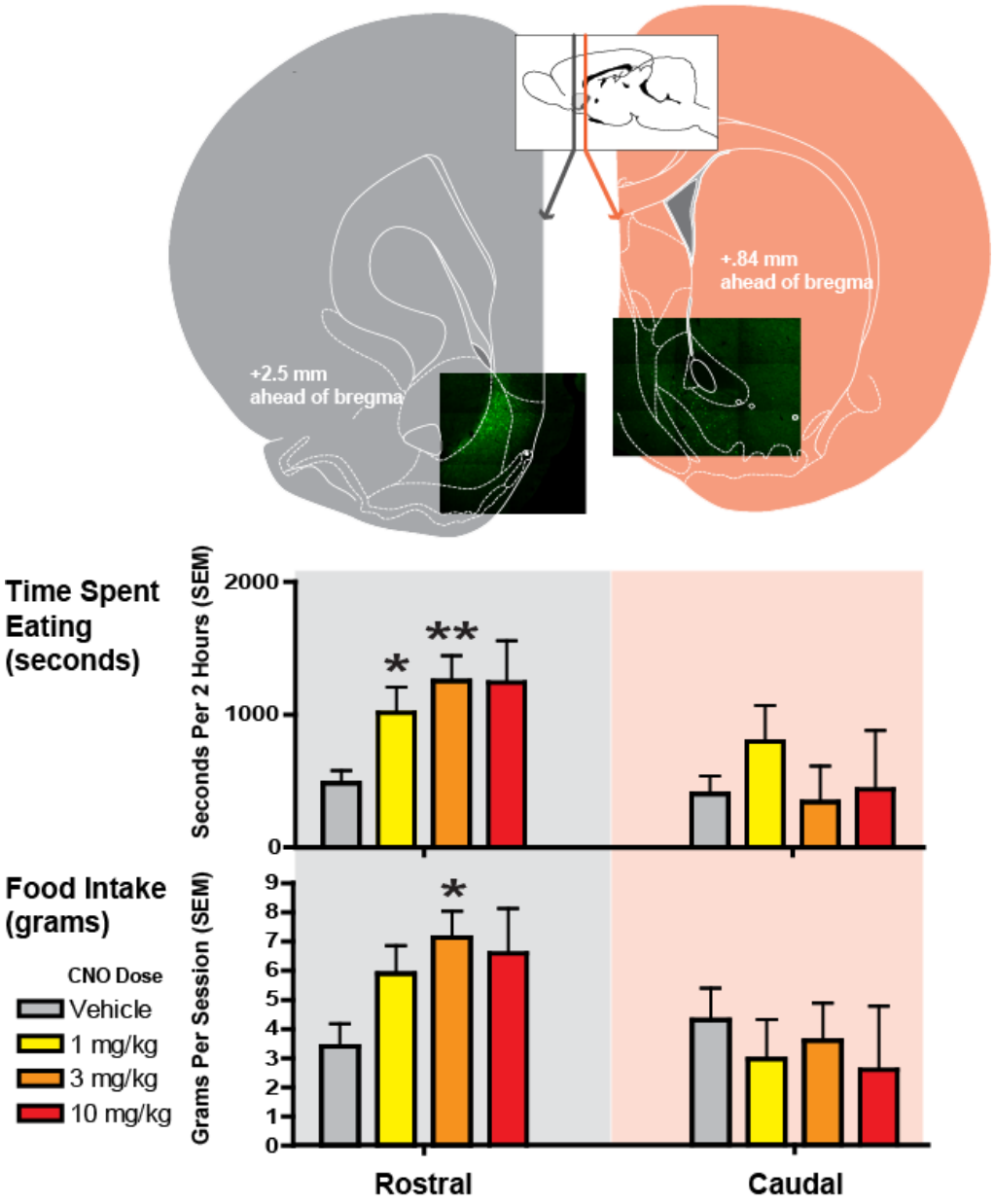


Figure 5.2. Graphs of effect of DREADD-mediated inhibition on food intake and eating behavior. Images (top) show example expression of DREADD virus in rostral and caudal medial shell, placed on the appropriate coronal slice. Graphs (bottom) show time spent eating (A), and amount of food intake (B) elicited by injections of CNO at doses of 0 (vehicle, grey), 1 (yellow), 3 (orange) and 10 (red) mg/kg. Injections of CNO generate increases in food intake and eating in rats expressing hM_4D DREADD receptors in rostral (n=8), but not caudal (n=4) medial shell. Errors bars indicate SEM, * $p < 0.05$, ** $p < 0.01$ change from vehicle, pairwise comparisons using Sidak corrections for multiple comparisons.

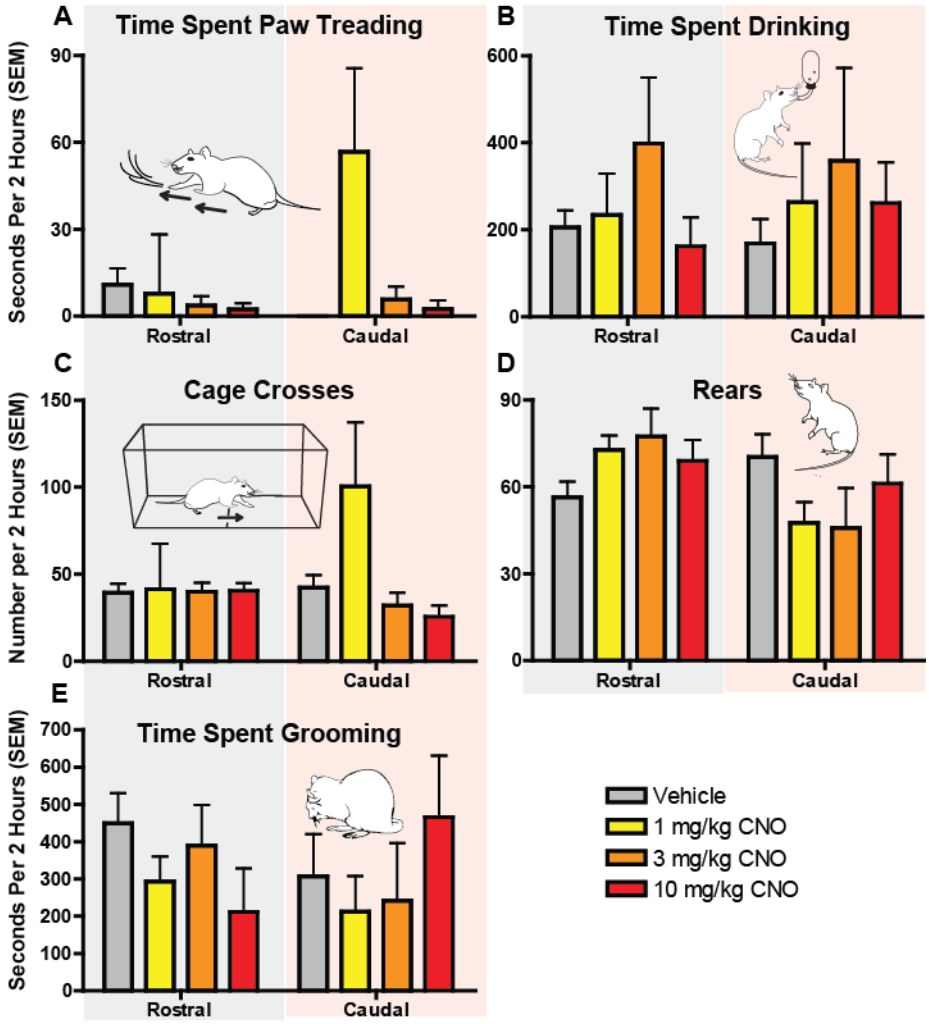


Figure 5.3. Graphs of the effect of DREADD-mediated inhibition on other behaviors. Bar graphs show time spent paw treading (A), time spent drinking (B), number of cage crosses (C), number of rears (D) and time spent grooming (E) elicited by injections of CNO at doses of 0 (vehicle, grey), 1 (yellow), 3 (orange) and 10 (red) mg/kg. Injections of CNO failed to generate significant changes in any of these behaviors, either in rats expressing hM_4D DREADD receptors in rostral (n=8) or caudal (n=4) medial shell. Errors bars indicate SEM, * $p < 0.05$, ** $p < 0.01$ change from vehicle, pairwise comparisons using Sidak corrections for multiple comparisons.

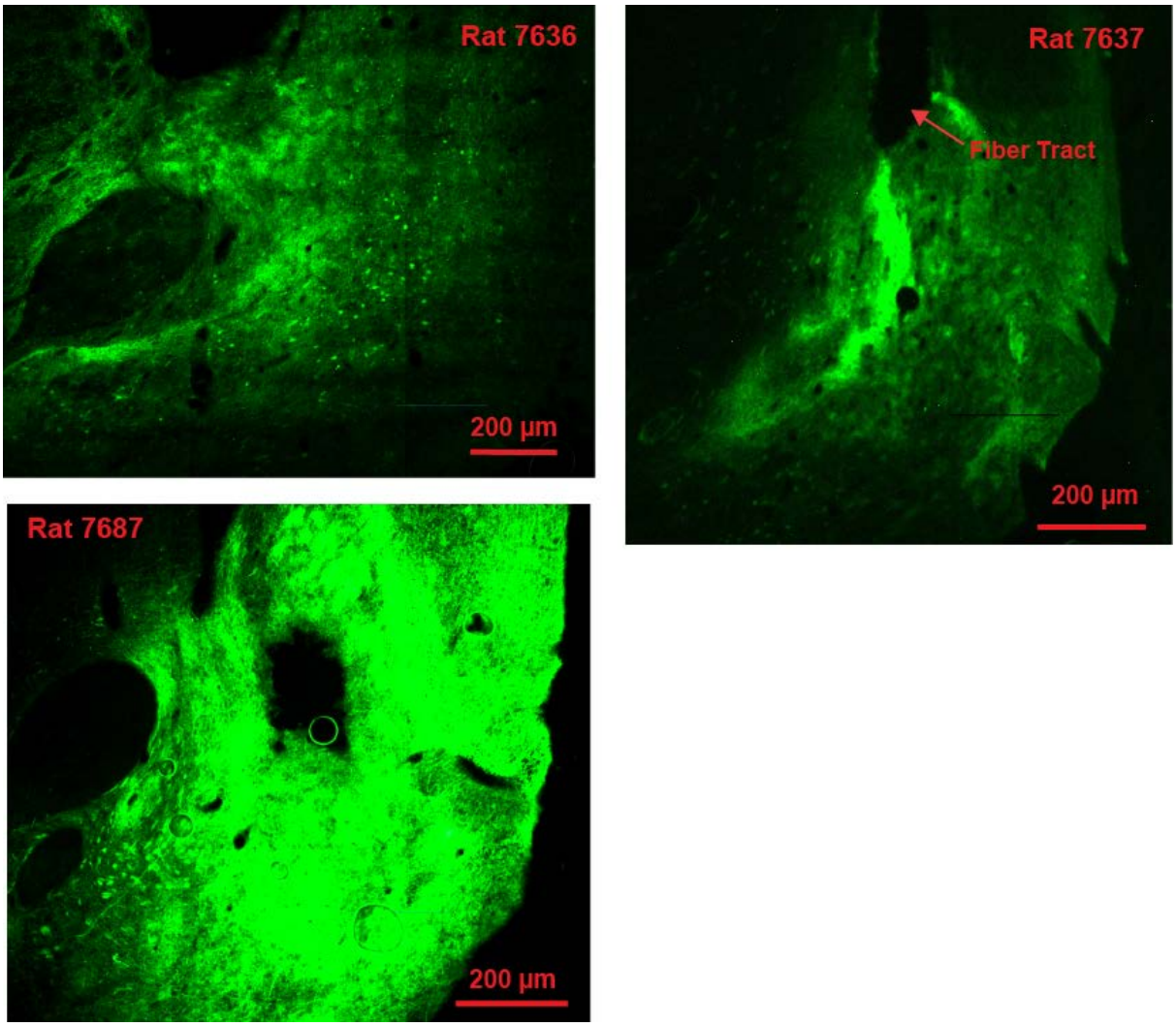


Figure 5.4. ArchT-GFP expression. Images of expression of ArchT virus in and around medial shell for rats 7636, 7637, 7687. Composite images are composed of tiled images taken at 10x magnification. Illumination of these sites with 635 nm light (1-10 mW) failed to generate detectable changes in motivated behaviors during online observations.

Chapter 6

General Discussion

The experiments described in this dissertation explored mechanisms that generate appetitive eating or fearful motivation along a rostrocaudal gradient in medial shell of nucleus accumbens (NAc).

Characteristics of GABAergic generation of fear and eating

In Chapter 2, I investigated whether desire and dread generated by subcortical GABAergic inhibition of NAc shell would share two characteristics with similar behaviors generated by corticolimbic glutamate disruptions (Faure et al., 2008; Reynolds and Berridge, 2008): 1) are eating and fear generated by GABAergic inhibition dependent on local endogenous dopamine signaling, and 2) can the valence of motivation generated by GABAergic muscimol be retuned by changes in the environment. To examine the potential dopamine-dependence of eating and fear generated by GABAergic muscimol, I combined a mixture of antagonists for D1-like and D2-like dopamine receptors (SCH23390 and raclopride, respectively) into the same microinjection as muscimol, to test whether muscimol would still generate intense eating in rostral shell, and defensive treading and fearful reactions to the experimenter after microinjections in caudal shell. I found that muscimol still robustly generated eating and fearful behaviors along the rostrocaudal gradient, even in the presence of local D1/D2 dopamine blockade.

To assess the second characteristic, of vulnerability to changes in environmental ambience, I measured the effects of corticolimbic glutamate blockade with DNQX and GABAergic inhibition with muscimol in three environments with very different emotional characteristics: 1) a "Standard" laboratory environment of relatively neutral valence with conventional fluorescent laboratory lighting and low ambient noise, 2) a "Stressful" laboratory environment intended to be over-stimulating and aversive, with additional bright lights illuminating the chamber and loud rock music playing continuously, and 3) the "Home" environment which was intended to be familiar and comfortable, taking place in the rats' actual home-room with low red lighting and ambient noise. As reported previously (Reynolds and Berridge, 2008), the valence of motivation generated by DNQX was powerfully shifted by changes in emotional ambience of the testing environment. Testing in the Stressful environment expanded the zone where DNQX generated fearful behaviors, nearly doubling the area where defensive treading was produced, and quadrupling the area where DNQX caused animals to react fearfully to the experimenter. Conversely, testing in the Home environment reduced the size of the defensive zone to just 10% of sites studied (one-third of those that produced fear in the Standard environment), nearly eliminated DNQX-induced defensive reactions to the experimenter, and more than doubled the zone where DNQX produced purely appetitive behavior to more than 75% of medial shell.

In contrast, the valence of motivation generated by GABAergic muscimol seemed relatively resistant to changes in emotional ambience, and remained consistent in its anatomical coding across the three environments. While defensive treading generated by muscimol was reduced by the Home environment, this may have been due to the removal of most of the visual cues that defensive treading is generally directed toward (Reynolds and Berridge, 2001). Testing

in the Home environment had no effect on the number of sites where muscimol caused rats to react defensively to the experimenter or on the number of sites where muscimol produces appetitive behaviors. Testing in the Stressful environment had no effects on the distribution of sites where muscimol generated appetitive or defensive behaviors, or on the intensity of these behaviors. Muscimol consistently generated appetitive eating in rostral areas of NAc shell and active fearful behaviors in caudal NAc shell. The resistance of eating and fear generated by GABAergic muscimol to both loss of local dopamine signaling and changes in emotional context can help give us insight in the neurobiological mechanisms by which appetitive eating can be flipped into aversive fear, and vice versa.

Corticolimbic glutamate and mesolimbic dopamine signals in environmental appraisal

The relative autonomy of GABAergic fear and eating implies that changes in motivational valence related to environmental appraisal may specifically require changes in "top-down" corticolimbic signaling. Conversely, GABAergic manipulations may tap into circuits that are more intrinsically subcortical, and more robustly coded by anatomy within the localized manipulation, changes in the emotional environment may not as powerfully modulate fear and desire generated by this subcortical GABAergic form of motivation release. As described in Chapter 2, potential mechanisms for the retuning of motivational valence include changes either in the pattern, intensity or anatomical distribution of corticolimbic glutamate afferent signals themselves, including from prefrontal cortex, amygdala and hippocampus (Beckstead, 1979; Christie et al., 1987; Fuller et al., 1987; Sesack et al., 1989; McDonald, 1991b; Gill and Grace, 2011), or in neuromodulator signals that influence medial shell glutamate, but not GABA function including dopamine as well as other signals, potentially including norepinephrine, corticotrophin-releasing factor, serotonin, or other valence-related neuromodulators (Chronister

et al., 1980; De Souza et al., 1985; Voorn et al., 1989; Jacobs and Azmitia, 1992; Berridge et al., 1997; Delfs et al., 1998). Potential differences in the dopamine requirements for eating and fear generated by glutamate disruption were investigated in Chapter 3.

D1 versus D2 dopamine receptors in the generation of eating and fear by corticolimbic glutamate disruption in medial shell of nucleus accumbens

In Chapter 3 of this dissertation (Richard and Berridge, 2011b), I focused on corticolimbic glutamatergic generation of motivations, and investigated whether D1 versus D2 dopamine receptors played different role in the dopamine/DNQX interaction involved in the generation of appetitive versus fearful motivated behaviors (via DNQX) in medial shell. I combined either D1 or D2 antagonists alone individually (or both combined together) into the same microinjection as DNQX, to test whether DNQX could still generate eating or defensive behaviors in the presence of selective local dopamine blockade. I found that while both eating and fear generated by corticolimbic glutamate blockade in NAc shell required local endogenous dopamine, as expected (Faure et al., 2008), appetitive eating generated by rostral glutamate disruption requires only D1-like dopamine signaling, whereas fearful motivated behaviors generated by caudal shell require both D1 and D2 dopamine neurotransmission.

Taken together with the rostrocaudal anatomical influence that biases the valence of DNQX-generated behavior, these results might suggest that rostral shell function is predominated by D1 signaling whereas caudal shell might involve more D2 signaling. But that conclusion would be too simple. Subsequently, I showed this using the flexibility of intermediate rostrocaudal sites, where environmental ambience can flip the valence of DNQX-induced behavior – from appetitive in a comfortable, familiar environment to defensive in a bright, loud, stressful environment (Reynolds and Berridge, 2008) – to tease apart valence and rostrocaudal

location. I found that the generation of defensive behavior (in a stressful environment) requires endogenous D2 activation, even at rostral sites, whereas hyperphagia produced at the same sites in the familiar environment was invulnerable to D2 blockade. In other words, the current local dopamine "mode" of a particular site of corticolimbic blockade in NAc shell can shift depending on the valence of motivated behavior being generated at a given moment: the same site that needs D2 dopamine transmission to enable DNQX to generate fear, does not need D2 dopamine receptors to enable DNQX to generate eating. These types of mode shifts may be critical for changes in motivational valence generated by corticolimbic glutamate blockade, but may have no effect on robust, hedonic subcortical generation of motivation, such as eating and fear generated by GABAergic muscimol, which does not depend on local dopamine signaling and is resistant to changes in the emotional valence of the current environment (Chapter 2).

Potential implications for accumbens shell glutamate inputs

While D1 versus D2 differences may initially bring to mind implications for the different NAc shell output pathways, which will be discussed below, D1 versus D2 dopamine receptors may also select for or inhibit particular sources of glutamate inputs coming into NAc shell. For instance, D1 receptor agonism on corticolimbic terminals in NAc is reported to presynaptically attenuate excitatory post-synaptic potentials (Pennartz et al., 1992; Nicola et al., 1996), including those evoked by activations of the amygdala and hippocampus, though excitations evoked by a subset of hippocampal neurons (14%) are actually potentiated by D1 dopamine stimulation likely via actions on hippocampal-NAc terminals (Charara and Grace, 2003). Similarly, D2 dopamine receptors are also reported to presynaptically attenuate some excitatory post-synaptic potentials (Bamford et al., 2004), including excitations evoked by prefrontal cortical activations (Brady and O'Donnell, 2004). We have previously hypothesized that the retuning of motivational valence of

behavior generated by NAc shell glutamate disruptions may be related to changes in the distribution or intensity of specific glutamate inputs to NAc shell (Chapter 2; Reynolds and Berridge, 2008). Therefore, I became interested in testing the influence of different glutamate inputs to NAc shell, in particular those from prefrontal cortex.

Prefrontal cortical modulation of desire and dread generated by glutamate disruptions in medial accumbens shell.

In Chapter 4 of this dissertation (Richard and Berridge, 2012), I investigated the effects of activation or inhibition of specific areas in medial prefrontal cortex on eating and fear generated by NAc shell glutamate disruption. Specifically, I focused on three regions of prefrontal cortex: infralimbic cortex, medial orbitofrontal cortex, and prelimbic cortex. If D2 receptor antagonism prevents DNQX-induced fear via effects on presynaptic glutamate, including the *potentiation* of prefrontal inputs, then one might expect activation of prefrontal inputs to NAc shell to selectively reduce DNQX-induced fearful behaviors, leaving DNQX-induced desire intact. Yet, prefrontal inputs to NAc have been primarily implicated in the positive and negative control of *appetitive* behaviors, including food and drug seeking (Ishikawa et al., 2008b; Peters et al., 2008; Bossert et al., 2012; Ghazizadeh et al., 2012; LaLumiere et al., 2012; Stefanik et al., 2012), potentially indicating that prefrontal cortex may exert top-down inhibitory control over appetitive behaviors generated by NAc inhibitions. I found that infralimbic activation, with the $GABA_A$ antagonist bicuculline, generally suppressed both appetitive and fearful motivated behaviors generated by NAc shell glutamate disruptions. In contrast, activation of medial orbitofrontal cortex, just rostral to infralimbic, selectively potentiated eating generated by NAc glutamate disruptions, in particular from those sites where DNQX generated less eating on its own (intermediate and caudal locations). Medial orbitofrontal

activation had no effect on DNQX-generated fearful behaviors. I found that activation of prelimbic cortex, just dorsal to infralimbic, had no effect on either appetitive or fearful behaviors generated by NAc shell DNQX. Additionally, *inhibition* of each prefrontal region had no effect on the ability of NAc shell DNQX to generate either eating or fear.

My infralimbic findings are consistent with the idea that infralimbic cortex activation might generally suppress the subcortical generation of intense motivations of either positive or negative valence, as described in Chapter 4. Infralimbic suppression of subcortical motivation is consistent with findings in rodent studies that infralimbic inhibition promotes behavioral reinstatement of motivated behaviors following their extinction, including appetitive seeking of cocaine and food rewards (Rhodes and Killcross, 2004; Ishikawa et al., 2008a; Peters et al., 2008; Ghazizadeh et al., 2012), and conditioned fear responses (Milad et al., 2004; Vidal-Gonzalez et al., 2006; Peters et al., 2009), but I extend these findings to infralimbic suppression of intense unconditioned appetitive and fearful motivations, which do not depend on learning. The non-selective suppression of both appetitive and fearful motivated behaviors by infralimbic activation may suggest that infralimbic inputs cannot act as a mechanism for the retuning of motivational valence by emotional environments. Yet, subsets of neurons within each of the structures projecting to NAc shell may be capable of generating seemingly opposite effects to that generated by the structure as a whole: for instance, re-activation of a subset of neurons in ventromedial prefrontal cortex (roughly corresponding to infralimbic cortex) which respond to a drug-associated context, is required for that same context to provoke drug relapse (Bossert et al., 2011), suggesting that some neurons in infralimbic cortex promote intense motivated behaviors, rather than police them.

My medial orbitofrontal findings are consistent with the putative role for this cortical area in reward and pleasure. In studies of human orbitofrontal cortex, mid-anterior regional activation has been associated with subjective pleasantness of a variety of rewards, including components of natural rewards like food, such as odor, taste, and fat content (de Araujo et al., 2003; de Araujo and Rolls, 2004; Kringelbach, 2005; Grabenhorst and Rolls, 2009), as well as the pleasurable effects of drugs (Vollm et al., 2004) and music (Blood and Zatorre, 2001). A selective role for orbitofrontal cortex in positive incentive emotion seems consistent with reports that mid-anterior orbitofrontal cortex activation in the human specifically codes positive hedonic impact of sensory pleasures (Kringelbach and Rolls, 2004; Kringelbach, 2005). Medial orbitofrontal cortex, and especially its mid-anterior zone, is an important node for registering hedonic processing that corresponds to subjective pleasure ratings (Kringelbach, 2005). Medial orbitofrontal activation may exert top-down positive control of medial shell responses in reward related tasks; for instance, functional connectivity between medial orbitofrontal cortex and medial shell predicts persistent responding for monetary reward in the face of uncertainty (Jung et al., 2010).

While I found no effect of prelimbic activation or inhibition in my study, it is important to note that prelimbic cortex has clearly been implicated in the generation of both appetitive and fearful conditioned behaviors in the reports of others (McFarland and Kalivas, 2001; Capriles et al., 2003; McFarland et al., 2004; Di Pietro et al., 2006; Vidal-Gonzalez et al., 2006; Corcoran and Quirk, 2007; Burgos-Robles et al., 2009) and may play a greater role in behaviors associated with its projections to the amygdala and NAc core (Sesack et al., 1989; Brog et al., 1993; Sutton et al., 2003; Gabbott et al., 2005; LaLumiere and Kalivas, 2008), as opposed to the intense unconditioned motivated behaviors generated here by NAc shell inhibitions. My findings do not

conflict with these reports. Additionally, while medial orbitofrontal and infralimbic activation only amplified or inhibited subcortically generated behaviors in this paradigm, other manipulations in these regions may be capable of generating motivation, rather than solely modulating behaviors induced by subcortical nuclei. For instance, opioid stimulation in ventromedial prefrontal cortex generates robust eating behavior (Mena et al., 2011), and human imaging results suggest that endogenous opioid release in medial orbitofrontal cortex may mediate the rewarding effects of alcohol consumption (Mitchell et al., 2012).

Role of other neuromodulators in eating and fear generated by accumbens shell inhibition

While the experiments in this dissertation focused on the neuromodulatory effects of dopamine on the rostrocaudal gradient, many other neuromodulatory systems act within NAc shell to generate changes in motivation and affect, potentially interacting with our rostrocaudal gradient. Many of these neurochemicals or receptors are organized differentially along the rostrocaudal gradient. Dopamine inputs from ventral tegmentum, which have been implicated in the generation of both incentive and fearful salience, are potentially much more homogenous in rostral shell, and weaker or more heterogenous in caudal shell (Chronister et al., 1980). Dopamine receptors may also have a lesser presence in caudal shell; D1 receptor mRNA levels are fairly consistent at more rostral and middle levels of shell, but abruptly drop off at the most caudal levels (.7 mm ahead of bregma), while the concentration of D2 receptor mRNA gradually declines from rostral to caudal shell (Bardo and Hammer, 1991). Levels of mRNA for the endogenous opioid enkephalin, associated with pleasure and reward (Peciña and Berridge, 2005; Katsuura et al., 2011), follow a similar pattern, with higher levels in rostral than caudal shell (Voorn and Docter, 1992). Caudal shell, in contrast, receives stronger innervation from midbrain cholecystokinin-containing neurons (Zaborszky et al., 1985), from serotonin neurons originating

in the dorsal raphe nucleus of the brainstem (Zhou et al., 1991; Jacobs and Azmitia, 1992) and from norepinephrine neurons originating in the locus coeruleus (Berridge et al., 1997; Delfs et al., 1998; Park et al., 2010). Caudal shell also exhibits higher levels of cholinergic neuronal markers, choline acetyltransferase and acetylcholinesterase (Meredith et al., 1989), as well as higher levels of vasopressin and oxytocin receptors (Tribollet et al., 1988; Veinante and Freund-Mercier, 1997).

Metabotropic glutamate receptors in nucleus accumbens desire and dread

Metabotropic glutamate (mglu) receptors emerged during the past decade as potential targets for the treatment of psychopathologies related to both appetitive and fearful motivation (Schoepp, 2001; Kenny and Markou, 2004). Agonists for the Group II subtypes of mglu receptors (which includes mglu receptors 2 and 3) are of interest for their ability to presynaptically regulate glutamate, dopamine, and GABA release (Cartmell and Schoepp, 2000; Schoepp, 2001; Chaki et al., 2006; Karasawa et al., 2006) and have been shown to exert effects relevant to the treatment of both addiction and schizophrenia (Markou et al., 2004; Bossert et al., 2006; Liechti et al., 2007; Patil et al., 2007). Potential rostrocaudal differences in the expression of these receptors have not been formally investigated, though mglu 2 receptor expression may be strongest in rostral shell (Ohishi et al., 1998). I previously found that infusion of a group II antagonist into medial shell of NAc shifts the affective and motivational valence of rats in a negative direction, reducing appetitive eating of tasty M&M chocolate candies while generating defensive treading, and reducing 'liking' for a sweet sucrose solution while enhancing 'disliking' disgust reactions to that same solution (Richard and Berridge, 2011a). Rather than generate both appetitive and fearfully motivated behavior, mglu blockade appeared to preferentially elicit aversive motivation and affect. While pilot testing revealed limited effects of an mglu agonist on

spontaneous motivated behaviors (unpublished observations), perhaps because baseline behaviors in a standard laboratory environment already skew relatively appetitive, I hypothesize that adding an mglu agonist into the same microinjection as DNQX might have a similar effect to testing in a familiar, comfortable environment: reducing DNQX-generated fearful behaviors and therefore expanding the zone where DNQX generated purely appetitive behaviors.

Mu opioid signaling: Appetitive motivation, pleasure, and rostrocaudal differences

Opioid signaling, especially at the mu-opioid receptor, is primarily implicated in positive motivational and affective processes, including eating of palatable foods and associated sensory pleasure (Gosnell and Majchrzak, 1989; Zhang and Kelley, 2000; Peciña and Berridge, 2005). Regarding the NAc and striatum in general, mu-opioid stimulation enhances appetitive eating throughout the rostrocaudal extent of medial shell (Peciña and Berridge, 2005), as well as throughout the rest of NAc and more dorsal areas of striatum (Bakshi and Kelley, 1993b, a; Zhang and Kelley, 2000; DiFeliceantonio et al., 2012) and in striatal-like areas (Swanson, 2005) such as the central nucleus of the amygdala (Mahler and Berridge, 2009). Critically, mu opioid stimulation can also increase positive taste reactivity, or 'liking', responses, to infusions of sweet sucrose taste, but only when said opioid stimulation occurs in a rostrodorsal "hotspot" in medial shell (Peciña and Berridge, 2005), largely restricted to the rostral half of shell where GABA inhibition and corticolimbic glutamate blockade most effectively generate eating (Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2008; Faure et al., 2010; Richard and Berridge, 2011b). Outside of this rostrocaudal hotspot mu opioid stimulation is either ineffective at altering 'liking' responses to tastes, or decreases 'liking' in small, caudally located "coldspot" (Peciña and Berridge, 2005). This rostrocaudal differentiation may be related to the pattern of expression of enkephalin, an endogenous opioid likely involved in the normal generation of pleasure via the

mu-opioid receptor (Mansour et al., 1995). Enkephalin expression is densest in rostral shell, and gradually decreases down a rostral-to-caudal gradient in medial shell (Voorn and Docter, 1992). The same pattern may hold true for mu opioid receptor expression (Tempel and Zukin, 1987).

Caudal shell norepinephrine: Important for aversive motivation?

Norepinephrine, which primarily targets the caudal portion of medial shell (Berridge et al., 1997; Delfs et al., 1998; Park et al., 2010), is primarily associated with the generation of aversive motivation, as well as the generation of arousal and responses to stress (Aston-Jones et al., 1999; Koob, 1999; Berridge et al., 2012). For this reason norepinephrine may be critical for the generation of fearful behaviors by caudal shell inhibitions. Yet limited experiments have been conducted on the role of norepinephrine within NAc shell. NAc shell norepinephrine levels are decreased by sham feeding with sucrose (Hajnal and Norgren, 2004) and may be important for consolidating aversive memories (Kerfoot and Williams, 2011). Agonism at Group II mglu receptors results in decreases in PCP-induced locomotor activation (a model of schizophrenia) which are correlated with decreases in norepinephrine release in NAc shell (Swanson and Schoepp, 2003), perhaps indicated a role of norepinephrine in behaviors relevant to schizophrenia. Examining the effects of stimulation or blockade of norepinephrine receptors in caudal shell, both on baseline behaviors and DNQX-generated behaviors, could provide valuable new information.

Corticotropin-releasing factor: Aversive motivational signal or general salience promoter?

While corticotropin-releasing factor (CRF) is primarily associated with stress and aversive motivation, it has been implicated in both appetitive and aversive processes within the NAc shell. CRF injections into NAc can accelerate partner preference formation in monogamous voles in a manner dependent on both CRF1 and CRF2 receptors (Lim et al., 2007). NAc shell

CRF also enhances Pavlovian-to-instrumental transfer, in which a cue previously paired with reward produces bursts of enhanced instrumental responding for reward (Pecina et al., 2006). Yet, infusions of CRF into NAc shell can produce changes in behavioral measures related to anxiety and depression, including increased immobility in a forced swim test, reduced sucrose preference, reduced time spent on the open arms of an elevated plus maze, and reduced time spent in the center of an open field (Chen et al., 2012). These effects do not seem easily differentiated based on the rostrocaudal location within NAc shell where they are elicited: Pavlovian-to-instrumental transfer is enhanced from the same caudal shell locations where CRF produces the anxiety or depression-like effects (Pecina et al., 2006; Chen et al., 2012). Perhaps CRF is similar to dopamine (especially D1 receptor signaling) in that it may promote behaviors related to both incentive and fearful salience, and may have no effect on the nature of the rostrocaudal eating to fear gradient described here. CRF signaling may still differentially promote desire versus dread, perhaps in an anatomically biased way, or via differential activation of CRF1 and CRF2 receptors, which may play different roles in motivation and affect (Bruchas et al., 2009). Recently, Lemos and colleagues (2012) demonstrated that CRF effects could flip from appetitive to aversive following severe stress, via a mechanism that may depend on changes in NAc dopamine. Future experiments could test the effects of enhanced CRF on the valence of behavior elicited by DNQX, or the effects of selective agonism/antagonism of CRF1 and CRF2 receptors to investigate the potential role of this polypeptide hormone in flips in motivational valence.

Oxytocin, vasopressin and serotonin in caudal accumbens shell

Other neurochemicals worth considering for their role in fear and feeding produced in NAc shell are oxytocin, vasopressin and serotonin, which may play a more dominant role in caudal shell, thanks to a greater density of receptors or greater neurochemical innervation by

these systems (Tribollet et al., 1988; Zhou et al., 1991; Jacobs and Azmitia, 1992; Veinante and Freund-Mercier, 1997). All three are generally implicated in positive affect and motivation, and work in the NAc has identified roles in maternal behavior and partner preference formation in the case of oxytocin (Liu and Wang, 2003; Olazabal and Young, 2006; D'Cunha et al., 2011) and drug and food seeking behavior in the case of serotonin (Pratt et al., 2012; Yoshimoto et al., 2012). Yet, their caudal shell dominance may indicate a potential role in aversive motivation as well; for instance, overexpression of serotonin-1B receptors in NAc shell potentiates both the appetitive and aversive properties of cocaine exposure (Barot et al., 2007).

Potential involvement of drug diffusion into neighboring structures: striatum, septum and the bed nucleus of the stria terminalis

Initial work by Kelley and colleagues found that intense eating generation by amino acid disruptions was very specific to medial shell of NAc. Basso and Kelley (1999) examined the effects of muscimol in various subregions of the NAc shell, and found that muscimol only generated eating when microinjected in medial, but not lateral or ventral shell. Eating induced by DNQX is also very specific to medial shell of NAc, as microinjections of DNQX in the NAc core, ventromedial striatum, ventrolateral striatum and dorsomedial striatum are all ineffective at generating the intense eating elicited by DNQX in medial shell (Kelley and Swanson, 1997). Additional experiments in our laboratory (Reynolds and Berridge, 2003) further demonstrated DNQX microinjections in the NAc core do not generate the intense rostrocaudal gradient of eating in rostral shell and fearful behaviors in caudal shell that is normally generated by medial shell DNQX microinjections, though milder levels of eating (along no gradient) and fear (in a reverse gradient) were generated at the highest doses. Thus, it is highly unlikely that the

rostrocaudal gradient effects reported here and previously are due to diffusion into neighboring regions of ventral or dorsal striatum.

It is also unlikely that drug diffusion into the neighboring medial and lateral septum is responsible for the production of eating versus fear along the rostrocaudal gradient. This possibility has been proposed based on the respective proximity of lateral septum to rostral shell and medial septum to caudal shell. If fearful behaviors elicited by caudal shell inhibition are due to diffusion to medial septum then one would expect medial septum inactivation to generate fearful behavior. Yet it appears almost the opposite is true: temporary medial septal inactivation is reported to decrease behaviors related to fear and anxiety, suppressing defensive burying and increasing open arm exploration in an elevated plus-maze (Degroot and Treit, 2004). In contrast, lateral septum lesions are purported to have anxiogenic effects, including decreasing licks during a water-lick conflict test (Yadin et al., 1993), potentially indicating that lateral septum is normally involved in the inhibition of anxiety. Overall, it is unlikely fearful behaviors induced by caudal shell inhibition are due to that subregion's greater proximity to medial septum.

Finally, the caudal most portion of the medial shell is continuous with the bed nucleus of the stria terminalis (BNST), a pallidal-like structure (Swanson, 2000, 2003, 2005) of the extended amygdala (de Olmos and Heimer, 1999; Alheid, 2003). Given that caudomedial shell may be a transition zone from the ventral striatopallidal macrosystem to the extended amygdala macrosystem (Zahm, 1998), it is not surprising that inhibitions of the BNST may elicit similar effects to that produced by NAc shell inhibition. In our lab, Eric Jackson (2009) found that muscimol microinjections in ventral BNST slightly decreased eating and generated intense defensive treading behaviors, somewhat similar to the effects of caudal shell muscimol. Additionally, muscimol microinjections in BNST suppressed hedonic 'liking' responses to

sucrose in a taste reactivity test, similarly to muscimol microinjections in caudal shell. Given these similarities, one might wonder whether the effects of caudal shell microinjections are simply due to drug diffusion into neighboring BNST. Yet, Fos plume mapping indicates that inhibitions that remain restricted to caudal shell (Faure et al., 2008; Faure et al., 2010) and that are in some cases primarily localized in rostral shell (Reynolds and Berridge, 2008; Richard and Berridge, 2011b), are capable of robustly generating fearful behaviors. Similarities between the effects of muscimol in NAc shell and BNST may simply be related to similar functional and neuroanatomical characteristics as members of the extended amygdala macrosystem.

What is the psychological nature of motivated behavior generated by amino acid disruptions in medial accumbens shell?

The desire and dread generated by DNQX or muscimol in NAc shell does not necessarily represent a prototypical, full emotional and motivational experience that we normally think of as fear or desire. Eating, defensive treading and fearful reactions to experimenter touch generated here may represent positive and negative affective or motivational "fragments". But these fragments may represent two sides of a generic motivational or affective process: for instance, unconditioned motivational salience. It is not clear that the motivational salience generated by these NAc shell inhibitions represents even a full version of normal motivational salience, such as may be produced by opioid or dopamine enhancements in NAc shell (Wyvell and Berridge, 2001; Pecina and Berridge, 2008). One reason for this discrepancy may be that while hyperpolarizations within NAc shell are *necessary* for the generation of motivational salience, excitation (or at least lack of inhibition) of discrete sets of NAc shell neurons may be necessary for the targeting of motivation towards learned cues or stimuli with particular sensory characteristics that may occur following learning or during particular appetite states.

Neuromodulators such as dopamine or opioids may be more capable of enhancing this selective mixture of inhibitions and excitations for the full generation of incentive salience (Nicola et al., 2004).

The ability of corticolimbic glutamate blockade or GABAergic inhibition to enhance incentive motivation in instrumental or Pavlovian paradigms remains controversial and insufficiently explored (Zhang et al., 2003; Wirtshafter and Stratford, 2010; LaLumiere et al., 2012; Stratford and Wirtshafter, 2012b). Under some circumstances, NAc shell muscimol may increase food seeking behavior in a progressive ratio test (Wirtshafter and Stratford, 2010), though not in others (Zhang et al., 2003). The effects of DNQX (or other AMPA antagonists) on food seeking in a progressive ratio paradigm remain to be explored, though NAc shell infusions of NBQX (another AMPA antagonist) are reported to have no effect on alcohol seeking on a progressive ratio schedule (Millan and McNally, 2011). This may be due to a selective effect of NAc shell amino acid disruptions on motivation related to food specifically: NAc shell muscimol microinjections that generate intense consumption of a sucrose solution actually decrease consumption of an ethanol solution (Stratford and Wirtshafter, 2011). The more flexible state of enhanced motivational salience potentially generated by DNQX may lend itself to broader potentiation of reward seeking (beyond simple consumption): for instance, pilot experiments by Sheila Reynolds in our laboratory indicated that NAc shell DNQX, but not muscimol, may potentiate the bursts of reward seeking observed in a Pavlovian-to-instrumental transfer paradigm (unpublished observations). NAc shell inhibition via $GABA_B$ receptor agonism may be similarly flexible, as these receptors primarily act by presynaptically inhibiting glutamate release (Meredith, 1999) or by modulating the effects of glutamate post-synaptically at NMDA receptors (Chalifoux and Carter, 2010, 2011). Consistent with this possibility, stimulation of $GABA_B$

receptors (via baclofen microinjection) potentiates second order instrumental responding for food reward, but $GABA_A$ receptor stimulation (via muscimol) does not (Pulman et al., 2012). Agonism at this receptor may generate more flexible motivational effects more similar to the effects of corticolimbic glutamate blockade with DNQX than to the robust, neuroanatomically rigid effects generated by $GABA_A$ stimulation (Chapter 2). In another comparison of muscimol and baclofen, Pulman and colleagues (Pulman et al., 2010) observed that animals that received muscimol appeared less responsive or vigilant to external cues, and remained in contact with food throughout almost the entire session, sometimes forcing "so much food into their mouths that they would often chew and gag for long periods before collecting the next pellet" (pg. 158). The intense fixation of behavior on the food pellets, at the expense of general activity, grooming and general vigilance, may result in an inability of rats that receive rostral shell muscimol to switch to defensive behaviors in an appropriate emotional context (Chapter 2). In contrast, rats that receive baclofen (and presumably corticolimbic glutamate blockade) may still retain enough flexibility in their behavior and attend to their environment, which may be critical for flipping between intense appetitive and defensive behavior across different environments.

How do we define the psychological components of motivation and affect?

Recent debate regarding the organization of emotions has used neuroscience research to pit brain-based basic emotions theories, or "locationist" theories of emotions (Panksepp, 1998; Calder, 2003), which assume that specific categories of emotions are respected by the brain and localized to discrete brain regions, against alternative theories, such as constructionist[2] or component/dimensional accounts of emotions (James, 1884; Wundt, 1897; Schacter and Singer,

[2] Constructionist, component and dimensional accounts of emotion have in common the assumption that brain areas involved in emotions are responsible for particular aspects, components or dimensions of emotional experience, and not for specific basic emotions.

1962; Russell, 1980; Sander et al., 2003; Oosterwijk et al., 2012). An important challenge to locationist theories, or brain-based natural kinds theories of emotion, is the large degree of overlap in brain systems mediating individual emotions (Barrett and Wager, 2006; Barrett et al., 2007b; Lindquist et al., 2012). Emotion related brain structures, including the anterior insula, amygdala, and NAc, have been reported to respond across multiple categories of basic emotions, in both humans and non-human animals (Sander et al., 2003; Barrett and Wager, 2006; Britton et al., 2006; Shabel et al., 2011). In a recent meta-analytical study, Lindquist and colleagues (2012) systematically examined whether activations of particular brain regions implicated in emotions, as indicated by changes in fMRI bold signal, were specific to particular basic emotions. They examined reported activations of structures implicated in emotion including the amygdala, anterior insula, orbitofrontal cortex, anterior cingulate cortex, and the periaqueductal gray, in comparison to regions implicated in executive function, language, and attention, including dorsomedial, dorsolateral and ventrolateral prefrontal cortex, anterior and medial temporal lobe, and retrosplenial cortex/posterior cingulate cortex. None of the brain regions studied were found to be activated specifically by one emotion category and not others (Lindquist et al., 2012).

LeDoux (2012) and others (Panksepp, 2007) have argued that similar responses to appetitive and aversive stimuli (or across other categories of stimuli) in fMRI studies do not necessarily indicate that the brain regions in question process these differently valenced stimuli in identical or even similar ways. Measuring activations at the brain structure level may obscure differences at the population or microcircuit level that can be observed with more sophisticated techniques (Lin et al., 2011). Panksepp (2000) has argued that more selective manipulations of the brain provide support for the existence of the following "basic" emotions: seeking/expectancy, rage/anger, fear/anxiety, lust/sexuality, care/nurturance, panic/separation,

and play/joy. In contrast, LeDoux argues that if we focus on distinct neuronal populations or microcircuits, then we should see evidence for specific "survival circuits" or circuits in the brain dedicated to particular immediate and long-term life-sustaining functions (LeDoux, 2012).

One such brain region described by LeDoux, where separate neuronal populations are purported to mediate separate survival circuits, is the basolateral amygdala. Yet while there is some evidence that distinct populations of neurons in the basolateral amygdala encode appetitive versus aversive events, it appears that a significant population of neurons respond to stimuli of both appetitive and aversive valence (Paton et al., 2006; Belova et al., 2008; Shabel et al., 2011). For instance, the activity of "fear responsive" neurons in the basolateral amygdala is more similar in response to oral infusions of a disgust-eliciting solution (previously paired with nausea-inducing lithium chloride), than it is to neuronal activity during hedonically pleasant infusions (Shabel et al., 2011). Yet, this difference only becomes significant when a population of neurons that responds to all three stimuli (fearful, disgusting and rewarding), perhaps encoding emotional salience, is removed from the analysis (Shabel et al., 2011). Critically, the activity of amygdala neurons in response to appetitive and aversive stimuli is more similar than would be expected by chance (e.g. more similar than what you might expect if separate populations of amygdala neurons were functioning as part of separate survival circuits or selectively mediating specific basic emotions) (Shabel and Janak, 2009). Even if the dominant pattern of activity in the amygdala (or other emotional-relevant brain structures) proves to separately encode appetitive and aversive stimuli, encoding of generic affective valence does not necessarily provide support for a survival circuit view. Given the very different behavioral responses required by animals in response to disgusting or aversive tastes (i.e. avoidance or rejection) versus actively dangerous or fearful stimuli (i.e. freezing, escape, active defensive

reactions) one might expect these stimuli to activate very different survival circuits. It is important to know not only how appetitive versus aversive stimuli are processed, but how different types of appetitive or aversive stimuli are processed, in order to understand whether differences in encoding are due to engagement of different survival circuits, encoding of valence, or other possibilities. For instance, are sexual and food stimuli processed more similarly than sexual and fearful stimuli? What about in comparison to fear and disgust stimuli? Some of the aforementioned studies have attempted to answer similar questions, but more work is needed.

Conclusions

Given the strong association of the NAc in general, and the medal shell in particular, with psychological processes related to positive motivation and affect (Ikemoto and Panksepp, 1999; McBride et al., 1999; Wyvell and Berridge, 2000; Kelley and Berridge, 2002; Peciña and Berridge, 2005; Mahler et al., 2007), it is still surprising that fearful behaviors can even be generated by manipulations of this brain region. Yet caudal shell inhibitions robustly generate defensive reactions to the experimenter as well as spontaneous defensive treading, and the rostrocaudal gradient of eating and fear generated by NAc shell amino acid disruptions has been repeatedly replicated (Reynolds and Berridge, 2001, 2002, 2003; Faure et al., 2008; Reynolds and Berridge, 2008; Faure et al., 2010; Richard and Berridge, 2011b, 2012). Yet many questions regarding *how* NAc shell DNQX or muscimol can generate eating versus fear remain. One category of potential inquiry concerns the role of general inhibition, which should release activity in downstream structures in the lateral hypothalamus, ventral pallidum, ventral tegmental area and other outputs (Taber and Fibiger, 1997; Stratford and Kelley, 1999; Meredith et al., 2008; Stratford and Wirtshafter, 2012a), versus the role of selective activation of a subset of NAc shell neurons via either lateral disinhibition or changes in the AMPA/NMDA ratio (Cull-Candy

and Leszkiewicz, 2004; Mao and Massaquoi, 2007; Faure et al., 2008; Tepper et al., 2008; Hull et al., 2009), perhaps resulting in a type of "contrast enhancement" (Nicola et al., 2004; Wolf et al., 2005; Moyer et al., 2007). While eating and/or fear are generated by a variety of inhibitory-type manipulations of NAc shell, including the glutamate blockade and GABA agonism described here, stimulation of mu-opioid receptors (Zhang and Kelley, 2000), and DREADD-mediated inhibition (Chapter 5), none of these manipulations definitively control for or rule out the importance of alterative mechanisms, including more selective excitations/inhibitions of particular neuronal ensembles or alternative pharmacological or network-related effects. More selective inhibitions of NAc shell efferents to particular output structures, using pharmacogenetic DREADDs or optogenetic techniques, could be useful in isolating the effects of inhibition of particular outputs from the disinhibitory effects these neurons may have on neighboring NAc shell neurons following manipulations within NAc shell.

Another important category of remaining inquiry concerns the role of particular neuronal ensembles in NAc shell in eating versus fear: do NAc shell inhibitions generate fear versus desire by acting on "labeled lines" or particular neurons, circuits or anatomical subregions specifically dedicated to appetitive versus incentive behaviors? Or do the same anatomical components of NAc shell mediate a shared psychological component that contributes to both fear and desire? The "flippability" of DNQX-generated behavioral valence, which can be flexibly remapped within the same anatomical area (Reynolds and Berridge, 2008), as well as the shared importance of endogenous dopamine for DNQX-generated eating and fear (Faure et al., 2008) hint at the likelihood of shared psychological or anatomical components. Yet the valenced anatomical rigidity of GABAergic muscimol-induced behaviors (Chapter 2), the different dopamine receptor requirements (D1 versus D1 plus D2) for eating and fear induced by DNQX

corticolimbic glutamate blockade (Chapter 3; Richard and Berridge, 2011b), and potential valence selection by specific glutamate inputs to NAc shell such as medial orbitofrontal cortex (Chapter 4; Richard and Berridge, 2012), indicate that at least some aspects of NAc shell eating versus eating may be mediated by separable anatomical substrates or circuits. Perhaps some generic psychological/anatomical inputs are integrated within NAc shell, but NAc shell efferents carry appetitive versus fearful signals along labeled line pathways, at least to some degree. Research aimed at determining which neuroanatomical components are shared across desire and dread (in addition to D1 dopamine signaling and reductions in infralimbic inputs), and which neuroanatomical components are differentially important (in addition to D2 signaling and perhaps medial orbitofrontal cortex), can help in narrowing down the potential psychological components that might combine or separate to form eating versus fear.

It is important to attempt to isolate those signals involved in generic motivational salience signals and those that may select for particular behavioral valences. Most research focused on identifying the roles of specialized microcircuits in different aspects of motivation and affect have focused on recording from these circuits during predefined behavioral tasks or in response to particular stimuli (a subset of which is described above). It is also critical to understand the *causal* role of these defined neuronal populations, by activating (or inhibiting) these select microcircuits or neuronal ensembles, using new technical tools such as optogenetics and pharmacogenetics, to see what emotional or motivational fragments they might produce. By not only determining what brain regions or circuits are necessary for predefined psychological processes, but also to testing what psychological events or fragments are generated by activation or manipulation of these circuits, perhaps we can allow the brain to show us how it organizes motivation and affect.

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