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Los Angeles

A Neurogenetic and Genomic Characterization of Reward-Seeking Behaviors

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of
Philosophy in Psychology

By

Alexander Scott James

2014

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ABSTRACT OF THE DISSERTATION

A Neurogenetic and Genomic Characterization of Reward-Seeking Behaviors

By

Alexander Scott James

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2014

Professor Dr. J. David Jentsch, Chair

Because Pavlovian and instrumental processes enabling the prediction and pursuit of desired rewards, as well as the avoidance of dangers and noxious stimuli, they are integral to a wide range of behaviors that we exhibit. The studies presented here combine hypothesis-based studies with hypothesis-free research in order to provide novel insight into the reward-seeking behaviors that shape human behavior, and importantly, when expressed in extreme forms, are components of and risk factors for a range of psychiatric conditions. The first series of studies in this dissertation sought to disentangle the complex role of NMDA-mediated dopamine cell burst firing in reward-related learning, and to test causally, for the first time, predictions made by the incentive salience account of phasic dopamine function. While instrumental learning was impaired in our genetic model of attenuated phasic dopamine release, we found no evidence to support a selective dependency of incentive salience attribution on phasic dopamine release. Rather, our data are congruent with an integrative perspective on the role of phasic dopamine release in reward-driven learning, rather unitary theories of dopamine as a prediction error or incentive salience signal. The second study sought new genomic determinants of variation in reward seeking, using association-level genome-wide scans in an inbred mouse strain panel combined with linear mixed effects modeling, and yielded several quantitative trait loci related to quantitative variation in instrumental responding for sucrose. Transcriptomics data was used to for prioritizing quantitative trait loci genes for future causal studies, and offered a resource for

the development of new hypotheses regarding the neurobiology that links genes to behavior in the context of reward-related learning. Moreover, the specific pattern of transcriptomics and genomics data acquired was suggestive of a common underlying genomic and transcriptomics basis for motivational and learning processes in instrumental behavior. The data collected in this dissertation offer new neurobiological understanding of behaviors that are germane to psychiatric conditions, and demonstrate the synergy offered by combining both hypothesis-based and hypothesis-free approaches to behavioral neuroscience research.

The dissertation of Alexander Scott James is approved.

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2014

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

The influence of basic forms of associative appetitive learning: human behaviors and clinical implications

Basic associative appetitive learning can be broadly classified into two types of learning: Pavlovian conditioning and instrumental conditioning. The former describes the learning about contingent relationships between one stimulus and another (including appetitive outcomes), while the latter describes the learning about the relationship between responses and the outcomes they produce. From an evolutionary perspective, non-associative learning offers significant responsivity to a changing environment; however, it is Pavlovian associations that allow organisms to make predictions about upcoming biologically-significant events in order to engage in preparatory behaviors that anticipate those events; instrumental conditioning affords organisms the ability to learn how their behaviors can manipulate the environment to control the presence of those biologically-significant events (Balleine and Dickinson, 1998).

Early psychologists believed that most organized behavior revolved around seeking positive stimuli and avoiding aversive stimuli (Spencer, 1880), and certainly, this perspective has intuitive value today. Presumably, in addition to non-associative processes, instrumental and Pavlovian learning form the building blocks upon which many, if not all, learned behaviors that humans exhibit in order to pursue desired stimuli and to avoid dangers. These goals occupy a significant amount of our time and likely critically shape our lives. Additional levels of behavioral complexity undoubtedly also depend upon cortically-based mechanisms that implement top-down cognitive control of motivated behavior (i.e., behavioral flexibility, response inhibition, selective attention, working memory, etc.); nevertheless, it can be argued that most learned

associative behaviors, when parsed into their most elementary components, involve instrumental and Pavlovian conditioning.

Therefore, the study of the neural mechanisms that control these forms of learning, and the genetic determinants of individual differences in these processes, is of great importance to the understanding of human behavior. This applies not only to “normal” variation in reward-seeking, but also to a range of pathological behaviors. Aberrant forms of reward-related behaviors are associated with a spectrum of psychiatric conditions, including addiction, autism, attention deficit hyperactive disorder, and depression (Neuringer, 2002; Everitt and Robbins, 2005; Martin-Soelch et al., 2007; Shiflett and Balleine, 2011).

Addiction is a useful example of the effects of maladaptive Pavlovian and instrumental conditioning. Drugs of abuse are themselves conceptualized as eliciting feelings of subjective reward by stimulating the brain regions that natural rewards activate (Wise, 1987). Though details of this perspective have been updated, its basic implications remain valid. Because drugs of abuse are rewarding stimuli – by virtue of activation of an endogenous reward circuit – both Pavlovian and instrumental learning play critical roles in the acquisition and maintenance of drug-taking behavior.

Not only does addiction involve appetitive associative learning, it invokes exaggerated and aberrant forms of it. Psychostimulant drugs with addictive liability directly induce alterations in reward-related learning. In rodents, repeated administration of these drugs enhances the acquisition of appetitive Pavlovian and instrumental conditioning (Taylor and Jentsch, 2001; Olausson et al., 2003; Olausson et al., 2006) and increases the ability of reward-paired cues to elicit behavioral responses (Harmer and Phillips, 1998; Wyvell and Berridge, 2001; Olausson et al., 2004b, a; Ostlund and Balleine, 2008; Ostlund et al., 2014). Changes in the output of neurotransmitter systems resulting from psychostimulant exposure appears to cause this

upward scaling of the degree to which rewards and reward-associated cues are able to exert forceful control over behavior (Robbins, 1978; Lett, 1989; Taylor and Jentsch, 2001; Wyvell and Berridge, 2001; Olausson et al., 2003, 2004b, a; Olausson et al., 2006; Steketee and Kalivas, 2011). This, in turn, facilitates rapid associative learning of drug-associated cues and instrumental responses that produced drug outcomes, contributing to the strength of desire for drugs, manifested as 'cravings' (Robinson and Berridge, 1993). Thus, the end result of psychostimulant-induced changes in reward learning processes includes unusually strong cue- and drug-related memories that lead to compulsive drug-seeking and -taking behavior, as well as chronic patterns of relapse characteristic of substance use disorders (Robinson and Berridge, 1993; Robinson and Berridge, 2008).

This is one conceptualization of behavioral addictions, amidst many that involve alterations Pavlovian and instrumental learning. Moreover, it is a member of the broader pool of theories, spanning a range of psychiatric conditions, that invoke Pavlovian and instrumental conditioning in their interpretation of different maladaptive behaviors. Advancements in the depth of our understanding of the neural circuitry that control Pavlovian and instrumental reward-seeking therefore directly influence the depth of our understanding of psychiatric disorders. What is currently known about this neural circuitry has largely been discovered through the study of animal behavior, and these results will be reviewed prior to presenting the research carried out in this dissertation.

Pavlovian conditioning: background

Pavlovian conditioning is the process by which an unconditional stimulus (US), which naturally elicits an unconditional response (UR), becomes associated with an initially neutral stimulus, rendering it a conditional stimulus (CS) that elicits a conditional response (CR). This association is formed by repeated presentations of the CS and US close together in time

(Pavlov and Anrep, 1927). These repeated pairings must have special characteristics: the relationship between the CS and the US must be one of contingency and predictive value, not mere contiguity. Pavlovian CRs are exhibited when the probability of the US occurring given the presence of CS is different than the probability of the US occurring in the absence of the CS, even if the absolute number of pairings between the CS and US is large (Rescorla, 1966; Rescorla, 1967). Thus, Pavlovian conditioning is the mechanism by which the stimuli are associated with other stimuli in service of generating expectancies (Bolles, 1972).

Though by no means the first influential formal quantitative theory, nor the most contemporary, the Rescorla-Wagner model remains today one of the most influential conceptualizations of Pavlovian conditioning. The Rescorla-Wagner model states that each time a CS is paired with a US, the strength of the association between the CS and the US is updated. The change in associative strength is equal to the difference between the motivational asymptote (i.e., the theoretical maximum associative strength the US can support) and the sum of the associative strengths of all stimuli present at the time of the pairing; this difference is scaled by two terms describing the learning rates that are specific to the CS and the US (Rescorla and Wagner, 1972). It belongs to a class of 'prediction error correction' models of Pavlovian learning, where learning occurs only when there is a difference between what outcome is expected based upon the stimuli present and what actually happens (i.e., the US or the lack of the US). Thus, over successive pairings, the CS comes to progressively acquire greater associative strength, and expectation of the US given the CS becomes greater. Learning stops when the CS is learned to fully predict the US because, at that stage, there is no difference between expected and actual outcomes. The predictions of the Rescorla-Wagner model are useful in providing mechanistic explanations of the conditioning phenomena that are mentioned in subsequent sections. One such phenomena is blocking: once the contingency between a CS and US becomes well learned, a second CS - presented in conjunction with the first- will not enter into an association

with the US (Kamin, 1969). Prediction error models have been useful in understanding the role of endogenously generated analgesia in Pavlovian fear conditioning (Bolles and Fanselow, 1980), and as will be discussed later, neural signals that reflect the parameters implemented by the models have been discovered (Schultz et al., 1997).

Instrumental conditioning: background

Instrumental conditioning occurs when a behavior elicits the occurrence of an outcome which, due to its reinforcing properties, changes the likelihood of the response occurring again (Thorndike, 1911; Skinner, 1938; Mackintosh, 1983; Balleine and Dickinson, 1998).

Instrumental learning was originally conceptualized as invoking a stimulus-response (S-R) reinforcement system. Through multiple reinforcement events, this set of environmental stimuli surrounding the instrumental action and contingent outcome delivery becomes capable of eliciting the instrumental response. This is the Law of Effect: through reinforcement events, the probability of the response, given a set of stimuli, increases (Thorndike, 1911).

In simple S-R theory, the association is made between the stimuli present and the response, while the reinforcing outcome itself is not encoded. Moreover, the S-R reinforcement system strengthens associations by way of the contiguity of the response and the outcome; that is, the contingent relationship between the two is not represented in S-R theory. Thus, S-R theory does not invoke the notion of causality; responses are habitual consequences of the set of present stimuli, and the actor has no explicit awareness of the consequences of its actions.

Later, a series of experiments demonstrated that animals performing instrumental actions are, in fact, aware of the causal relationship between actions and outcomes, and that the outcome itself is encoded in the associative structure of the behavior (Balleine and Dickinson, 1998).

When the contingency between an instrumental action and its outcome is degraded by increasing the probability of non-contingent presentations of the reward, rats decrease their

performance of the degraded instrumental response, but continue to perform other instrumental responses (Hammond, 1980; Dickinson and Mulatero, 1989).

Pavlovian conditioning: neuroanatomy and neurochemistry

The form of Pavlovian learning studied here, Pavlovian approach conditioning, is a simple conditioning procedure in which animals are trained to associate a CS with an appetitive outcome, such as the delivery of a food pellet, and conditioning is measured by an increase in approach to the location of delivery of this reward elicited by presentation of the CS. While this procedure may best be thought of as having a Pavlovian component, as well as an instrumental component (Holland, 1979) – the approach to the magazine and subsequent food consumption required to experience the US – it has proven useful in understanding the neuroanatomical and neurochemical basis behaviors modulated by learned CS-US associations. Pavlovian approach behavior is controlled by a network of nuclei associated with the ventral striatum (Belin et al., 2009a). For example, lesions of the central nucleus of the amygdala (CeA) and the nucleus accumbens core lesions impair Pavlovian approach learning and performance of already learned responses, respectively (Parkinson et al., 1999; Parkinson et al., 2000a). Disconnection of the nucleus accumbens core and anterior cingulate cortex impairs the acquisition of a variant of Pavlovian approach, termed autoshaping, in which conditioning is measured as discriminated approach to a reward-paired CS (as opposed to an un-paired CS), both of which are spatially distinct from the location of reward retrieval (Parkinson et al., 2000b). These studies have led to the formulation of a circuitry underlying approach conditioning consisting of anterior cingulate inputs conveying discriminative components of Pavlovian approach to the nucleus accumbens core. The CeA is thought to convey motivational signals for Pavlovian approach to the nucleus accumbens core, possibly indirectly via its connections to the ventral tegmental area (VTA) (Ahn and Phillips, 2003; Belin et al., 2009a). The basolateral

amygdala and orbitofrontal cortex may further mediate outcome encoding in appetitive Pavlovian conditioning procedures (Hatfield et al., 1996; Ostlund and Balleine, 2007).

Dopamine signaling plays a critical role in Pavlovian approach behavior. Systemic administration of dopamine receptor antagonists reduce conditional food magazine approach (Wassum et al., 2011), as well as the expression, rather than the acquisition, of Pavlovian approach responses (Flagel et al., 2011). These systemic effects are likely mediated by the above proposed neural circuit, as dopamine depletions of the nucleus accumbens and dopamine antagonists delivered to this region impair discriminative Pavlovian approach learning and performance of Pavlovian responses (Di Ciano et al., 2001; Dalley et al., 2002; Parkinson et al., 2002). On the other hand, dopamine neurotransmission in the amygdala appears to participate in the acquisition, but not performance, of Pavlovian approach behavior (Harmer and Phillips, 1999).

NMDA receptors, through their ability to detect coincident synaptic inputs, have long been known to be key regulators of synaptic plasticity, and by extension, learned behaviors (Morris et al., 1986; Bliss and Collingridge, 1993; McHugh et al., 1996; Kandel, 2001; Malenka and Bear, 2004). The NMDA receptor's influence on learning can be extended to include Pavlovian approach conditioning: NMDA antagonist administration into the amygdala, nucleus accumbens core, or VTA impairs the acquisition of approach behavior (Burns et al., 1994; Di Ciano et al., 2001; Stuber et al., 2008). NMDA-dependent increases in AMPA receptor currents in the VTA are observed during Pavlovian approach conditioning, highlighting a critical role for excitatory transmission and its interaction with dopamine neurons in acquisition of Pavlovian approach responses (Stuber et al., 2008). NMDA and dopamine receptors also been implicated in consolidation of Pavlovian approach learning, as infusions of antagonists after daily training sessions slows learning (Dalley et al., 2005).

More recently, with the advent of inducible and conditional genetics models, our understanding of Pavlovian approach has become more refined: for example, NMDA receptors in the striatum and in prefrontal cortex neurons which project to the ventral tegmental area have been shown to be highly important to approach learning (Parker et al., 2011a), while optogenetic studies have implicated dopamine neuron burst firing activity, as will be discussed in greater depth below (Steinberg et al., 2013).

Instrumental conditioning: neuroanatomy and neurochemistry

While Pavlovian responses require the nucleus accumbens, and it affects motivational components of instrumental responding, it does not appear to encode the central 'response-outcome' association that defines instrumental learning (Corbit et al., 2001; de Borchgrave et al., 2002; Kelley, 2004). Rather, specific compartments of the dorsal striatum, via parallel cortico-striatal-thalamic loops, have emerged the key regulators of instrumental response acquisition, performance of learned responses, action-outcome contingency sensitivity, goal-directed response-outcome representation, and habit formation (Robbins and Everitt, 2002; Kelley, 2004; Balleine et al., 2007; Robinson et al., 2007; Yin et al., 2008; Shiflett et al., 2010; Groman et al., 2011; Ostlund et al., 2011). Representations relating to the causal effects of particular instrumental responses (i.e., the discrimination between contingent and non-contingent rewards presentation) and outcome encoding appear to be situated in the prelimbic prefrontal cortex and the posterior dorsomedial striatum (Corbit and Balleine, 2003; Yin et al., 2005a; Yin et al., 2005b). The dorsolateral striatum and the infralimbic PFC, conversely, control well-learned instrumental response habits that are no longer sensitive to action-outcome contingencies (Coutureau and Killcross, 2003; Yin et al., 2004, 2006a; Corbit and Janak, 2010). Thus, over the course of learning a response, the translation of goal-directed behavior into S-R habitual behavior is paralleled by a transfer of control of the action from the posterior dorsomedial striatum to the dorsolateral striatum. The basolateral amygdala may also contribute

to outcome encoding in instrumental responding by associating the sensory features of appetitive rewards to the affective consequences of their consumption (Balleine et al., 2003).

Like Pavlovian conditioning, NMDA receptor activation and NMDA receptor-related plasticity throughout the nuclei that comprise a distributed midbrain – striatum – limbic – cortical network have been implicated in the development of instrumental responses. Infusion of an NMDA antagonist into the VTA, nucleus accumbens core, basolateral amygdala, or medial prefrontal cortex impairs instrumental learning (Kelley et al., 1997; Baldwin et al., 2000; Zellner et al., 2008). NMDA activity within the CeN and posterior lateral striatum has also been implicated in instrumental behavior, albeit with less specificity toward learning phases (Andrzejewski et al., 2004). There is also much evidence to suggest that dopamine D1 receptors are a crucial substrate for instrumental learning (Beninger and Miller, 1998), and variation in dopamine efflux has been associated with variation in instrumental learning and motivation (Cheng and Feenstra, 2006; Ostlund et al., 2011). Moreover, several studies indicate that an interactive effect of concurrent stimulation D1 and NMDA receptors in the facilitation of this learning. For example, D1 antagonism within the nucleus accumbens core impairs learning but also impacts basic motor behaviors, but concurrent infusion of small doses of D1 and NMDA antagonists, which when given apart are without effect, selectively impair instrumental learning and leave motor behavior intact (Smith-Roe and Kelley, 2000). D1 receptor activation within the mPFC is also required for instrumental learning and performance, and again, the concurrent infusion of sub-threshold doses of D1 and NMDA receptor antagonists into the mPFC impair instrumental learning (Baldwin et al., 2002). D1 antagonism within the BLA and the CeN amygdala also dose-dependently impairs instrumental learning (Andrzejewski et al., 2005).

Within the striatum, it is thought that dopamine exerts a permissive role for NMDA-related changes in cortico-striatal and amygdala-striatal synapse efficacy, whereby synapses whose

glutamatergic activity is time-coincident with bursts of dopamine release are selectively strengthened (Beninger and Miller, 1998; Kelley, 2004; Horvitz, 2009; Shiflett and Balleine, 2011). Thus, when a response elicits a reward, long-term potentiation of striatal synapses representing recently elicited actions provides a temporal association mechanism that connects the response with the reward and thereby shapes new instrumentally-conditioned behavioral patterns (Horvitz, 2009). Recent studies have used response-contingent optogenetic techniques to demonstrate that burst stimulation of dopamine neurons can facilitate instrumental responses and/or support instrumental behavior their own right (Adamantidis et al., 2011a; Witten et al., 2011b), which likely enables the striatal plasticity processes that encode response-outcome learning.

Pavlovian-instrumental interactions

Though distinct in many ways, Pavlovian and instrumental processes have been demonstrated to interact in motivational aspects of reward-seeking. In addition to modulating food-directed behaviors, interactions between Pavlovian and instrumental processes are thought to play a critical role in the acquisition of drug dependence and facilitate its maintenance by enabling highly motivated drug-seeking behavior (Belin et al., 2009b). The manners in which Pavlovian cues modulate the vigor of an instrumental response have been extensively studied (Balleine, 2005). Transfer of motivational influence from the CS to the instrumental response can be controlled through association between the CS and sensory properties of the US with which it was paired, or through association between the CS and the general motivational properties of the US (Dickinson and Dawson, 1987; Balleine, 1994; Colwill and Motzkin, 1994; Corbit and Balleine, 2003). The nucleus accumbens shell and basolateral amygdala are implicated in the former, and the orbitofrontal cortex, accumbens core, and the central nucleus of the amygdala in the latter. (Corbit et al., 2001; Hall et al., 2001; Corbit and Balleine, 2005; Ostlund and Balleine, 2007).

Another manifestation of Pavlovian-instrumental interactions is derived from conditioned reinforcement tests: here, appetitive CSs can themselves come to acquire their own incentive properties (Bindra, 1974; Bolles, 1975) that can directly reinforce novel instrumental responses (Mackintosh, 1974). The neuroanatomical substrates of conditioned reinforcement considerably overlap with those which control the invigorating effects of CSs, involving the basolateral amygdala, nucleus accumbens core, and orbitofrontal cortex (Cador et al., 1989; Parkinson et al., 1999; Pears et al., 2003; Burke et al., 2008). The same is true for neurochemistry: responding for conditioned reinforcement is enhanced by facilitation of nucleus accumbens shell dopamine transmission (Robbins, 1978; Taylor and Robbins, 1984, 1986; Robbins et al., 1989), and blocking dopamine receptors prevents the response invigorating effects of cues (Dickinson et al., 2000; Wassum et al., 2011).

The interaction between Pavlovian cues and instrumental behavior is thought to depend upon basolateral amygdala neurons' glutamateric afferents to the nucleus accumbens; these respond to reward-predictive cues, which in turn drive cue-evoked firing in the nucleus accumbens (Ambroggi et al., 2008; Tye et al., 2008). This, in combination with dopamine signaling, is thought to enable the influence of Pavlovian cues on instrumental reward-seeking and conditioned reinforcement (Yun et al., 2004; Ambroggi et al., 2008; Jones et al., 2010; Stuber et al., 2011).

The debate over dopamine's role in reward-related learning

The midbrain dopaminergic system has long been recognized as a key player in a variety of reward-seeking behaviors (Bozarth, 1994), and literature presented above strongly supports this assertion. Despite it being perhaps the most studied molecule in the field of reward-related learning, the precise nature of its involvement has been highly contentious.

Of the 11 known dopaminergic cell groups, A9 and A10, corresponding to the substantia nigra pars compacta and the VTA, have received the most attention from researchers. Substantia nigra pars compacta dopamine neurons project primarily to the caudate and putamen, via the nigrostriatal pathway, while VTA dopamine neurons innervate limbic structures (e.g., nucleus accumbens, amygdala, hippocampus), via the mesolimbic pathway, and cortical structures, via the mesocortical pathway. Dopamine receptors are segregated into two classes: D1/D5 receptors, which are $G_{s/oif}$ -coupled, and primarily excitatory effect on striatal medium spiny neurons; and D2/D3/D4 receptors, which are $G_{i/o}$ -coupled, and therefore exhibit approximately opposite effects on signal transduction pathways and cell excitability. Though many exceptions have been observed, on net, D1-family receptor activation is associated a cascade of signaling events that leads to induction of long term potentiation (LTP), while D2-family receptor activation is associated with LTD-induction (Shen et al., 2008; Wall et al., 2011).

Dopamine and reward

Initial conceptualizations of the role of dopamine in appetitive learning centered on the notion that dopamine signals controlled the positive subjective effects of rewards; that is, dopamine signals were thought of as being the neural instantiation of reward itself. Studies identified mesolimbic dopamine transmission as the most important component of reinforcing medial forebrain bundle stimulation (Wise, 1978; Gallistel and Davis, 1983; Wise and Bozarth, 1984; Gallistel et al., 1985; Bozarth, 1994). Thus, VTA dopamine transmission was theorized to be the principle neural mechanism responsible for reward-elicited hedonic experiences (Wise, 1980; Wise and Bozarth, 1984; Bozarth, 1987; Koob and Le Moal, 2001). A variety of lines of evidence, each implicating dopamine in a domain of rewarding experience, support this claim (Wise and Rompre, 1989). Food rewards elicit responses from dopamine cells and increase dopamine efflux in the nucleus accumbens (Hernandez and Hoebel, 1988; Apicella et al., 1991; Smith et al., 1995). Moreover, all drugs of abuse, through their mechanisms of action, either

directly or indirectly increase brain dopamine levels (Wise and Bozarth, 1987; Di Chiara and Imperato, 1988)

However, dopamine depletion studies in mice demonstrate that in the context of very small brain concentrations of dopamine, reward preference, pleasurable responses to palatable stimuli, or deprivation-induced changes in palatability are preserved (Berridge et al., 1989; Peciña et al., 1997; Cannon and Palmiter, 2003; Berridge and Aldridge, 2008; Wassum et al., 2011).

Dopamine is also not required for the reinforcing effects of some drug rewards, such as morphine (Hnasko et al., 2005), and even within the intracranial self-stimulation paradigm, dopamine cell firing does not track well with the rates of behavioral output that presumably reflect brain reward (Kilpatrick et al., 2000). Evidence against a selective role in reward is also derived from numerous demonstrations of aversive stimuli increasing (or inhibiting) dopamine neuron activity or extracellular dopamine levels, and furthermore, dopamine antagonists or reducing dopamine neuron activity can impair some forms of learning about aversive events (Schultz and Romo, 1987; Sorg and Kalivas, 1991; Salamone, 1994; Horvitz, 2000; Ungless et al., 2004; Brischoux et al., 2009; Matsumoto and Hikosaka, 2009; Lammel et al., 2011; Zweifel et al., 2011).

Dopamine and behavioral invigoration and effort

Striatal dopamine is known to modulate locomotor activation and the arousal of behavior and attention by reinforcing events (Schultz, 1988; Delfs et al., 1990; Albin et al., 1995; Redgrave et al., 1999; Correa et al., 2002; Dauer and Przedborski, 2003). Consequently, dopamine has been conceptualized as a mediator of the “activational” or “energetic” processes that enable animals to engage in vigorous responses and other highly motivated behaviors (Salamone, 1994; Salamone and Correa, 2002; Robbins and Everitt, 2007). Here, dopamine is seen as a regulator of activational state, which has the primary function of amplifying, encouraging, or

organizing preparatory behavior in light of an anticipated reinforcing event (Robbins and Everitt, 1992; Salamone and Correa, 2002; Barbano and Cador, 2007; Robbins and Everitt, 2007). Consistent with this approach, nucleus accumbens dopamine depletions do not affect primary motivation for food, nor instrumental responding when the number of responses required to obtain reinforcement is low, but do impair performance when those requirements are large (Aberman and Salamone, 1999; Salamone et al., 2001; Correa et al., 2002). In intact rats, moderate fixed ratio schedules of reinforcement normally enhance response rates, but this effect is lost in dopamine-depleted animals (Salamone et al., 2003; Mingote et al., 2005).

Dopamine and reward prediction error

Dopamine cells exhibit three modes of firing: an inactive/hyperpolarized state, a tonic, background level of discharge of approximately 2-4 Hz, and a transient, “phasic” bursting mode where cells fire at 10-20 Hz with inter-spike intervals of less than 80 ms (Grace and Bunney, 1984a, b; Hyland et al., 2002; Grace et al., 2007; Zhang et al., 2009; Wall et al., 2011) which is particularly efficacious at increasing dopamine release (Gonon, 1988; Bean and Roth, 1991). A considerable body of evidence derived from electrophysiological recordings of midbrain neurons in primates suggests that phasic dopamine signals are the neural instantiation of the prediction error signal as implemented by temporal difference learning algorithms and the Rescorla-Wagner model (Rescorla and Wagner, 1972; Schultz et al., 1993; Schultz et al., 1997; Hollerman and Schultz, 1998; Sutton and Barto, 1998). Here, phasic dopamine signaling represents the difference between predicted and actually received rewards (Schultz, 2002). Before a neutral cue is paired with a reward, the cue itself does not elicit a dopamine spike, but unanticipated presentations of the reward do. This firing represents a “positive prediction error”, in that a reward was delivered that was not expected given the set of cues present (Hollerman and Schultz, 1998). Over successive trials, when contingent pairings of cue and reward are provided, the cue itself comes to elicit dopamine cell firing, while the reward elicits a lesser

degree of dopamine activity, as it is partially expected. At the end stages of learning, the dopamine signal has fully back-propagated to the time of the CS that predicts it. However, when an expected reward is omitted, a “negative prediction error” occurs, and dopamine cell firing rates drop below baseline (Hammond, 1980; Mirenowicz and Schultz, 1994; Schultz et al., 1997; Schultz, 2007). Prediction error signals with these properties have also been observed in humans using functional brain imaging (McClure et al., 2004; O'Doherty, 2004; Pessiglione et al., 2006) and in rodents using dopamine-detecting fast-scan cyclic voltammetry (Day et al., 2007; Clark et al., 2010). More generally, dopamine signals comply well with classic behaviorists' theories; for instance, because transient reward-related dopamine signals occur only when deviations from expectancies occur, dopamine prediction error signals track with the behavioral effect of blocking, wherein conditional stimuli that fully predict a reward ‘block’ the ability of other subsequently trained stimuli to acquire predictive strength (Kamin, 1969; Waelti et al., 2001). Transient optically-elicited dopamine release has recently been shown to ‘unblock’ conditioning to a stimulus that would normally undergo blocking, suggesting a causal role for phasic transmission in blocking (Steinberg et al., 2013), and by proxy, a causal role in learning from prediction error.

Dopamine as an incentive salience signal

Incentive motivational accounts of goal-directed behavior describe behavior as being “pulled” towards stimuli imbued with incentive motivational properties (Bolles, 1972; Bindra, 1978; Toates, 1986; Balleine and Dickinson, 1998). These motivational properties have been thought of as being conveyed by dopamine signaling. That is, stimuli – both USs and CSs – acquire incentive motivational properties that can attract an animal towards them, via coincident presentation during phasic dopamine release. And it is this signal that is considered to deploy these motivational properties: dopamine is thought to transform rewards and cues from being merely pleasurable, or ‘liked,’ to being ‘wanted,’ attractors of behavior with enormously

significant motivational properties (Berridge and Valenstein, 1991; Robinson and Berridge, 1993; Berridge and Robinson, 1998).

Here, dopamine is explicitly hypothesized to not mediate associative learning per se (i.e., it has no role in the acquisition of the CS-US or response-US contingency), but instead controls attachment of incentive value to stimuli, which in turn facilitates associative learning (Berridge, 2007; Flagel et al., 2011). Conditional stimuli that have acquired incentive properties through their association with unconditional incentive rewards are claimed, under this theory, to carry several properties over and above simple associative information. Three properties of incentive conditional stimuli that differentiate them from outcome-predictive conditional stimuli have been proposed: the cue acts as a conditioned reinforcer, in that its presentation can support acquisition of a novel instrumental response; the cue can elicit 'wanting' of the US (i.e., Pavlovian-instrumental transfer); and the cue can attract behavior towards itself (Berridge et al., 2009). The enhancement in the ability of Pavlovian cues to themselves support instrumental responding after amphetamine administration is taken as evidence for dopamine's central role in the the first property of incentive conditional stimuli (Robbins, 1978; Taylor and Robbins, 1984, 1986; but see Winterbauer and Balleine, 2007). The dependence on dopamine receptors on the ability of Pavlovian stimuli to invigorate instrumental responding (i.e., cue-triggered 'wanting') and the heightening of process by dopamine releasing drugs, as well as its tracking with phasic dopamine signals (Dickinson et al., 2000; Wyvell and Berridge, 2000, 2001; Ostlund et al., 2014) are taken as evidence for the second. . These findings also have been suggested to refute the prediction error theory of dopamine in so far as the effects of dopamine manipulations on changes in cue-elicited behavior are immediate: they do not require re-exposure to their associated rewards, and therefore do not require the experience of prediction errors (Berridge, 2007)

Support for a role of dopamine in the third property of incentive conditional stimuli, the gravitation of behavior towards the stimulus itself, comes from the study of individual differences in CRs to reward-predictive cues. It has long been known that considerable variance in CRs does exist; in the conditional salivary reflex in dogs, some dogs tended to approach the location of food delivery, whereas others tended to approach the predictive stimulus itself (Zener, 1937). These individual differences in direction of approach also exist in rats, as detected by 'autoshaping' procedures, which differ from Pavlovian magazine approach procedures described above in a subtle but significant way. Upon CS presentation, some animals will approach the location of reward delivery ('goal-tracking' behavior), while others will forego that location in order to interact with the CS itself ('sign-tracking' behavior), often in a manner that resembles food consummatory behavior, such as biting or gnawing (Jenkins and Moore, 1973; Boakes, 1977; Tomie, 1996). In rats, this tends to take place when CSs are manipulable (i.e., operandi such as movable levers) or are easily localized visual cues (e.g., stimuli on a touchscreen monitor). Because the rate at which sign-tracking animals and goal-tracking animals increase the expression of their respective CRs over the course of training does appear to not differ, it has been argued that their cue-outcome contingency learning rates are equivalent (Robinson and Flagel, 2009). Rather, because it is the degree to which the CS pulls behavior towards directly toward it that differs, sign-tracking is taken to reflect stronger attribution of incentive salience to the cue, relative to goal-tracking (Flagel et al., 2009). Under the incentive salience theory of addiction, these individual differences in tendency to attribute strong incentive salience to reward-associated cues are thought to reflect risk-markers for the development of addiction (Flagel et al., 2010).

Evidence for a differential role of dopamine in sign-tracking and goal-tracking comes from the observation that sign-trackers exhibit differences in dopamine system-related transcript levels (Flagel et al., 2007), and repeated amphetamine treatment increases sign-tracking, but not goal-

tracking (Doremus-Fitzwater and Spear, 2011; but see also Simon et al. 2009). Sign-tracking animals also display greater responding in tests of conditioned reinforcement that are known to be sensitive to perturbations of the mesolimbic dopamine system (Robbins et al., 1989; Robinson and Flagel, 2009). Most recently, it has been shown that dopamine receptor antagonism inhibits learning of a sign-tracking response, but not a goal-tracking response (Flagel et al., 2011), strongly suggesting a dissociation between incentive motivational and contingency learning processes. Moreover, over the course of learning, sign-trackers develop CS-evoked dopamine prediction error signals that are of greater intensity, and the full back-propagation of the dopamine signal from the US to the CS – that is, the loss of US-evoked firing that has been observed for well-learned associations in primates (Schultz et al., 1997) – is more common in sign-trackers than in goal-trackers (Flagel et al., 2011). Because goal-trackers' dopamine signals appear to violate tenets of traditional prediction error signals (i.e., US to CS back-propagation of the signal as prediction errors converge to zero when contingency is well-acquired), this result is taken to mean that phasic dopaminergic responses recorded by Schultz and colleagues during learning are not at all teaching the predictive relationships between CSs and USs, but rather play a role in instructing the attribution of incentive salience to the reward-predictive cues.

Seeking new insight into reward-related learning

Though the precise nature of its involvement is still controversial, the aforementioned literature has made a strong case for the involvement of dopamine in reward-seeking behaviors. In sum, though aspects of appetitive reward and learning are possible in the absence of dopamine (Cannon and Palmiter, 2003; Robinson et al., 2005), dopamine is clearly thought to play a modulatory role in driving acquisition and performance of conditioned behaviors (Taylor and Robbins, 1984; Parkinson et al., 2002; Kelley, 2004; Berridge, 2007; Salamone et al., 2007; Schultz, 2007; Cohen and Frank, 2009; Tsai et al., 2009; Bromberg-Martin et al., 2010).

Nevertheless, the finding of reward preference and reward learning in the absence of dopamine remains a compelling caveat which has important implications for the study of these behaviors. While learning in the absence of dopamine by no means confirms that learning in intact animals does not normally utilize dopamine, it does highlight the fact that dopamine is not the end all and be all of reward-related learning. This conclusion highlights the importance of seeking new molecular substrates of reward-seeking behavior in order to make meaningful progress in our understanding of the neural basis of these behaviors and the psychiatric conditions in which they are dysregulated.

Hypothesis-based approaches to the discovery of regulators of reward-related learning have indeed led to the discovery of new genes that control instrumental learning (Lobo et al., 2007), and targeted genetic knockouts and models of single-gene human disorders have long been used in identifying the genetic basis of learning (Dudai et al., 1976; Tang et al., 1999; Kushner et al., 2005; Roubertoux and Carlier, 2010). However, another avenue for discovery of novel molecular regulators of reward-seeking behaviors is through the hypothesis-free study of individual differences in the traits. We argue that reward-based instrumental and Pavlovian learning are the foundation for many learned behaviors in humans, and clearly, there is tremendous variation in reward-seeking behaviors. Model organisms also exhibit quantitative variation in these traits. Measurement of these traits in genetically heterogeneous sets of model organisms, such as panels of inbred mouse strains, allows one to harness the power of mouse-based genome-wide association scanning for the discovery of novel neurogenetic determinants of naturally-occurring variation in reward-related learning. Importantly, this natural variation includes both quantitative variation within the range of 'normalcy' and also variation outside that range; therefore, this approach allows simultaneous study of genetic mechanisms controlling basic human reward-seeking behaviors and also extreme forms of reward-seeking that are associated with psychiatric conditions.

It is important to state that several major criticisms have levied against genetic screens using mice; perhaps the most serious is that mouse genome scans have not resulted in the discovery of many genes that control complex traits (Bennett et al., 2010). This is owed primarily to two factors. The first is that low genetic recombination rate results in low chromosomal mapping resolution in mouse linkage studies (Bennett et al., 2010). The second is that genetic inheritance patterns of complex traits are themselves complex. More often than not, the phenotype is regulated by many genes, each of which have small effect sizes (Crusio, 2004b; Valdar et al., 2006). Genome-wide association studies using sets of commonly studied inbred mice sought to address the first limitation. Unfortunately, these methods failed to address the second limitation, in that they have poor power to detect low (i.e., realistic) effect size genetic signals (Bennett et al., 2010; Su et al., 2010). Moreover, mouse genome-scans are susceptible to spurious findings because unequal relatedness amongst different strains results in complex population genetic substructures; failure to control for these effects leads to violation of independence assumptions, which dramatically inflates Type I error rate (Helgason et al., 2005; Payseur and Place, 2007; Kang et al., 2008; Bennett et al., 2010; Su et al., 2010). Several strategies, including genomic control and principle components analysis, have been developed to correct for population substructure, but for a variety of reasons, they are inappropriate for mouse genome-wide association scans (Kang et al., 2010).

However, recent advancements in design of mouse panels and statistical techniques have made great strides in addressing these problems. The Hybrid Mouse Diversity Panel (HMDP) combines classical inbred strains with recombinant inbred mice to form a set of mice that affords significantly greater genomic resolution and statistical power to detect small effect genes than traditional panels (Bennett et al., 2010), and mixed models capable of handling population substructure are readily available (Kang et al., 2008; Lippert et al., 2011). Consequently, utilizing mouse genomics approaches in concert with systems genetics tools presently

represents an exciting avenue towards the discovery of new genetic regulators of reward-seeking behaviors. Because inbred mice constitute a renewable genetic resource population, they are also ideal for systems genomics approaches which combine information gathered from multiple studies. These tools can be used to narrow the list of candidate genes garnered from genome-wide association studies, but also to discover novel molecular, cellular, and inter-cellular mechanisms related to quantitative traits. As such, they have the potential to elucidate genomic interactions at multiple levels of analysis that relate to reward-seeking behaviors. Identifying such loci may not only inform the structure of genetic risk for psychopathologies in which reward-related learning is aberrant, but given the degree to which reward-seeking pervades human behavior, may yield insight into the genetics of a wide range of behaviors: higher order learning processes, and responsivity to feedback, impulsive behavior, and many other aspects of temperament are all plausible candidate phenotypes for which genetic landscape could be illuminated.

AIMS OF THE DISSERTATION

Aim 1: Broaden our understanding of the role of dopamine in reward-related learning by characterizing the effects of attenuation of phasic dopamine on instrumental learning, Pavlovian approach, and incentive salience attribution

Phasic dopamine signaling is thought to play a critical role in aspects of reward-related learning. Using a conditional knockout strategy, we have found that attenuation of phasic dopamine release impairs acquisition of food-reinforced instrumental responding, but does not influence appetitive Pavlovian approach conditioning. Recent evidence suggests that quantitative variation in the magnitude of phasic dopamine release relates to individual differences in the degree to which appetitive Pavlovian cues become desired and attract behavior (sign-tracking). Such attribution of incentive value, in its extreme form, is thought to play a critical role in addictive disorders by allowing drug-associated stimuli to exert tremendous control over behavior, and therefore elicit craving and drug-seeking behavior. However, causal evidence linking phasic dopamine release to incentive salience attribution to reward-paired cues is lacking. Using a conditioning paradigm sensitive to individual differences in incentive value attribution, we aimed to extend our characterization of the role of phasic dopamine using our conditional knockout mouse model by examining whether genetic suppression of the amplitude of phasic dopamine release elicits a reduced tendency of reward-associated cues to attract behavior. Together with our already collected data, this aim seeks us to create a comprehensive picture of the role of phasic dopamine in reward-related learning and will contribute to the understanding of its impact in addictive behaviors and other psychiatric disorders characterized by distortions in instrumental and Pavlovian reward-seeking behaviors.

Aim 2: Reveal new genetic and transcriptomics determinants of reward-seeking behaviors by leveraging genome-wide association strategies with systems genetics tools

This aim will apply the instrumental learning paradigm that that we have used to characterize instrumental learning in Aim 1, but here, rather than testing a hypothesis about dopamine, we will use hypothesis-free genome-wide discovery methods to identify novel molecular regulators of this behavior. Extreme variations in the motivation to exploit the environment to obtain rewards through reward-based learning may underlie maladaptive behaviors that characterize various psychiatric disorders, namely substance dependence and morbid obesity. However, little is known about the genetic markers that modulate these individual differences in reward-related behaviors. The combination of a recently developed hybrid mouse panel with novel statistical methods that can control for population substructure effects, which have historically confounded mouse-base genome-wide scans, now permits mouse genome-wide association studies that have considerable improvements in power, resolution, and validity over previous approaches. Utilizing these methods, a genome-wide association study of appetitive instrumental learning is the aim of this study. Moreover, this aim sought to deepen our understanding of these behavioral processes by application of the array of systems genetics tools available by virtue of studying a continuously available inbred panel. This approach will allow identification of novel genetic determinants of variation in multiple components of reward-related behaviors that are not only germane to psychiatric conditions, but also intricately related to a broad spectrum of goal-directed human behaviors.

CHAPTER 2

Compromised NMDA/Glutamate receptor signaling in dopamine neurons impairs instrumental learning, but not Pavlovian goal-tracking or sign-tracking

Abstract

Dominant theories regarding of the role of phasic dopamine (DA) transmission in learning include its conceptualization as the vehicle by which incentive salience is conveyed to rewards and associated cues, and as a contingency teaching signal reflecting reward prediction error. Nevertheless, a causal role for phasic DA cell firing in reward-related learning is not yet fully established. Because some forms of plasticity, and the occurrence phasic bursting events in dopamine neurons are mediated by the NMDA/glutamate receptors (NMDAR), we utilized the cre-recombinase system to generate mice that either fully or partially lacked NMDARs in DA neurons exclusively, as well as appropriate controls, to test the premise that phasic DA transmission modulates 1) goal-directed instrumental learning processes, 2) the ability to learn the predictive value of cues during Pavlovian approach learning, and 3) the likelihood of Pavlovian cues to become highly motivating incentive stimuli that directly attract behavior. Loss of NMDARs in DA neurons did not significantly affect locomotor behavior or free reward consumption levels. On the other hand, animals lacking NMDARs in DA cells exhibited a selective reduction in active lever responses that emerged over the course of instrumental learning. Loss of receptor expression did not, however, influence Pavlovian approach learning, nor the likelihood of an animal exhibiting a conditional response associated with elevated attribution of incentive salience to reward-paired cues (sign-tracking). These data endorse a dissociation in the role of DA neuron NMDARs, and by extension, quantitative reductions in phasic DA neuron firing patterns, in reward-related learning. These data do not support

hypotheses suggesting that the biological significance of the phasic DA signal strictly conforms to incentive salience or prediction error perspectives.

Introduction

The electrical activity of dopamine neurons, and associated activity-dependent synaptic release of dopamine, is thought to be critical to reward-related learning and behavior (Wise and Rompre, 1989; Robbins and Everitt, 1992; Robinson and Berridge, 1993; Salamone, 1994; Schultz et al., 1997; Redgrave et al., 1999; Frank et al., 2004; Kelley, 2004). A considerable body of evidence, derived mostly from electrophysiological recordings of midbrain neurons in primates and voltammetry recordings of dopamine release in rodents, implicates burst firing (brief event-related, high-frequency discharge activity) and associated non-linear, phasic increases in the quantity of transmitter released of dopamine cells (Grace and Bunney, 1984b; Gonon, 1988; Bean and Roth, 1991) as the neural instantiation of the prediction error signal that figures in both classical and modern mathematical learning models (Rescorla and Wagner, 1972; Schultz et al., 1993; Schultz et al., 1997; Hollerman and Schultz, 1998; Sutton and Barto, 1998; Day et al., 2007; Clark et al., 2010). In these models, phasic aspects of dopamine signaling represent the difference between predicted and actually received rewards (Schultz, 2002), information used to update expectancies of the organism as it experiences the contingent relationships between stimuli that predict biologically significant outcomes, and the responses that produce them.

An alternate perspective regards phasic dopamine as the mechanism by which stimuli and internal representations of those stimuli become attractive and focused upon, which is termed incentive salience attribution (Crow, 1976; Berridge and Valenstein, 1991; Robinson and Berridge, 1993; Berridge and Robinson, 1998). This process transforms rewards and cues that

predict them from being merely pleasurable, or 'liked' stimuli, to being 'wanted' attractors of motivated behavior and attention.

Both the prediction error and incentive salience perspectives are supported by the results of experiments that evaluate the behavioral effects of manipulations of dopamine transmission, such as optogenetic reinforcement of instrumental and Pavlovian behavior (Tsai et al., 2009; Witten et al., 2011a). However, the study of individual differences in the degree to which appetitive Pavlovian cues become desired and attract behavior (measured using sign-tracking behaviors), rather than elicit approach to the locale of imminent reward delivery, offers a unique paradigm in which the two perspectives make distinct predictions. Contingency learning via prediction error signals, expressed by goal-tracking, can be distinguished from contingency learning that also involves incentive salience attribution, expressed as sign-tracking (Robinson and Flagel, 2009). Recent evidence suggests that quantitative variation in the magnitude of cue-evoked phasic dopamine release relates to individual propensity to engage in sign-tracking rather than goal-tracking (Flagel et al., 2011): sign-tracking subjects exhibited greater conditional stimulus-elicited dopamine transients than goal-trackers. However, causal evidence linking phasic dopamine release to incentive salience attribution to reward-paired cues remains lacking.

Because NMDA/glutamate receptors are a critical determinant of phasic firing activity of dopamine neurons (Suaud-Chagny et al., 1992), phasic dopamine release is attenuated in a mouse model lacking NMDA receptors in dopamine neurons (Zweifel et al., 2009; Luo et al., 2011). This mouse model represents a useful system with which to directly interrogate the effects of quantitative reduction of endogenously-generated phasic dopamine signaling on behavioral processes. Here, we use this system to evaluate the role for phasic dopamine signaling in instrumental learning, Pavlovian magazine approach, and sign-tracking/goal-tracking. These studies are an effort to offer a comprehensive characterization of the role of

phasic dopamine in reward-related learning, and in particular, to ascertain variation evoked phasic dopamine release is causally related to incentive salience, but not prediction error-based contingency learning, as suggested by Flagel et al. 2011.

Methods

Mouse lines

B6.SJL-*Slc6a3*^{tm1.1(cre)Bkmn}/J (Jackson Laboratory stock number 006660; referred to here as DATcre+) mice, each heterozygous for mutated dopamine transporter (DAT) gene with an internal ribosomal entry site cre recombinase allele, and B6.129S4-*Grin1*^{tm2Stl}/J (stock number 005246, referred to here as NR1^{flox/flox}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). In DATcre+ mice, cre recombinase cDNA was inserted into the 3' untranslated region of the DAT gene for bicistronic mRNA translation; cre-mediated recombination is detectable in this line as early as E15 and is primarily restricted to the substantia nigra, VTA, and retrorubral field (Backman et al., 2006). Recombination is also observed in prefrontal cortex and amygdala-projecting dopamine neurons, which are known to express minimal levels of DAT (Luo et al., 2011). NR1^{flox/flox} mice have a loxP site between exons 11 and 12 and another loxP site, along with a neomycin resistance gene, at the 3' end of the *Grin1* gene (Tonegawa et al., 1996). The NR1 gene is an obligatory component of the functional NMDA receptor (Forrest et al., 1994), which regulates dopamine cell phasic firing by facilitating temporal summation of excitatory inputs, a consequence of its slow inactivating kinetics (Suaud-Chagny et al., 1992; Overton and Clark, 1997). Conditional inactivation of NR1 blocks NMDA (Tsien et al., 1996a), thereby resulting in attenuation of the magnitude of phasic dopamine release to approximately 30% that of controls (Zweifel et al., 2009; Parker et al., 2010).

Male DATcre⁺ mice were bred with female NR1^{flox/flox} mice; the DATcre⁺ males in the resulting F1 generation were further bred with a different set of female NR1^{flox/flox} mice to create DATcre⁻;NR1^{flox/wt}, DATcre⁻;NR1^{flox/flox}, DATcre⁺;NR1^{flox/wt}, and DATcre⁺;NR1^{flox/flox} mice (collectively referred to as “DATcre;NR1 mice”). Male DATcre⁺ mice were also crossed to female B6.129S4-GT(ROSA)26Sor^{tm1Sor}/J (stock number 003474; referred to as ROSA26-LacZ) reporter mice (Soriano, 1999), obtained from Dr. Alcino Silva’s laboratory at UCLA. DATcre, NR1, and LacZ zygosity were determined using conventional PCR methods. Mice were approximately 60-120 days old when introduced into the study. All subjects were housed in cages of between two and four mice with Sani-Chip cage bedding (PJ Murphy Forest Products, Montville, NJ), in temperature and humidity controlled rooms with 14/10 hour light/dark cycles (on 5:00 A.M. and off 7:00 P.M.). Behavioral testing was conducted during the light cycle. Food was available *ad libitum* during locomotor behavior and free reward consumption testing, but was restricted during other experiments, as detailed below. All experimental protocols were consistent with the US Public Health Service *Guide for the Care and Use of Laboratory Animals* and were approved by the Chancellor's Animal Research Committee at University of California at Los Angeles.

LacZ X-Gal staining

Successful cre-driven transfection cleaves the floxed polyadenylation site that precedes the beta-galactosidase gene in ROSA26-LacZ mice, which is visualized by staining for the enzymatic substrate of beta-galactosidase, the chromogen 5-bromo-4-chloro-3-indolyl- β -d-galactopyranoside (X-gal) (Zhuang et al., 2005). DATcre⁺ mice either carrying, or not carrying, the ROSA26-LacZ gene were transcardially perfused with freshly mixed 4% cold paraformaldehyde. Brains were stored in paraformaldehyde for one day, then switched to a 30% sucrose/PBS solution and stored until they sank in the solution. Slices of 40 μ m width were cut in a cryostat and rinsed in phosphate buffered saline (PBS). The staining solution contained

85.33 mg potassium ferrocyanide, 64 mg potassium ferricyanide, 4 mL of 20 mM MgCl₂, 36 mL PBS, 60 mg X-gal, and 800 µL dimethylformamide. Solution was allowed to react with brain slices at 37° C for 48 hours, then rinsed again with PBS and mounted on slides.

Quantification of monoamine utilization in the striatum

DATcre;NR1 mice (males and females, n = 8 – 10 per genotype) were euthanized by rapid decapitation and tissue punches were taken from the ventral striatum. Tissue samples were rapidly frozen for subsequent analyses of monoamines and their metabolites using high pressure liquid chromatography (HPLC) as described in detail in Jentsch et al. (1997): tissue was homogenized in 0.1 M perchloric acid, and 200 µL of supernatant was quantified acid by reverse-phase column HPLC (BAS, West Lafayette, IN) at 0.7V applied using a 7% acetonitrile-based mobile phase. Protein homogenates were quantified using the Lowry method (Lowry et al., 1951).

Locomotor activity in a novel context

165 DATcre-NR1 mice were used (both males and females, n = 31 to 36 per genotype; locomotor data for 36 mice lost due to equipment failure). Mice were placed in Opto M3 locomotor activity monitors (Columbus Instruments, Columbus, OH, USA) with 1" spaced x- and z-axis infrared beam emitters. Locomotor behavior was monitored for 30 minutes (data collected in 5-min time bins) in standard Plexiglas rat-sized (25 x 45 cm) cages with a thin layer of bedding. A second rat-sized cage was inverted upon the first to prevent animals from jumping out of the monitoring area.

Free consumption of a palatable reward

Subsequently, the same mice used in the locomotor experiment (n = 40 to 42 per genotype) were habituated to a two bottle free choice palatable reward consumption procedure over the

course of two days. Mice were exposed to 2-h sessions of individual housing with access to two Lixit tube-equipped water bottles, one filled with water and the other filled with a 10% v/v sweetened condensed milk solution (Kroger, Cincinnati, OH). Bottle positions (i.e., left side of the cage versus right side, order counterbalanced across genotypes) were switched on the second day of habituation. Testing began the following day: bottle positions were again switched, and data were collected for two days; a final switch, followed by two days of data collection concluded the procedure. Data presented are averages of consumption levels on the second day of placement on each side (i.e., post-habituation days two and four); this strategy was used to mitigate any innate side preferences and also give time for animals to adapt to the switching of reward location.

Instrumental conditioning

A new set of 112 experimentally-naïve mice (all male, $n = 22$ to 27 per genotype, reflecting 11 mice excluded due to pellet dispenser or lever failure) were provided limited access to chow in their home cages in order to achieve body weights approximately 85% of their free-feeding levels. Mice were exposed to 0.5 grams of the reinforcer pellets (14 mg Dustless Precision Pellets; Bio-Serv, Frenchtown, NJ) used for subsequent behavioral experiments in their home cages during the first day of food restriction. Body weight was maintained at this level by providing a customized amount of standard rodent chow, at least 1 hr after testing finished. Mice were trained on sequential days in extra-wide aluminum and polycarbonate Med Associates (St. Albans, VT) modular mouse testing chambers, each stationed inside a sound-attenuating chamber and equipped with a white noise generator, house light (both on during all experiments), and a tone generator. A horizontal array of five illuminable nose-poke apertures formed one side of the box, and on the other resided an illuminable pellet delivery magazine with an entry-detection photocell. Chambers also contained two retractable ultra-sensitive

mouse levers (2 g force requirement for actuation; Med Associates, St. Albans, VT); these levers were positioned one each on either side of the food magazine.

Training began with two days of familiarization to delivery of food pellets to the magazine. Fifty pellets were delivered to the magazine on a fixed-time 30-s schedule, each followed by a 2-sec illumination of the magazine. Ten daily 30-min sessions of instrumental training followed.

Sessions began with the extension of both levers, and responses on the active lever (designated left versus right in a counterbalanced fashion across genotypes) that completed the ratio schedule resulted in a 50-ms tone pulse, along with a pellet delivery and a 2-sec illumination of the magazine light. The first 10 pellets per session were delivered on a fixed-ratio 1 schedule; subsequently, pellets were delivered on a variable-ratio 2 schedule.

Responses to the inactive lever were recorded but had no programmed consequence. A 0.5-s time-out followed each pellet delivery, though responses made during this period did count towards completion of the next reinforcement schedule (but could not elicit another pellet delivery).

Pavlovian approach conditioning

After 14 days without testing, but during which food restriction was maintained, 62 of the same subjects ($n = 13$ to 19 per genotype) underwent 10 days of Pavlovian magazine approach conditioning. During these sessions, three of the five nose poke apertures were lit in order to provide ambient light during the test. In each session, 10 compound CSs (extinction of the house light illumination and a 5 Hz pulsing tone) were presented on a variable-time (VT) 120-s schedule. During each 20-s CS presentation, pellets were delivered on a random-time 5-s schedule (i.e., on average, 4 pellets delivered per CS).

Sign-tracking / goal-tracking task

Methods for sign-tracking / goal-tracking Pavlovian learning were modeled after Flagel et al. (2011). A set of 63 experimentally-naïve animals was used (all male, $n = 31$ to 32 per genotype); in these subjects, the same schedule of food restriction was initiated prior to behavioral training. Animals first underwent two days of magazine training, in which 30 food pellets were delivered to the magazine on a VT60-s schedule. Fifteen daily sessions of sign-tracking / goal-tracking training began the next day. These sessions consisted of 15 presentations, on a VT180-s schedule, of a CS (“lever-CS”), the termination of each of which was associated with the delivery of two food pellets in the magazine. Each lever-CS involved the extension of the lever to the right of the food magazine. Presses on the lever-CS were recorded but had no programmed consequence (i.e., no effect on delivery of food).

The day following the last conditioning session all mice underwent a single test of conditioned reinforcement, wherein the two most lateral nose-poke apertures were illuminated. Responses to the active aperture (designated left versus right in a counterbalanced fashion across genotypes) resulted in a 5-s extension of the lever-CS, while responses to the inactive aperture were recorded but were without programmed effect. No food was delivered during this session. The session ended 60 min after the first active aperture response or after 90 mins had elapsed, whichever came first.

Data analysis: Statistical tests were conducted using SPSS 21 (IBM Corp., Armonk, NY) and Stata 13 (StataCorp LP, College Station, Texas). In all omnibus tests, DATcre (+ vs –) and NR1 (*flox/wt* vs *flox/flox*) zygosity were entered as between subjects factors, except for the sign-tracking / goal-tracking data, where only DATcre+ animals were studied. Day treated as a quadratic continuous covariate.

All datasets were inspected for compliance with assumptions of the general linear model. Where assumptions were met, data were analyzed by univariate or repeated measures ANOVA,

with t-tests where appropriate. For locomotor and learning experiments, we found significant departures from assumptions of traditional repeated measures ANOVA, including violations of sphericity, and heterogeneous, correlated residuals; this is not unexpected, given that subjects' behavior typically correlates across successive days of learning. Given that population-level analysis often does not accurately characterize individual learning curves (Lashley, 1942; Estes, 1956; Gallistel et al., 2004; Verbeke and Molenberghs, 2009) a subject-specific data analysis method (i.e., one uses random effects to model individual variation in learning curves not captured by measured covariates) is an appropriate strategy for addressing these assumption violations. To better model the data and meet assumptions, we therefore utilized generalized linear mixed model.

Models were fitted via maximum likelihood by mean-variance adaptive Gauss-Hermite quadrature. Non-nested models were chosen on the basis of Akaike Information Criterion and Bayesian Information Criterion. Random subject-specific intercepts and linear slopes across days (entered as a factor variable), and their covariance were included on the basis of significant likelihood ratio testing of nested models. Distribution and link functions were chosen on the basis of properties of the variable being studied and the properties of model residuals. Continuous data were analyzed in Gaussian identity-link models (i.e., linear mixed models); heavily skewed continuous data were modeled as log-Gaussian or as gamma distributed log-linked; log-link negative binomial models were applied to overdispersed count data; and binomial logit models were applied to probability data.

Statistics presented are tests of fixed effects. Wald χ^2 tests of main effects and interactions were followed by contrasts of simple effects and, where appropriate, Bonferroni-adjusted tests of means. Locomotor behavior (x-axis beam breaks and z-axis beam breaks) were analyzed across five-minute time bins entered as linear covariate. Free reward consumption (mL/kg consumed) was analyzed with day of measurement as a repeated measure; water consumption

data were not analyzed, as levels were negligible, falling within the range of measurement error. Monoamines were analyzed as utilization rates, calculated as the ratio of metabolite to monoamine content (ng/mg tissue); secondary analyses of monoamines and metabolites separately were consistent with turnover measures and are not presented here.

For instrumental learning, reinforcers earned across days were analyzed, as were active and inactive lever presses across days (analyzed separately so that differing variance and covariance parameters could be appropriately modeled). Number of active lever presses and inactive lever presses across two days of extinction learning were also analyzed

The primary measures of discriminated Pavlovian approach behavior was the 'conditioning ratio:' a daily average of the number of head entries during CS presentations divided by that number plus the number of entries during a time period of equivalent duration immediately preceding the CS (the latter termed the pre-CS period). We also analyzed latency to enter the food magazine after CS onset; latencies less than 5 sec (because rewards were delivered on a random time 5-s schedule during the CS) and conditioning ratios greater than 0.5 are considered evidence of conditioning. Secondly, we also analyzed separately the number of head entries made during CS periods and number of head entries made during the pre-CS period.

For sign-tracking / goal-tracking data, we analyzed genotype effects on behavioral data across successive sessions mirroring the analysis in Flagel et al. (2011): a) the probability of making a lever press during a lever-CS presentation, b) numbers of lever presses, and c) latency to press the lever were the primary measures of sign-tracking behavior, while a) probability of making a head entry into the magazine during the lever-CS presentations, b) number of head entries during lever-CS presentations , and c) latency to enter the magazine after lever lever-CS presentation were primary measures of goal-tracking behavior. For comparison with the

Pavlovian magazine approach conditioning data, we also calculated conditioning ratio (also a measure of goal-tracking behavior). Data from six subjects on day 8 was lost due to technical failure, and treated as missing at random in mixed model analysis.

We also calculated proposed 'response bias' measures described by Meyer et al. (2012), wherein phenotypic tendency towards sign-tracking versus goal-tracking is quantified by a) discrimination index of contact behaviors ($[\text{number of lever-CS presses} - \text{number of food magazine entries}] / [\text{number of lever-CS presses} + \text{number of food magazine entries}]$), b) the difference between probabilities contact behaviors, and c) difference in averaged latencies to contact behavior after lever-CS onset divided by its duration. These three measures were also averaged to form a unitary approach phenotype 'summary score' ranging from +1 to -1 (fully sign-tracking to fully goal-tracking); scores were averaged across the last two sessions to form a final index measure for each subject. In order to test whether any genotype effects were obscured by analysis of all subjects' behavior simultaneously, we also used the summary score to assign mice as sign-trackers or goal trackers, on the basis of whether their score was positive or negative, respectively. We then analyzed behavior in analyses in which sign-tracking animals were segregated from goal-tracking animals.

Measures extracted and analyzed during responding for conditioned reinforcement included number of lever-CSs earned, and active and inactive aperture nose pokes.

Results

Cre-mediated recombination is detected in dopamine neuron nuclei in the DATcre+ mouse, and dopamine utilization is unchanged after genetic loss of NMDA receptors in dopamine neurons

In Figure 1A, cre-mediated gene recombination can be seen prominently in the substantia nigra pars compacta and the ventral tegmental area of the DATcre+ mouse, consistent with its initial

characterization (Backman et al., 2006). No recombination was observed in DATcre– littermates (not shown). Quantification of monoamine utilization by HPLC indicated that neither the DATcre construct nor the floxed NR1 gene affected basal dopamine utilization within the striatum (Figure 1B; all F 's < 1.51, p 's > 0.228). No effects on serotonin utilization or norepinephrine were detected (data not shown).

A mouse model of reduced phasic dopamine release does not exhibit altered preference for palatable rewards or locomotor response to a novel environment

DATcre–;NR1^{flox/flox}, DATcre–;NR1^{flox/flox}, DATcre+;NR1^{flox/wt}, and DATcre+;NR1^{flox/flox} mice were initially characterized for total ambulatory activity in a novel environment. All mice exhibited reduced locomotor behavior over time, as depicted in Figure 2A (time bin: p < 0.001); however, no main effects or interactions involving DATcre genotype or NR1 genotype were detected (all p 's > 0.374), indicating that general locomotor behavior was unaffected by genetic manipulation of NR1 in dopamine cells. These findings are in agreement with others using similar mouse models (Engblom et al., 2008; Zweifel et al., 2008).

The following day, mice were tested for differences in volume of free reward consumption. Data depicted in Figure 2B are consumption levels averaged over the final 2 sessions. All mice increased consumption of the sweetened condensed milk solution across successive days of access (day: $F_{1,320} = 12.38$, p < 0.001), but no effects of genotype were detected (all F 's < 0.64, p 's > 0.423).

Genetic attenuation of phasic dopamine release impairs instrumental behavior

A set of DATcre;NR1 experimentally naïve animals were food restricted, familiarized with the food reinforcer, and after two sessions of magazine training, began 10 consecutive days of instrumental learning. Reinforcers earned each day are depicted in Figure 3A. Here, mixed

model revealed significant DATcre x day ($\chi^2_{(1)} = 8.72, p = 0.003$), NR1 x day ($\chi^2_{(1)} = 9.31, p = 0.002$), and DATcre x NR1 x day interactions ($\chi^2_{(1)} = 16.02, p < 0.001$). The NR1 x day interaction was significant within DATcre+ animals only (within DATcre+, $\chi^2_{(1)} = 22.07, p < 0.001$; within DATcre–, $\chi^2_{(1)} = 0.51, p = 0.46$), and successive contrasts revealed that while initial behavior did not differ, DATcre+;NR1^{flox/flox} mice earned fewer reinforcers than DATcre+;NR1^{flox/wt} mice on days 3 - 6 ($\chi^2_{(1)} = 9.01, p = 0.027, \chi^2_{(1)} = 13.69, p = 0.002, \chi^2_{(1)} = 16.89, p < 0.001, \chi^2_{(1)} = 9.49, p = 0.021$, respectively). Similar findings were obtained when the omnibus interaction was explored via simple effects within NR1 genotypes (within NR1^{flox/flox}, $\chi^2_{(1)} = 22.17, p < 0.001$; within NR1^{flox/wt}, $\chi^2_{(1)} = 0.61, p = 0.434$). DATcre+;NR1^{flox/flox} earned fewer reinforcers than DATcre–;NR1^{flox/wt} mice on day 5 ($\chi^2_{(1)} = 10.14, p = 0.014$), with a similar trend on day 6 ($\chi^2_{(1)} = 7.41, p = 0.065$). Moreover, no differences between DATcre–;NR1^{flox/flox}, DATcre–;NR1^{flox/wt}, and DATcre+;NR1^{flox/wt} mice were detected (all p 's > 0.330).

To provide evidence that this difference in instrumental responding reflected differences in associative behavior, similar analyses were performed on number of active lever (Figure 3B) and inactive lever (inset) presses. A DATcre x NR1 x day interaction for active lever presses ($\chi^2_{(1)} = 8.02, p = 0.005$) was decomposed (within DATcre+, $\chi^2_{(1)} = 25.50, p < 0.001$; within DATcre–, $\chi^2_{(1)} = 0.57, p = 0.448$) to reveal that fewer active lever presses were made by DATcre+;NR1^{flox/flox} mice relative to DATcre+;NR1^{flox/wt} mice, again on days 3 - 6 ($\chi^2_{(1)} = 9.36, p = 0.022, \chi^2_{(1)} = 14.88, p = 0.001, \chi^2_{(1)} = 18.45, p < 0.001, \chi^2_{(1)} = 9.72, p = 0.018$, respectively). Fewer active lever presses were also made by DATcre+;NR1^{flox/flox} mice relative to DATcre+;NR1^{flox/wt} on day 5 ($\chi^2_{(1)} = 11.55, p = 0.007$) and near-significant differences were found for day 6 ($\chi^2_{(1)} = 7.72, p = 0.054$). Analysis of inactive lever presses revealed no effects

of or interactions with genotypes (no main effects of genotype nor any interactions per genotype (all p 's > 0.102)

Genetic attenuation of phasic dopamine release does not impact acquisition of a Pavlovian magazine approach behavior

Prediction error signals in the mesencephalon have been most extensively characterized within the context of appetitive Pavlovian conditioning (Ljungberg et al., 1992; Schultz et al., 1997); these studies, however, are by and large correlational in nature. With this in mind, we examined whether genetic reduction of phasic dopamine activity impacted the ability of conditional stimuli to elicit Pavlovian magazine approach.

After a 14-d period, during which no testing occurred but food restriction was maintained, 64 of the same subjects underwent 10, daily Pavlovian magazine approach conditioning sessions. Across these conditioning sessions, as shown in Figure 4A, number of head entries during the CS period progressively increased (day: $p < 0.001$), while head entries during an equivalent period of time prior to CS onset – termed the pre-CS period (day: $p < 0.001$) diminished. ‘Conditioning ratio,’ a quantitative measure of discriminative Pavlovian responding (CS period / CS+Pre-CS period magazine head entry counts), depicted in Figure 4B, increased progressively as training continued (day: $p < 0.001$). No interactions involving DAT^{cre} or NR1 genotypes for entries during the CS, entries during the pre-CS, or for conditioning ratio were significant (all p 's > 0.374). Similarly, rate of decreases in latencies to enter the food magazine after the CS onset was unaffected by genotypes (Figure 4C; all p 's > 0.162).

Individual variation in tendency to attribute incentive salience to reward-predictive cues is unaffected by genetic attenuation of phasic dopamine release

A new cohort of DATcre;NR1 mice were food restricted, given two sessions of magazine training, and 15 sessions of pairings between a lever-CS and food delivery. Here, all subjects were DATcre+. Because we consistently failed to find phenotypic effects in DATcre+;NR1^{flox/wt} animals, we treated them as controls in this experiment, and compared their behavior to that of DATcre+;NR1^{flox/flox} animals.

Acquisition of sign-tracking and goal-tracking conditional responses are depicted in Figure 5, using the dependent measures of Flagel et al. (2011). Note that the range of sign-tracking and goal-tracking response graphs differ; though we reliably detected vigorous sign-tracking to the lever-CS, this conditional response occurred in fewer mice than did the goal-tracking conditional responses. Additionally, because we present quantitative goal-tracking and sign-tracking measures from the same subjects in Figure 5, rather than segregating subjects as expressing one response or the other, goal-tracking learning curves appear as less steep, because this response tended to be learned first, as can occur in rats (Meyer et al., 2012). This goal-tracking is then diminished as it undergoes response competition in animals that transition to sign-tracking. Notably, however, magazine entry (i.e., goal-tracking) conditioning ratio (Supplemental Figure 1) develops and stabilizes in a manner similar to the Pavlovian approach learning presented in Figure 4.

No genotype effects on magazine approach/goal-tracking behavior, as measured by probability of entering the magazine (at once per lever-CS), number of magazine entries during the lever-CS and a preceding pre-CS period, and magazine entry latency, were found (Figure 5A-C, left side; all NR1, DATcre effects and interactions p 's > 0.103). Moreover, no genotype differences on the development of corresponding lever-CS approach/sign-tracking behaviors were observed: animals with lacking NMDA-dependent phasic dopamine release were as likely as controls to learn to press the lever-CS as conditioning sessions progressed, and made similar number of presses at similar latencies (Figure 5A-C, all p 's > 0.239).

Because sign-tracking responses tend to come at the expense of goal tracking responses, and vice versa, we calculated relative response bias scores (Meyer et al., 2012) to better understand the distribution of conditional responses expressed. In Figure 6, scores above zero are indicative of a tendency to sign-track rather than goal-track, and negative values correspond to goal-tracking bias. Biases in the probability of making a sign-tracking versus a goal-tracking response was relatively similarly distributed in both genotype groups; similar results can be seen for number of responses and response latency in Supplemental Figures 2 - 3.

As in Meyer et al. (2012), we calculated the average of these three bias measures (see Supplemental Figure 4). Summary scores averaged across the last two sessions are depicted in Figure 7A (left side). No significant differences between genotypes were found ($t_{61} = 0.772$, $p = 0.443$). We designated mice sign-trackers if their final summary scores were positive, and goal-trackers if negative (Figure 7A, right side). Frequency of expression of each phenotype did not differ between genotypes (Fisher's exact test, $p = 1.00$). The acquisition of each conditional response in mice designated sign-trackers and mice designated goal-trackers is shown in Figures 7B and 7C. We analyzed, in an attempt to detect differences in the rate of learning of their respective conditional response, the acquisition of lever approach in sign-trackers selectively and the magazine approach in goal-trackers selectively; however, again, no genotype interactions across conditioning sessions were detected (Figure 7B; all p 's > 0.404 ; see Supplemental Figure 5 for similar figures for response latency and number of response).

Finally, we studied the ability of the lever-CS to support new learning in a single conditioned reinforcement test, which has previously been shown to be elevated in animals who predominantly exhibited a sign-tracking response (Robinson and Flagel, 2009; Flagel et al., 2011; Lomanowska et al., 2011). Mice were allowed to make nose poke responses to elicit brief presentations of the lever-CS during a single session that followed the last day of conditioning. The number of lever-CS presentations earned, along with number of active nose

poke aperture responses and inactive nose poke aperture responses made, shown in Figure 8, were similar across genotypes (lever-CSs: $t_{61} = 0.526$, $p = 0.601$; active aperture: $t_{61} = 0.559$, $p = 0.551$; inactive aperture: $t_{61} = 0.553$, $p = 0.581$).

Discussion

In a series of experiments aimed at characterizing the effects of genetic excision of the NMDA receptor from dopamine neurons, as a model of attenuated dopamine neuron phasic firing, we demonstrated a distinct configuration of effects on associative learning. Lower phasic dopamine release impaired instrumental learning but was without effect on Pavlovian magazine approach learning, or on the acquisition or probability of goal-tracking and sign-tracking. These results are presented against the backdrop of normal exploratory locomotion behavior and reward consumption, eliminating these ancillary phenotypes as likely explanations for the observed learning effects. Moreover, the observation of unaffected basal dopamine utilization in the striatum indicates the absence of any gross differences in the dopamine system that would complicate interpretation of results.

Phasic dopamine contributes to acquisition of an appetitive instrumental response

Here, loss of NMDA receptors in dopamine neurons caused impaired instrumental responding, in general agreement with results gathered earlier using a similar mouse model (Zweifel et al., 2009). The particular pattern of results, with impairments in spontaneously acquired instrumental behavior not observed until the middle of the learning curve, is most consistent with an impaired learning process, rather than any baseline differences in lever press responses themselves. Additionally, groups converged by the end of training. The absence of asymptotic differences in instrumental behavior, which might reflect motivation to obtain the reinforcer, may indicate that the phenotype in DAT^{cre+};NR1^{flox/flox} animals relates to altered learning.

This result would appear to implicate a causal role for phasic dopamine in instrumental learning. These findings are consistent with a number of optogenetic studies wherein response-contingent optical activation of dopamine neurons either facilitated an appetitive instrumental or was sufficient to support responding alone (Adamantidis et al., 2011a; Witten et al., 2011a; Kim et al., 2012). Most recently, a particularly compelling strategy was used to support the notion that dopamine activity functions as a prediction error signal: after asymptotic acquisition of the relationship between CS that was predictive periods of response-contingent reward availability (i.e., a discriminative stimulus), transient co-activation of dopamine neurons upon compound CS presentation dramatically reduced the normally observed blocking effect (Steinberg et al., 2013). Moreover, the behavioral impact of unexpected negative shifts in outcome value was also diminished by activation of dopamine neurons. These findings are particularly consistent with phasic dopamine acting as prediction error signal that plays a causal role in reward-related learning.

The optogenetic results are convincing. One caveat, however, is that it remains unclear how the creation of action potentials via optogenetic stimulation influences dopamine cells, aside from the intended attempt to replicate phasic burst patterns. For example, whether optogenetic induction of phasic events causes artifacts such as differential cell activity during peri-phasic event periods, or whether the simulated phasic firing truly reproduces normal stimulus-elicited phasic events and causes post-synaptic effects that replicate those of stimulus-evoked events, remains unclear. Here, we were able to demonstrate that instrumental learning is affected by attenuation of natural phasic dopamine release, signals that were endogenously generated in response to environmental stimuli. Consequently, while we were not able to directly control the action potentials of dopamine cells, these data may more accurately reflect role for phasic dopamine in reward-seeking.

Pavlovian magazine approach behavior is unaffected by attenuated phasic dopamine capacity

Given that prediction error signals in the mesencephalon have been observed during Pavlovian conditioning, across a wide variety of task conditions and parameters most extensively characterized within the context of appetitive Pavlovian conditioning (Ljungberg et al., 1992; Schultz et al., 1993; Schultz et al., 1997; Waelti et al., 2001; Fiorillo et al., 2003), we expected reductions in NMDA receptors in DA neurons would impair this behavior. However, we detected no effects, nor have several others using a similar mouse genetics approaches (Parker et al., 2010; Parker et al., 2011a).

One potential explanation is that during instrumental performance, attenuated incentive salience attribution to instrumental outcomes associated with diminished capacity for phasic dopamine release resulted in diminished levels of reward-seeking; on the other hand, the Pavlovian approach paradigm used may have emphasized contingency learning rather than incentive salience attribution. Alternatively, it may be that the failure to detect effects on Pavlovian approach behavior is a consequence of the residual phasic dopamine release.

NMDA receptor loss in dopamine neurons results in attenuation of the magnitude of phasic dopamine release to approximately 30% that of controls (Zweifel et al., 2009; Parker et al., 2010). It is probable that this remaining signal may be sufficient to support Pavlovian appetitive learning, given that reward preference and reward learning is possible even after massive dopamine depletions (Cannon and Palmiter, 2003; Robinson et al., 2005). What is left may be entirely sufficient signal to noise for acquisition and representation of Pavlovian delay conditioning, especially when associative contingencies are binary and deterministic (i.e., $P(US|CS)=1$, $P(US|\sim CS)=0$). The magnitude of midbrain neuron burst responses encodes the relative value of predictive stimuli (Fiorillo et al., 2003); perhaps a behavioral impairment would be revealed in a scenario where 30% of the normal to signal to noise signaling in dopamine neurons is not sufficient (e.g., discriminating between two stimuli with marginal differences in predictive value). Curiously, though, impairments in fear conditioning (measured with

conditional fear potentiated startle) have been reported in mice lacking NMDA-dependent phasic dopamine release (Zweifel et al., 2009; Zweifel et al., 2011), suggesting valence and/or the degree to which the US supports rapid conditioning may also be significant factors.

Interestingly, other phenotypes that were historically thought to require NMDA receptors in dopamine neurons, such as sensitization to psychostimulants (Kalivas and Alesdatter, 1993; Wolf et al., 1994; Wolf, 1998; Vanderschuren and Kalivas, 2000), have also turned out to be unaffected in their absence (Zweifel et al., 2008; Beutler et al., 2011; Luo et al., 2011). Given that the degree of NMDA receptor dependent plasticity – expressed as an increase AMPA receptor expression or current – that is induced by drugs of abuse correlates with degree of behavioral sensitization observed (Ungless et al., 2001; Borgland et al., 2004) and is expressed selectively during periods of active learning of in Pavlovian conditioning (Stuber et al., 2008), these results are especially surprising. Several studies have implicated NMDA receptors in other cell-types or brain regions as responsible for these phenomena (Beutler et al., 2011; Luo et al., 2011; Parker et al., 2011a); further discussion of this can be found in the conclusion chapter of this thesis.

Genetic model of attenuation of phasic dopamine release does not impact frequency or acquisition of a sign-tracker conditional response

The incomplete loss of phasic dopamine release in this genetic model offers a unique opportunity to distinguish between the prediction-error and the incentive salience perspectives of dopamine in learning. In many cases, learned behavioral phenomena can be equally explained by the acquisition of the contingencies between stimuli using phasic dopamine prediction error signals, or the combination of non-dopaminergic contingency learning with attribution of incentive properties to associative stimuli via phasic dopamine release, rendering them non-dissociable.

Sign-tracking rats – those that approach and interact with a predictive cue (often the extension of a lever-CS) , often at the expense of approaching the location of reward delivery (Williams and Williams, 1969)– are thought of as exhibiting a form of incentive salience attribution that is over and above the Pavlovian contingency learning exhibited by goal-tracking rats – those that approach the location of food delivery the location of food delivery. Because sign-tracking is thought to rely upon a form of Pavlovian incentive salience attribution, it is unsurprising that sign-tracking animals display more prominent CS-evoked dopamine as they learn their conditional response than do goal-trackers (Flagel et al., 2011). Therefore, both goal-trackers and sign-trackers learn the contingency between the CS and the US, but it has been suggested that sign-trackers' phasic dopamine signals also imbue the CS with incentive motivational properties, effectively utilizing dopamine as an 'incentive salience attribution prediction error signal. If this is a causal relationship, we hypothesized that the quantitative reduction in phasic dopamine release amplitude should reduce the frequency of sign-tracking behavior or the rate of its acquisition. Here, we found no evidence to support this conclusion: in all dependent measures, no behavioral differences were observed in our mouse model of diminished phasic dopamine activity.

Locomotor sensitization to psychostimulants is a phenomenon considered to be linked, with respect to dopaminergic substrates and behavioral co-presentation, to heightened or sensitized incentive salience attribution (Robinson and Berridge, 1993; Wyvell and Berridge, 2001; Tindell et al., 2005; Olausson et al., 2006), and locomotor sensitization to amphetamine has been shown to enhance sign-tracking behavior (Doremus-Fitzwater and Spear, 2011). The preponderance of studies mentioned above indicating that locomotor sensitization is readily inducible in the absence of NMDA receptors in dopamine neurons (Engblom et al., 2008; Zweifel et al., 2008; Beutler et al., 2011; Luo et al., 2011) are consistent with our finding that attenuation of phasic dopamine activity via elimination of NMDA receptors on dopamine

neurons did not affect incentive salience attribution during Pavlovian conditional approach. While other neurobiological, genetic, and environmental factors likely distinguish animals that develop a goal-tracking conditional response from those that develop a sign-tracking conditional response (Flagel et al., 2007; Flagel et al., 2010; Lomanowska et al., 2011; Fitzpatrick et al., 2013; Perez-Sepulveda et al., 2013; Haight and Flagel, 2014), our data indicate that quantitative variation in phasic dopamine release, at least within the range we can manipulate it with this model system, does not.

A recent voltammetry study of sign-tracking behavior offered some new insight into sign-tracking behavior. Over the course of long term sign-tracking training, US-evoked dopamine diminished, and as training further continued, eventually the CS-evoked response diminished as well (Clark et al., 2013). A similar result was found in early midbrain prediction error signal studies (Ljungberg et al., 1992). This phenomenon is by no means inconsistent with incentive sensitization, which focuses on how exaggerated increased control over behavior by reward-predictive cues is acquired rather than maintained (Robinson and Berridge, 1993). Indeed, dependence of behaviors upon dopamine diminishes as actions are over-trained and transition to habits (Choi et al., 2005). However, Clark et al. (2013) were able to demonstrate that the diminished CS-evoked dopamine was a consequence of animals learning the timing of inter-trial intervals; when a CS was presented after an unusual inter-trial interval duration, CS-evoked dopamine returned to baseline. This is consistent with prediction error accounts: the unexpected stimulus presentation can be seen as a positive prediction error. It is more difficult to reconcile with incentive salience attribution, however. Once a cue has acquired incentive motivational properties – coincident with the development of CS-evoked phasic dopamine release – why presenting it at a slightly different time would impart further incentive salience is unclear. Thus, phasic dopamine signaling resembling prediction error is indeed observable within the context of sign-tracking behavior, consistent with our failure to find group differences

in our genetic model of diminished phasic release.

Indeed, it is possible that the difference between sign-tracking and goal-tracking is less related to incentive salience attribution as it may be related to Pavlovian versus Pavlovian *and* instrumental conditioning: while components of conditional responses that resemble sign-tracking are not reduced by omission schedules of reinforcement (a test of responsivity to instrumental contingencies), goal-tracking responses are suppressed (Holland, 1977; Holland, 1979). Thus, the former may be a more 'pure' Pavlovian behavior and the latter a Pavlovian cue triggered instrumental response directed towards reward collection, rather than an incentive salience-driven behavior versus one that is not.

Sign-tracking in mice

Sign-tracking behavior comparable to that observed towards a lever-CS in rats has been difficult to reproduce in C57Bl/6J mice: mice either show no lever-CS directed behavior (Zweifel et al., 2009; Parker et al., 2011a), or only demonstrate conditional locomotion in the vicinity of the lever-CS (Gore and Zweifel, 2013). Sign tracking in the form of actual lever presses, however, have not been reported previously.

Of interest was the considerable individual variation in whether a sign-tracking or goal-tracking CR emerged. Mice generally began with goal tracking (presumably due to previous magazine training), but 20-25% then developed overt sign-tracking conditional responses without any accompanying goal-tracking (see bias distribution figures); others continued goal-tracking, and others performed both behaviors. Given that these mice, aside from the genetic manipulations that were without effect, are genetically homogenous, this variation in response type suggests considerable influence of (unmeasured) environmental or other non-heritable genetic factors, as has been observed in rats (Lomanowska et al., 2011). However, it is possible that the C57Bl/6J strain has a relatively low tendency to sign-track, and the phenotype is under strong

heritable control. Sign-tracking in different strains of inbred mice has not been systematically explored, and the genetic underpinnings of individual variation in this behavior remain an open question.

Here, the sign-tracking conditional response appeared to be less common than in published data on rat behavior, and its onset is likely delayed relative to rats as well. However, video monitoring of testing chambers revealed that the sign-tracking phenotype is very much present in mice (data not shown): those that engaged in this behavior did it consistently and vigorously, engaging in the same consummatory-like rapid biting, gnawing, and invigorated approach to the lever-CS reported in rats (Zener, 1937; Jenkins and Moore, 1973; Boakes, 1977; Tomie, 1996; Flagel et al., 2010). Behavior was many times observed to be intensely focused towards the lever-CS, either expressed as stereotypic sniffing and various other interaction that did not necessarily result in a lever actuation; consequently, it's likely that mice sign-track more often than we report here.

Design of cre-loxP experiments

Many previous studies of genetic deletion of NR1 from dopamine neurons have used only cre+ and cre- animals, or only heterozygote and homozygote floxed animals (Zweifel et al., 2008; Zweifel et al., 2009; Parker et al., 2010; Parker et al., 2011a). Here, we used a 2x2 genotype design: animals carried either one or two floxed alleles, and were cre-positive or cre-negative. This design allows enables us to make stronger conclusions. In particular, the statistical tests for interactions between DATcre genotype and NR1 genotype lend credibility to the conclusion that the effect observed is due to cre-mediated gene excision, rather than various confounding factors that can affect transgenic mouse research (e.g., background effects and flanking genes from a non-isogenic donor strain traveling with the modified gene to a significant degree even after extensive backcrossing). Examples of these effects are numerous (Wade, 1987; Le Roy et

al., 2000; Spyropoulos et al., 2003; Chen et al., 2004; Pekarik and Izpisua Belmonte, 2008). These factors are especially significant for studies utilizing the NR1^{flox/flox} mouse, which was generated using 129S4/SvJae strain embryonic stem cells (Tsien et al., 1996b; Tsien et al., 1996a), followed by a moderate level of backcrossing to a C57Bl/6N background.

Limitations

The conditional inactivation of NR1 blocks NMDA currents, which reduces phasic firing, but also eliminates NMDA receptor-mediated LTP (Engblom et al., 2008; Zweifel et al., 2008; Luo et al., 2011), which is well known to associate with learning (Morris et al., 1986; McHugh et al., 1996). This does present some difficulties with respect to interpreting our results strictly from the perspective of phasic dopamine release. Thus, although we cannot rule out a role for phasic dopamine release in Pavlovian approach conditioning, we can conclude that it does not rely upon NMDA-related plasticity in dopamine neurons, irrespective of whether it takes the form of goal-tracking or sign-tracking. However, we argue that the effects observed here are unlikely to be related to loss of LTP given that a) loss of NMDA receptor LTP does not affect locomotor sensitization, which has been linked to incentive sensitization, b) were it to be a critical neural mechanism, loss of LTP in dopamine neurons would likely be expected to diminish sign-tracking, and c) in addition to Pavlovian learning, instrumental learning is also impaired by infusion of an NMDA antagonist into the VTA (Stuber et al., 2008; Zellner et al., 2008). Yet when NMDA receptors were removed from dopamine neurons entirely, Pavlovian behaviors were unimpaired, while instrumental responding was affected, suggesting differences in phasic dopamine release, rather than plasticity, were responsible for the observed effects.

Conclusions

Because aberrant reward-related behaviors found in a range of psychiatric conditions, including addiction, autism, attention deficit hyperactive disorder, and depression (Robinson and

Berridge, 1993; Taylor and Jentsch, 2001; Neuringer, 2002; Everitt and Robbins, 2005; Olausson et al., 2006; Martin-Soelch et al., 2007; Flagel et al., 2009; Groman et al., 2009; Shiflett and Balleine, 2011; Groman et al., 2012), understanding the intricacies of phasic dopamine release in these reward learning is of particular clinical significance.

Here, we utilized a mouse model of compromised NMDA-dependent phasic dopamine release to study reward-related associative learning. The quantitative reduction endogenously generated phasic dopamine signals in the mouse model represents a unique biologically plausible model of alterations in phasic dopamine neurotransmission. In addition to lending external validity to the data gathered here, this characteristic makes this mouse model well positioned to answer many other questions about dopamine in reward-related behaviors. Most recently, for example, *reductions* in phasic dopamine release have been implicated in escalation of cocaine use (Willuhn et al., 2014), while another implicated *augmented* signals in cocaine's ability to heighten cue-evoked reward seeking (Ostlund et al., 2014). By virtue its incomplete depletion of signals that are not programmed by optical or electrical stimulation, extending the DAT^{cre};NR1 mouse to these paradigms in the future may allow parsing the causal contribution of phasic dopamine to the significant aspects of addiction they model.

The series of experiments presented here suggest that reduction in phasic dopamine release impairs instrumental, but not Pavlovian learning or the expression of conditional responses, regardless of whether are expressed as goal-tracking or sign-tracking. Our results are not necessarily uniformly consistent with a prediction error account of dopamine, nor are they fully consistent with incentive salience perspectives. Rather, we argue they are more congruent with multifaceted conceptualizations of dopamine, such as those that posit dopamine acts as a behavioral invigorator, with phasic dopamine release directing attention towards seeking biologically significant stimuli, resulting in behavioral and cognitive focus on the relationships between stimuli and the responses that produce these stimuli, which are learned, in some cases, by prediction error signals.

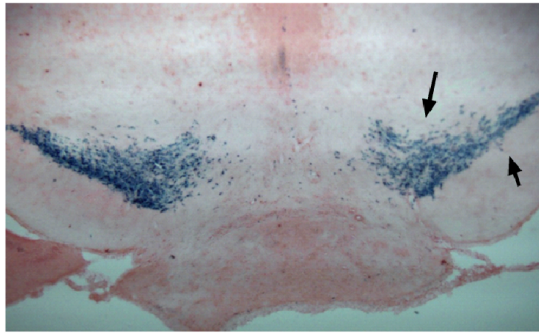
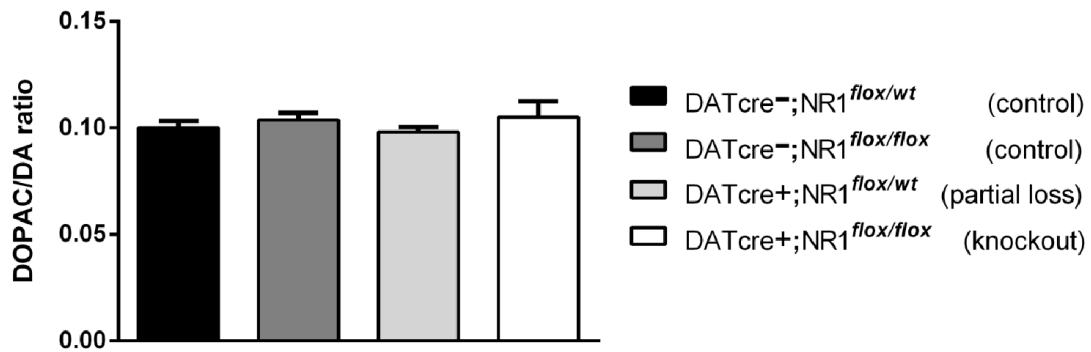
A**B**

Figure 1: Cre-mediated recombination is detected in dopamine neuron nuclei in the DATcre⁺ mouse, and dopamine utilization is unchanged after genetic loss of NMDA receptors in dopamine neurons. (A) Prominent recombination is seen in the midbrain of DATcre⁺ mice crossed with ROSA26-LacZ mice; arrows indicate ventral tegmental and substantia nigra pars compacta nuclei. (B) Nucleus accumbens dopamine turnover are indistinguishable amongst the 4 combinations of DATcre and NR1 genotypes.

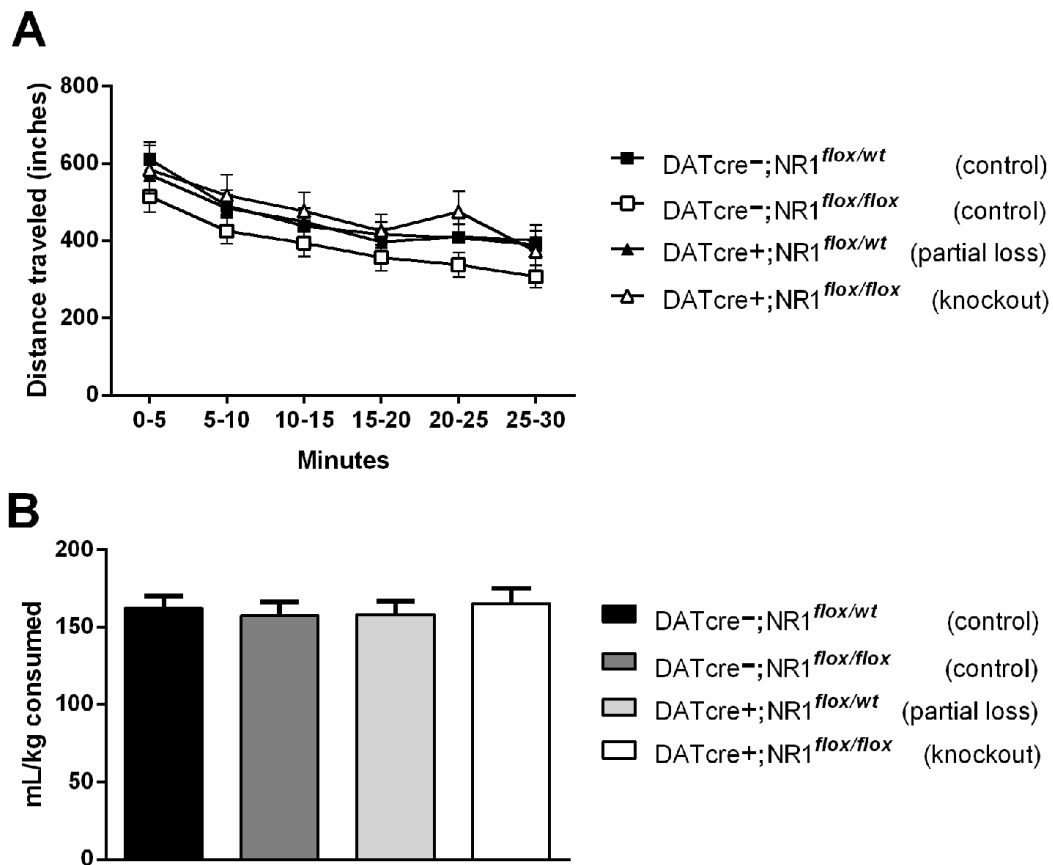


Figure 2: A mouse model of reduced phasic dopamine release does not exhibit altered locomotor response to a novel environment or preference for palatable rewards. (A) No genotype effects were found over successive 5-min bins, and (B) levels of consumption of a 10% sweetened condensed milk solution were similar across all genotypes.

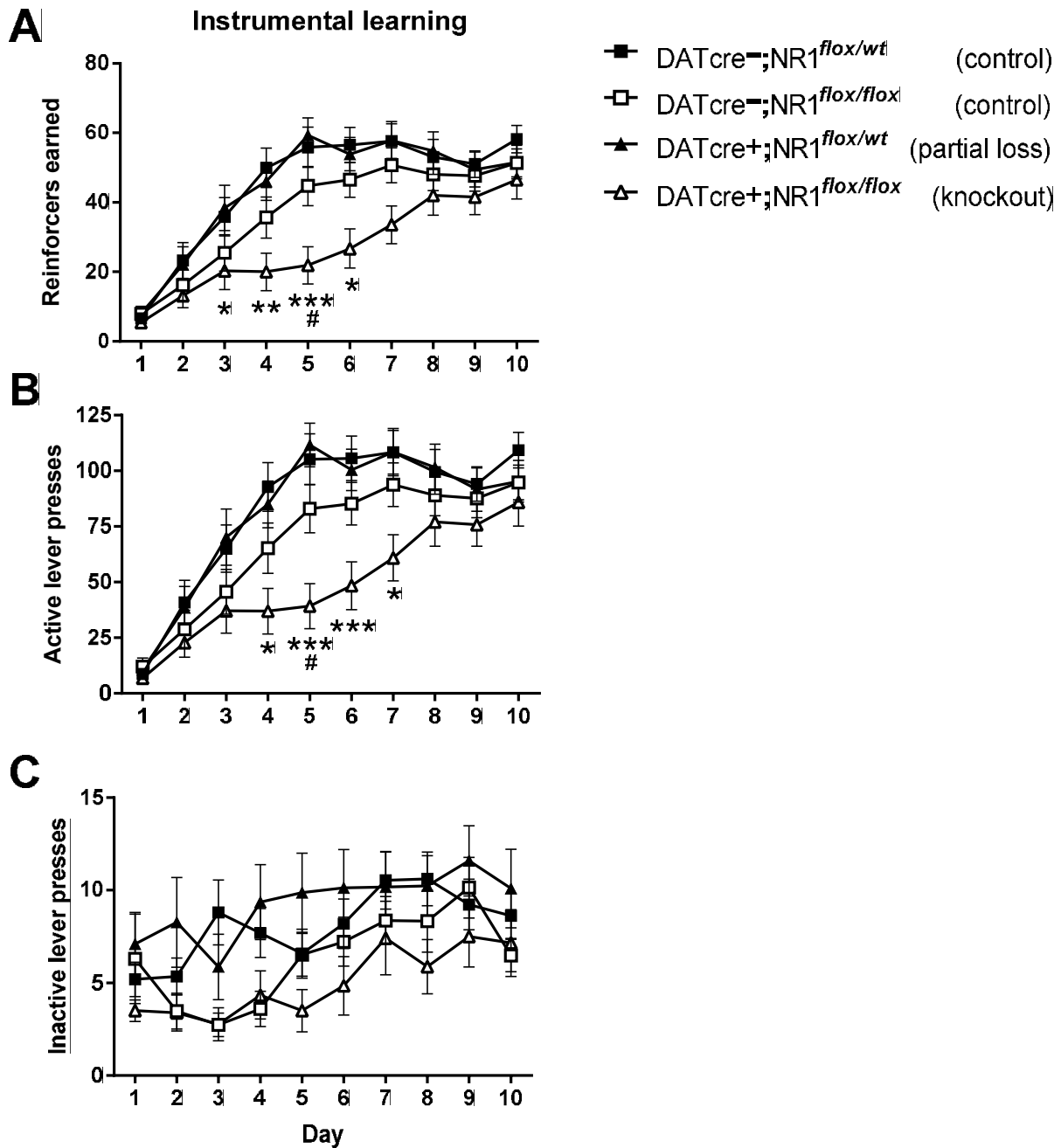


Figure 3: Loss of NMDA receptors in dopamine neurons impairs instrumental learning.

(A) DATcre⁺;NR1^{flox/flox} earn less reinforcers over 10 days of instrumental learning, and (B) make less active lever presses, but (C) press the inactive lever at levels similar to the three other genotypes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ DATcre⁺;NR1^{flox/flox} vs DATcre⁺;NR1^{flox/wt}; # $p < 0.05$ vs. DATcre⁻;NR1^{flox/flox} mice.

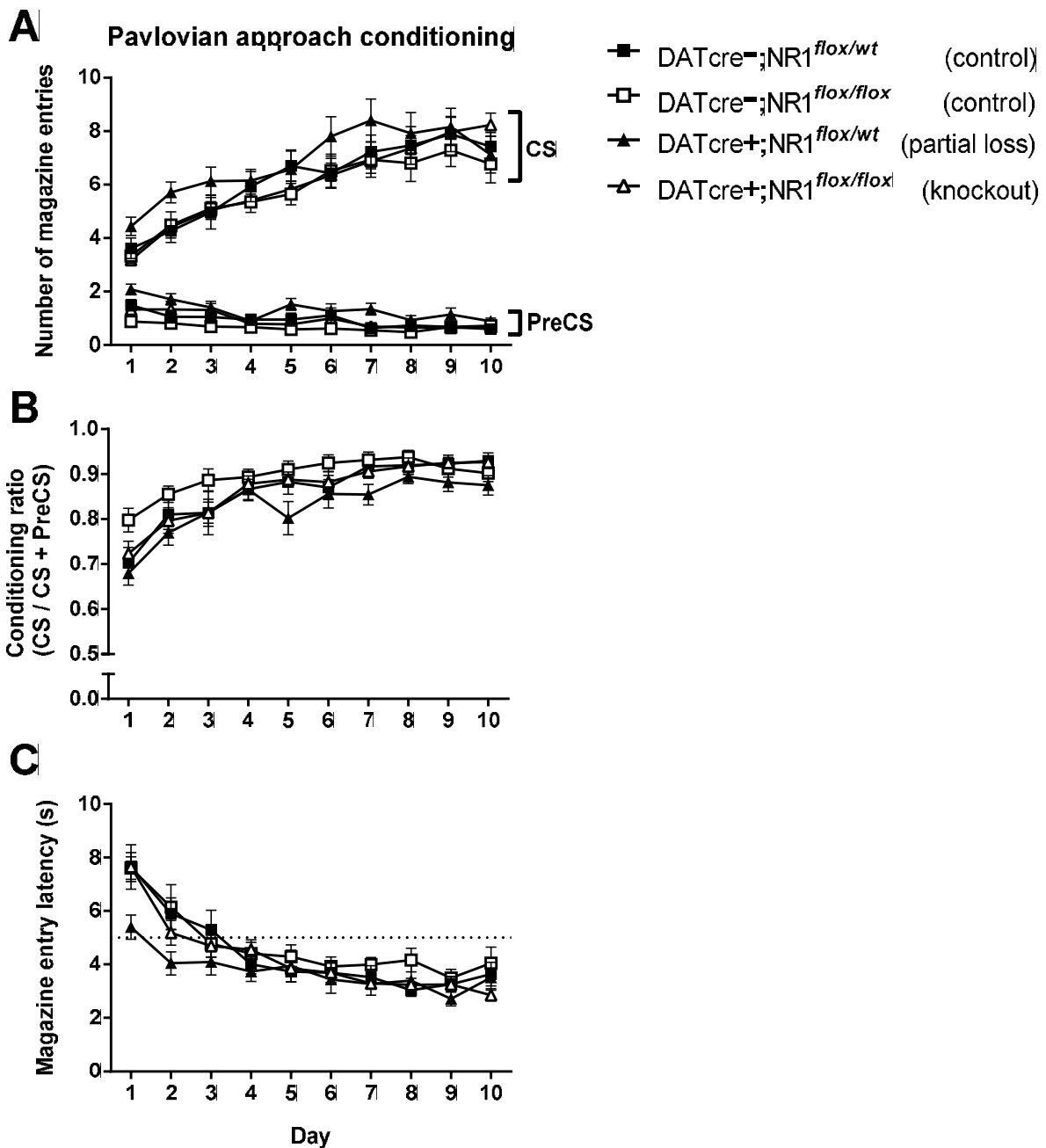


Figure 4: Loss of NMDA receptors in dopamine neurons leaves Pavlovian magazine approach conditioning intact, as measured by A) number of entries into the food magazine during the CS and a pre-CS period of equivalent length, B) the conditioning ratio of CS period to CS + pre-CS period head entries, and C) latency to enter the food magazine upon the onset of the CS. Dashed line indicates the cross-over point between potentially US-elicited behavior (> 5-sec) and CS-elicited magazine approach (< 5-sec).

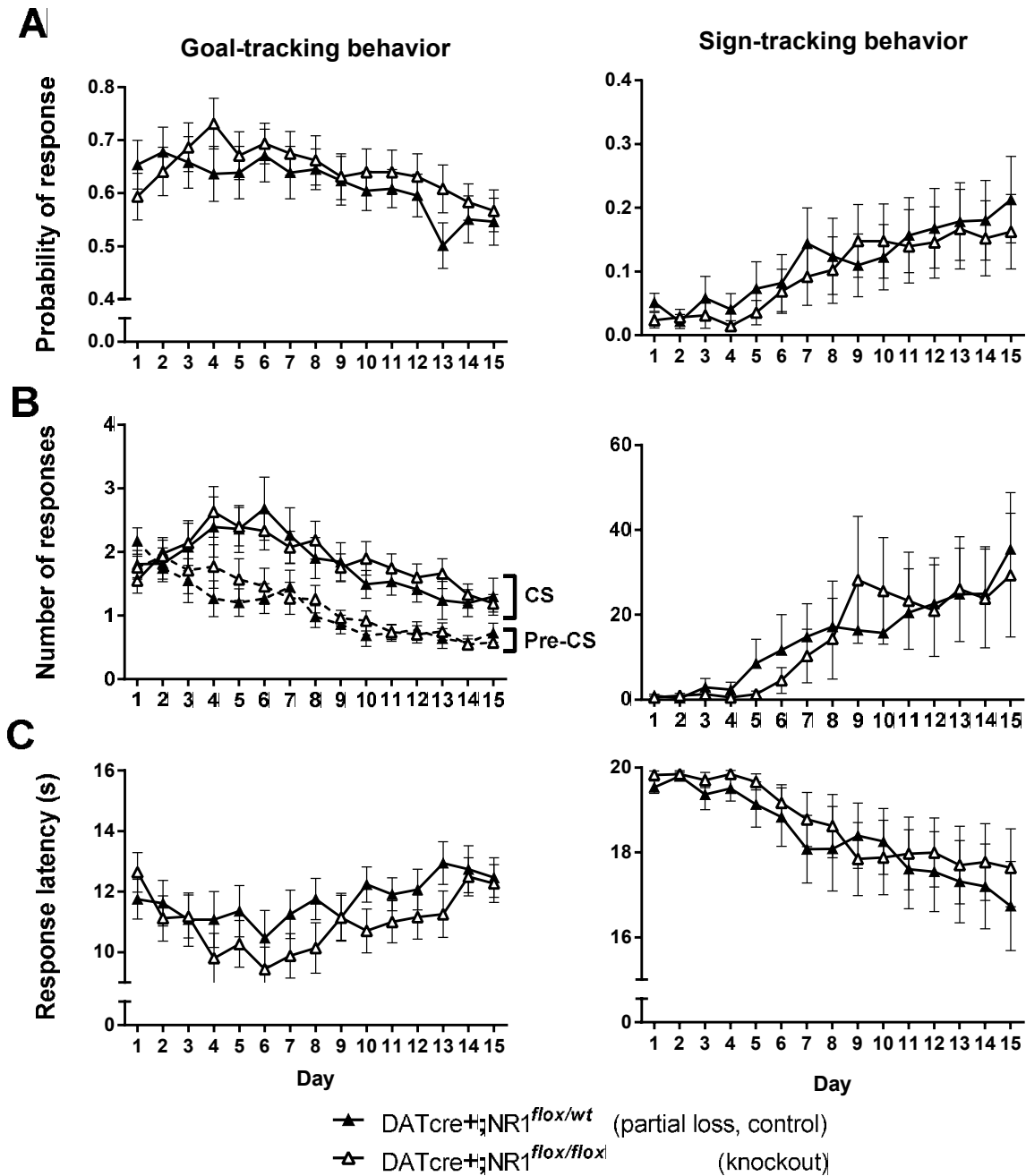


Figure 5: Genetic deletion of NMDA receptors in dopamine neurons is without effect on incentive salience attribution. Mice fully with two floxed NR1 alleles engage in goal tracking and sign-tracking behaviors at levels similar to heterozygote controls, as measured by (A) probability of a single magazine entry (left) or lever press (right) during lever-CS presentation; (B) Number of magazine head entries (left) and lever presses (right) during lever-CS presentation and (C) latency to enter the magazine (left) or contact the lever-CS (right) upon its extension.

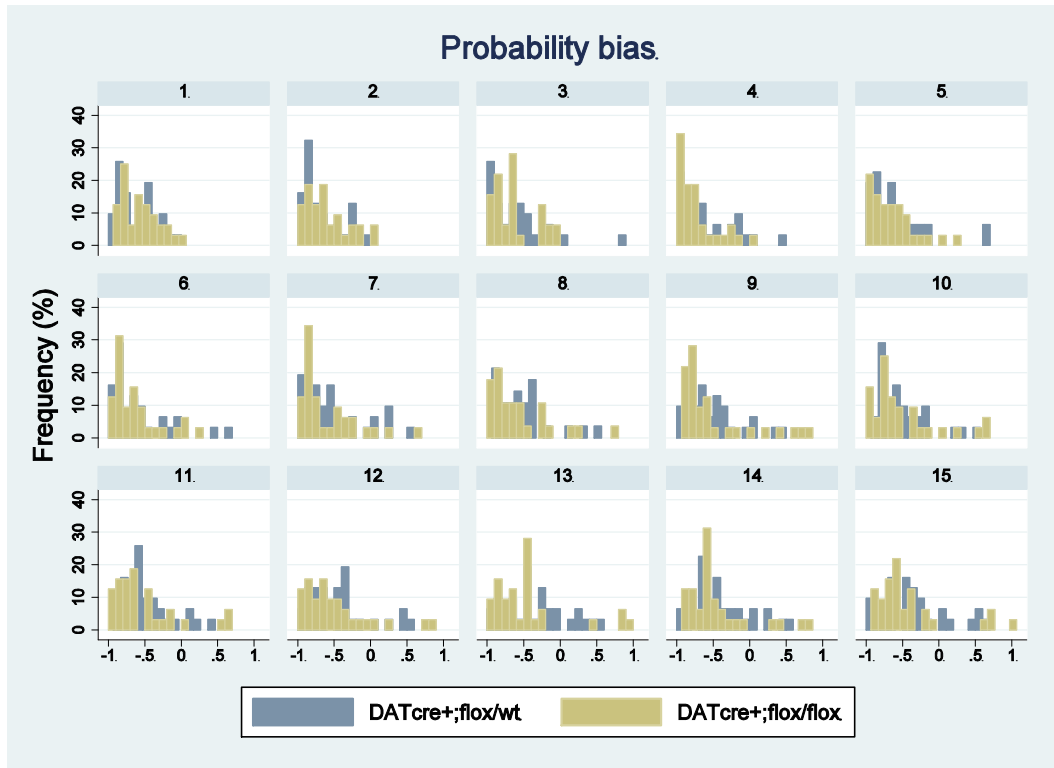


Figure 6: A closer look at the distribution of sign-tracking and goal-tracking behavior of DATcre+;NR1^{flox/wt} and DATcre+;NR1^{flox/flox} mice. Histograms depict sign-tracking versus goal-tracking biases with respect to response probability. Positive values indicate a tendency to sign-track, and negative values a tendency to goal-track. Goal-tracking is dominant early in training, but emerges progressively across successive days. The distributions of both genotypes are highly overlapping at all stages of training.

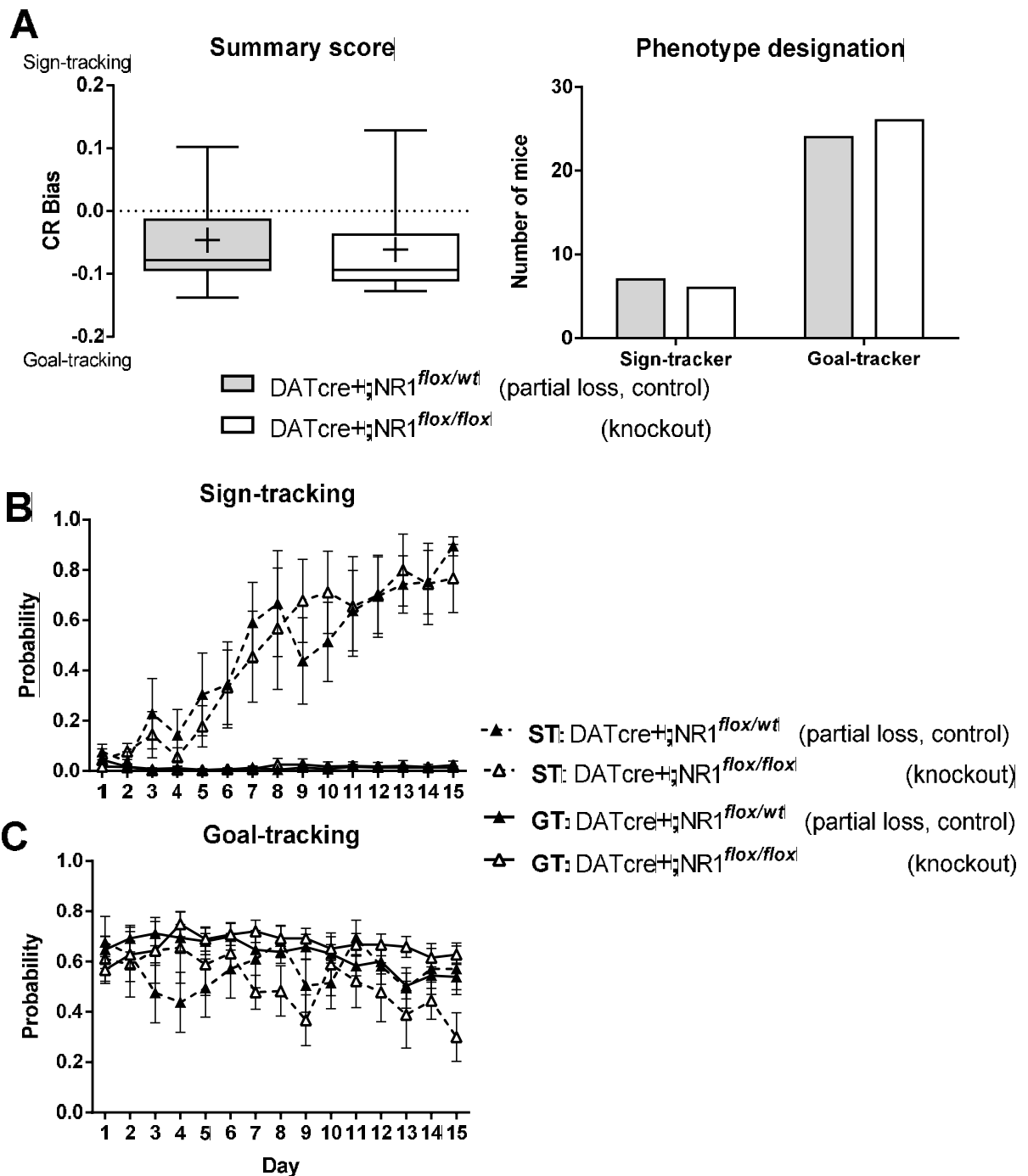


Figure 7: Analyses within groups according to conditional response designation. (A)

Box-and-whisker plots for conditional response bias summary scores (left) averaged across the last two days of training. Positive and negative values were used to

designate mice as sign-trackers and goal-trackers (right), respectively. (B) Sign-tracking

behavior, expressed as response probability plotted according to designation. (C) Goal-

tracking behavior, with mice designated goal-trackers plotted according to designation.

Behavior did not differ as a function of genotype in either case.

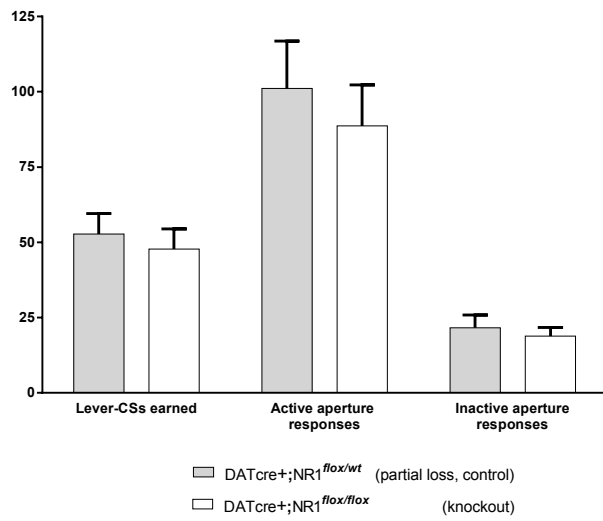
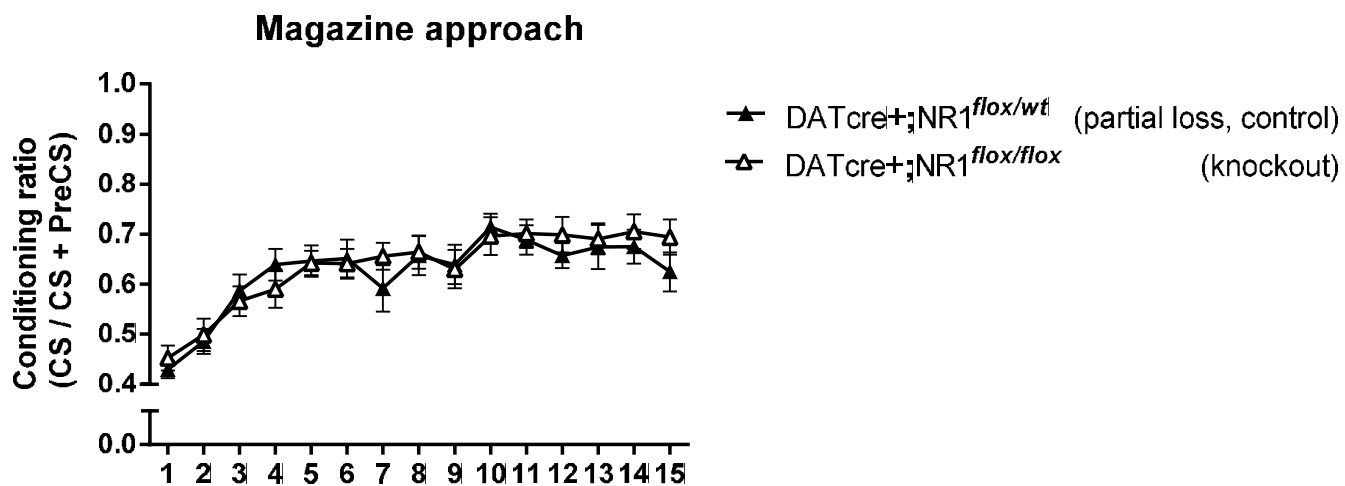
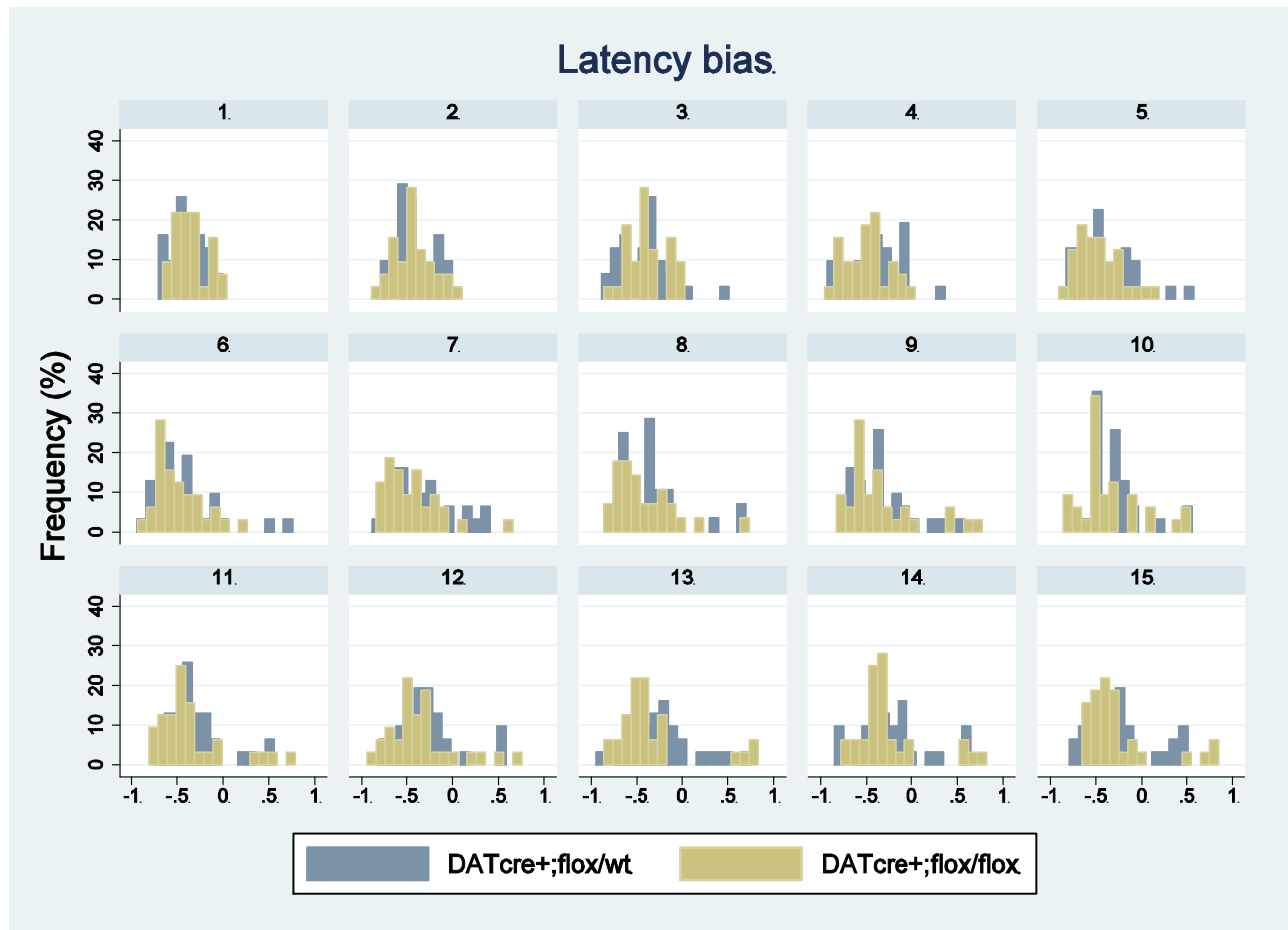


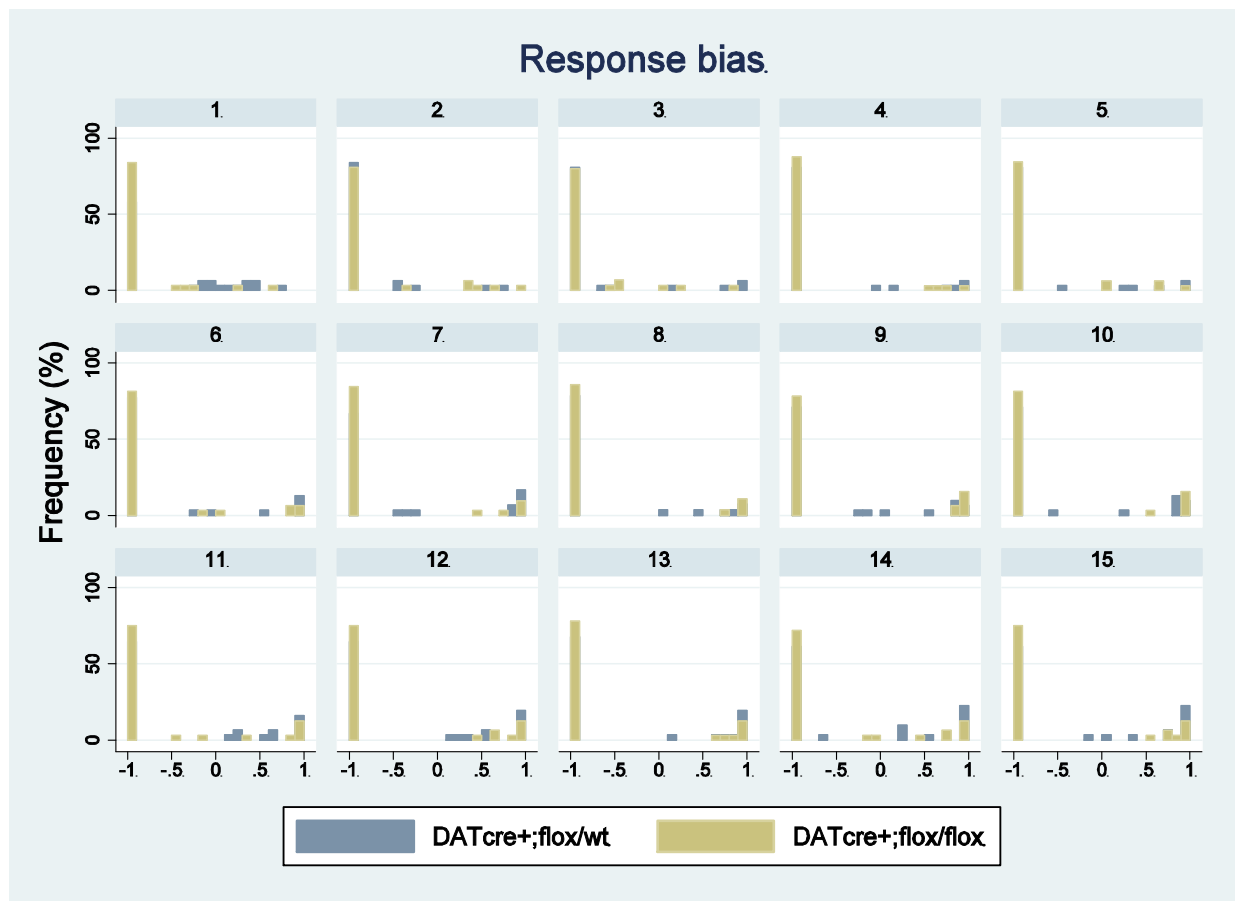
Figure 8: Test of conditioned reinforcement. Behavior from a single session in which mice were allowed to earn brief presentations of the lever-CS by performing a novel instrumental response. No genotype effects were found.



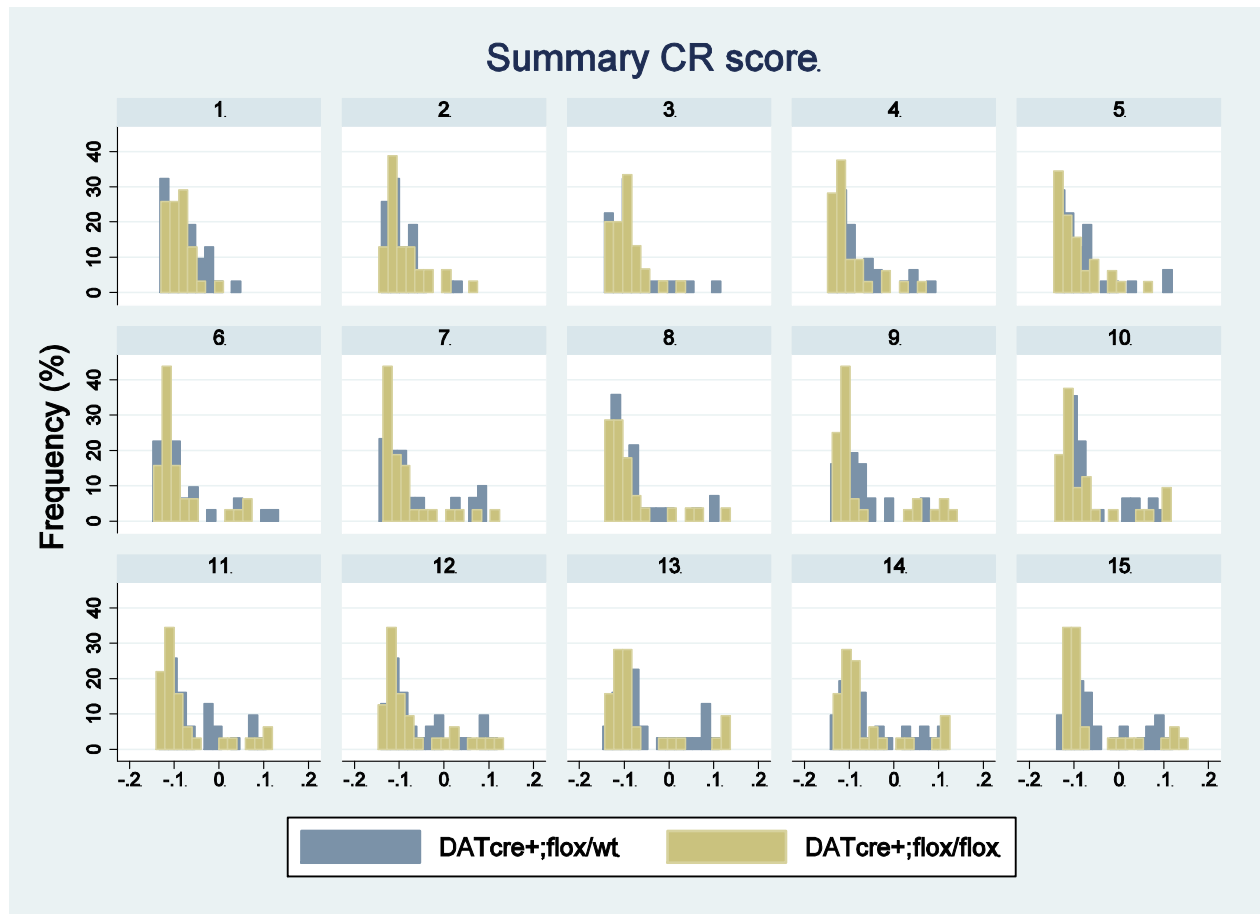
Supplemental Figure 1: Magazine head entry conditioning ratio in the sign-tracking / goal-tracking experiment. Animals showed robust acquisition of goal-tracking, similar to that observed in the Pavlovian magazine approach experiment. No genotype effects were detected.



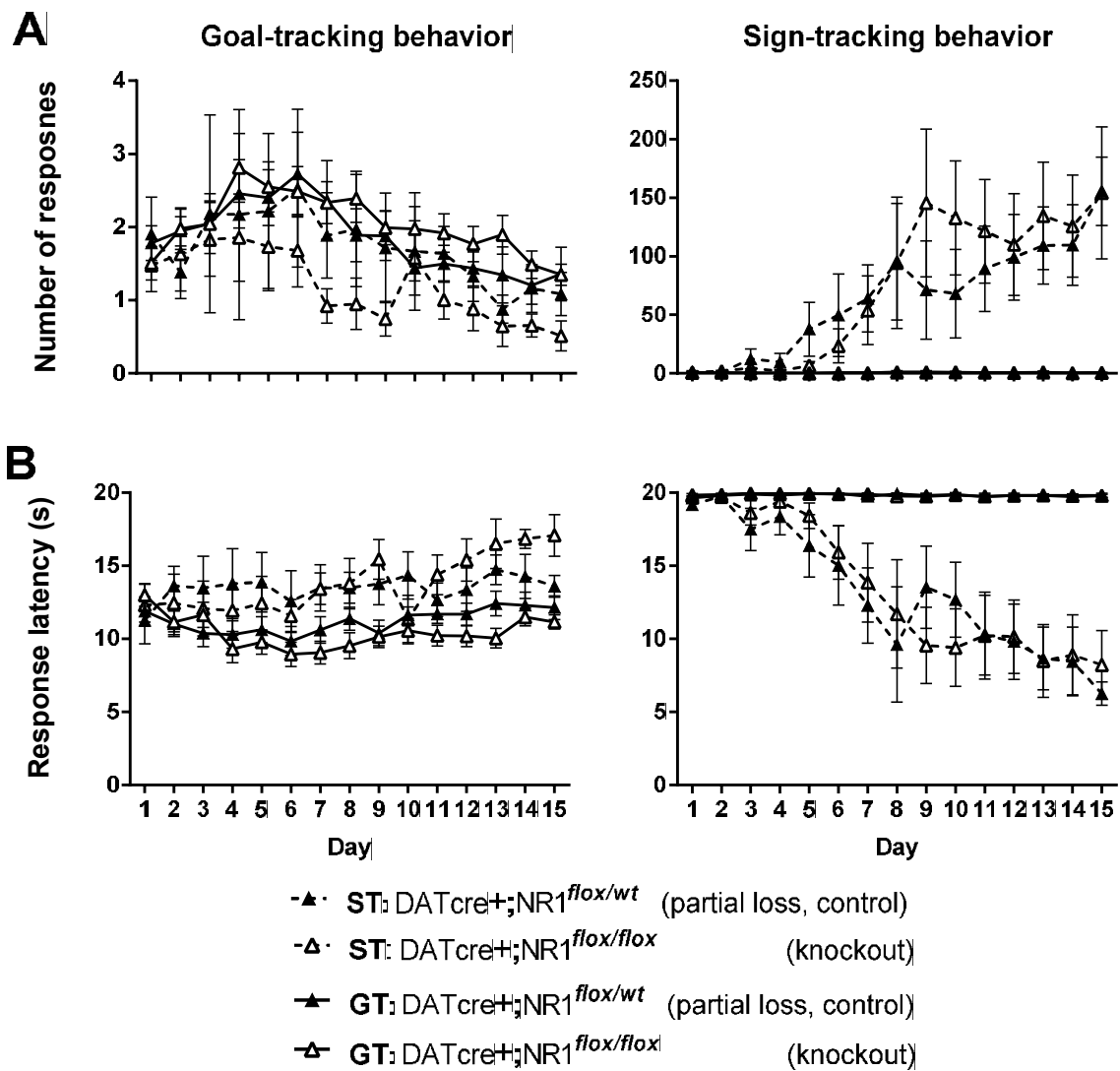
Supplemental Figure 2: Distributions of latency bias scores across 15 days of sign-tracking/goal-tracking training, expressing differences in average latency to actuate the lever-CS and average latency to enter the food magazine. Positive values indicate a sign-tracking bias and negative values indicate a goal-tracking bias.



Supplemental Figure 3: Distribution of response bias scores across 15 days of sign-tracking/goal-tracking training, expressing quantitative differences in number of interactions with the lever-CS versus number of head entries into the food magazine. Positive values indicate a sign-tracking bias and negative values indicate a goal-tracking bias.



Supplemental Figure 4: Distribution of summary scores across 15 days of sign-tracking / goal-tracking training; scores calculated as average of probability (Figure 6), latency, and response biases (Supplemental Figures 2 – 3), with positive values indicating a tendency to engage in sign-tracking and negative values a tendency to goal-track.



Supplemental Figure 5: Goal-tracking and sign-tracking behaviors plotted separately by conditional response designation. (A) Response quantities: head entries (left) and lever-presses (right). (B) Response latencies: head entry latency (left) and lever press latency (right).

CHAPTER 3

Multiple novel genomic and transcriptomics determinants of variation in appetitive reward-driven instrumental behavior, revealed by the genome-wide association and systems genetics approaches

Abstract

Learning to exploit the environment in the pursuit of desired outcomes is a central to a wide array of behaviors that humans and other animals exhibit in order to achieve internal goals. In its extreme forms, variation in rates of learning about rewards, and motivation to seek them, may underlie risk for psychiatric disorders (e.g., substance dependence). However, little is known about the genetic markers that modulate these individual differences in reward-related behaviors. Therefore, we sought to delineate novel genomic influences on variation in learning to perform an instrumental response to produce an appetitive outcome, and in motivation to perform this reward-seeking behavior. We performed a genome-wide association study (GWAS) of instrumental learning using the Hybrid Mouse Diversity Panel (HMDP) in conjunction with linear mixed model analysis, which together offer improvements in domains of power, resolution, and type I error rate relative to traditional genetics approaches. Spontaneous acquisition and performance of instrumental behavior was assessed in 70 inbred strains during three 8-hour daily sessions in which lever responses were reinforced by sucrose; schedule of reinforcement advanced from 1 press to an average of 5 presses to elicit reward deliver. Eight quantitative genome-wide significant trait loci (QTL) for instrumental behavior were identified, situated on chromosomes 3, 4, 10, 11, 12, 16, and 19. Weighted correlational techniques were applied to gene expression measures from the striatum of HMDP mice, a brain region critical for instrumental behavior. We identified networks of highly co-expressed genes statistically associated with strain-level variation in instrumental reward-seeking. Ontology analyses of these gene networks and their highly intra-connected ‘hub’ genes uncovered novel regulatory pathways related to reward-seeking behaviors. These results were combined with expression-

QTL data to prioritize genes within the identified instrumental behavior QTLs. We also found that measures of learning rate and measures of motivation – processes often thought of as dissociable at a behavioral level – may in fact depend upon common underlying genetics and genomics. These data together yielded novel neurobiological insight into molecular pathways related to instrumental learning, with potential utility in the generation of novel research hypotheses, and provided a fundamentally data-driven prioritization of QTL genes for future causal studies of the genomic basis of variation in reward-seeking behavior.

Introduction

Learning to predict the occurrence of rewarding and aversive stimuli, and to exploit the environment in the pursuit of desired outcomes is central to a wide array of behaviors that humans and other animals exhibit to achieve internal goals. Consequently, individual differences in rates of learning of these processes, or in the motivation to emit them in the pursuit of goals may have far-reaching importance for human behavior. Individual differences in reward-seeking may contribute to variation in temperament: everyday behaviors involve the pursuit of rewards, response-outcome learning, and motivation. However, in its extreme forms, variation in reward-seeking behavior play a central role in range of psychiatric conditions, including addictive disorders, attention deficit hyperactivity disorder, depression, obesity, and autism (Robinson and Berridge, 1993; Neuringer, 2002; Everitt and Robbins, 2005; Volkow et al., 2012). Indeed, altered sensitivity to rewards and motivation to obtain them – whether blunted (e.g., as in schizophrenia and depression)–or sensitized (e.g., as in substance dependence) -- represent clinically significant components of psychiatric disorders. Therefore, understanding the biological basis of fluctuations in these behaviors is intrinsically linked to the understanding neurobiology of these psychiatric disorders, as well as the confluence of risk factors that lead to their presentation.

Animal model organisms have proven enormously useful in identifying the neural basis of reward learning, with a targeted genetic knockouts and models of single-gene human disorders yielding significant insights (Dudai et al., 1976; Tang et al., 1999; Bourtchouladze et al., 2003; Kushner et al., 2005; Brigman et al., 2008; Morice et al., 2008; Pavlowsky et al., 2010; Roubertoux and Carlier, 2010; Shilyansky et al., 2010; Krueger and Bear, 2011), and many genes required for learning have been identified in this manner. However, few studies have utilized the power of mouse-based genome-wide linkage and association analysis to identify naturally occurring genetic variants that control individual differences in associative learning.

The study of phenotype differences in inbred mouse strains has yielded many QTL related to psychiatric disorders (Oliverio et al., 1973; Eleftheriou et al., 1974; Fuller, 1974; Crabbe et al., 1980; Reith et al., 1981; Schoemaker et al., 1982; Crabbe et al., 1983; Goldman and Crabbe, 1986). However, few causal genes have been identified. Many efforts to use inbred mice to perform genome scans have been hindered by either low genomic resolution, owed to low rates of recombination, or low power to detect small effect sizes (Bennett et al., 2010; Su et al., 2010); these are significant problems for identifying loci related to complex traits such as reward seeking, which likely have complex patterns of genetic inheritance, and regulation by many genes of low effect size (Crusio, 2004b; Valdar et al., 2006). Moreover, mouse genome-scans are susceptible to type I errors in the same manner that human genome scans are, with unequal genetic relatedness of subjects leading to spurious associations (Helgason et al., 2005; Payseur and Place, 2007; Kang et al., 2008; Bennett et al., 2010; Su et al., 2010).

Recent advancements in design of mouse panels and statistical techniques have made great strides in addressing these problems (Churchill et al., 2004; Kang et al., 2008; Churchill et al., 2012; Svenson et al., 2012; Chesler, 2014). The Hybrid Mouse Diversity Panel (HMDP) combines classical inbred strains with recombinant inbred mice to form a set of mice that affords significantly greater genomic resolution and statistical power to detect small effect genes than

traditional panels (Bennett et al., 2010). Here, we combined this mouse panel with recently developed mixed model analyses that afford control over confounding by population stratification to perform a genome-wide association study of instrumental reward-seeking behavior. We also integrated gene expression data collected from the striatum, a brain region implicated in the control of multiple components of reward-driven instrumental behavior (Graybiel, 1995; Kelley et al., 1997; Packard and Knowlton, 2002; O'Doherty et al., 2004; Yin et al., 2004, 2006a; Balleine et al., 2007; Groman et al., 2011) by using systems genetics tools that synergize with GWAS results (Kang et al., 2008; Lippert et al., 2011), in an effort to elucidate novel genomic and transcriptomic determinants of naturally-occurring variation in reward-seeking behavior.

Methods

Subjects

300 male mice drawn from the HMDP strain set were studied. We collected data from 81 strains, of which 39 were classical inbred strains, and 42 were recombinant inbred strains, $n = 1 - 11$ per strain (see Table 1). Mice were acquired either from Jackson Laboratories, from the breeding colony of Dr. Aldons J. Lusis at UCLA, or by transfer from University of Tennessee Heath Science Center (laboratory of Dr. Robert Williams). Three BXD strains were also bred on site. Mice were housed in sets of 1- 3 cage-mates which were always of the same strain. Mice were habituated to the 12h:12h on/off light cycle vivarium for at least 2 weeks prior to initiation of behavioral testing, at which time they were between 55 and 160 days old. Mice were tested between 7:00 AM and 7:00 PM (light cycle). Water was available *ad libitum* except during 4 hour sucrose familiarization sessions (see below). All experimental protocols were consistent with the US Public Health Service *Guide for the Care and Use of Laboratory Animals* and were approved by the Chancellor's Animal Research Committee at UCLA.

Two bottle free choice sucrose consumption procedure

Prior to food restriction, a subset of the subject population was phenotyped for strain-level differences in consumption of sucrose solution to a) ascertain to what degree any inter-strain differences related to or could account for inter-strain variance in instrumental learning, and b) to establish the optimal sucrose solution concentration for instrumental learning (i.e., the concentration for which inter-strain differences was at a minima). For this procedure and all further procedures, sucrose solution was made fresh daily. Subjects were habituated to 1 hr sessions of individual housing in a laboratory space room, during which time they had free access to two bottles: one contained water and the other contained sucrose (initially, 20% w/v). The positions of the sucrose-containing and water-containing bottles relative to each other in each cage (left versus right side) were alternated each day, and standard rodent chow was always available. On three subsequent days, intake of 0.2%, 2%, or 20% (w/v) sucrose (and water) was measured by weighing each bottle before and after the consumption session. The order that concentrations were presented was counterbalanced across mice and strains.

Food restriction / sucrose familiarization

Mice were then provided limited access to chow in their home cage in a manner sufficient motivation to acquire and engage in an instrumental behavior in the morning, while maintaining close to that level of body weight throughout the 8 hr learning sessions. Pilot studies indicated that adjusting food to achieve weights that were 90-95% of free-feeding body weights in the morning, and 80-85% in the evening offered optimal results. Over 6 days, chow was titrated to reach this range. Chow was provided in the evening, at least 60 mins after instrumental learning sessions. The second day after food restriction was imposed, mice were provided access to 20% sucrose (concentration chosen on the basis of results from the consumption procedure; mice from whom sucrose consumption dose-response data were acquired did not

receive these exposure sessions) in their home cage for 4 hrs; three exposure sessions on consecutive days were given, the first of which occurred in the vivarium and second and third of which occurred in a laboratory room (to habituate mice to transport from the vivarium).

Instrumental learning

On the 7th day after food restriction began (2 days after the last home-cage sucrose exposure session), mice were introduced extra-wide aluminum and polycarbonate Med Associates (St. Albans, VT) modular mouse testing chambers, each stationed inside a sound attenuating chamber and equipped with a white noise generator, house light (both on always), and a tone generator. A horizontal array of five illuminable nose-poke apertures formed one side of the box, and the opposite wall was fitted with an illuminable pellet delivery magazine with an entry-detection photocell. The magazines also contained reservoirs for sucrose solution. A 5mL syringe (BD and Co, Franklin Lakes, NJ) containing sucrose was connected via PE-50 tubing (Stoelting Co., Wood Dale, IL) to a 26 gauge metal inlet fashioned underneath the magazine; sucrose was delivered to a connected reservoir by activation of a PHM-100 Med-Associates syringe pump situated on top of the sound attenuating chamber. Adjacent to the magazine, on both sides, were retractable ultra-sensitive mouse levers (2 gram force requirement for actuation). Water was made available in the chamber via a lixit-sipper which was extended to approximately 2" above the grid floor, angled facing away from the magazine.

Mice first underwent a single 1-h session of magazine training, during which 10 μ L of 20% sucrose solution was delivered to the magazine by a 0.85 second pump activation. A total of sixty rewards, coincident with a 3-s activation of the magazine light, were delivered on a variable-time 60-second schedule. This session, like subsequent instrumental learning sessions, began in the AM, and mice were provided their daily allotted chow in the PM.

Next, mice began a series of three days of instrumental learning sessions. Each started with a non-contingent delivery of 10 uL of sucrose and 3-s magazine illumination, followed by the extension of a single lever into the box (location relative to the magazine was counterbalanced within and across strains). Consequently, the first 50 rewards were delivered on a fixed ratio 1 schedule of reinforcement; the next 50 were delivered under a variable ratio 2 (VR2; range of 1 to 3 presses required) schedule; the schedule was then switched to variable ratio 5 (VR5; range 1 – 9 presses) for the remainder of the session, which terminated after 8 hours or after delivery of 500 rewards. Rewards were delivered coincident with a 3-sec magazine illumination. To facilitate response acquisition, the proprioceptive feedback of a successful lever actuation was supplemented by a 0.05 sec tone pulse. All presses, except those made during the 0.85 second pump activation during reward delivery, counted towards completion of the next response schedule. Mice were allowed to freely organize their instrumental response patterns: there was never a time-out period and multiple rewards could be earned prior to their retrieval.

Phenotypes

On each of the three days of instrumental learning, we collected data on all lever press and magazine entry events; each was time-stamped. Summary measures of spontaneous acquisition of instrumental behavior included total number of lever presses (across all 3 days) and presses per reinforcement schedule per day. The latter set was transformed using time data into measures of response rate within each of the three reinforcement schedules within each day. We also calculated a binary phenotype termed ‘responder’ which segregated subjects according to whether they made at least 50 lever presses across the 3 days; this divided animals into those who participated in lever press behavior during the total 24 hours of time spent in the chamber from those that that did. The cutoff was chosen on the basis of task parameters (number of reinforcers earned prior to schedule advancement), which were

determined on the basis of pilot studies indicating that on average, relative disorganization gave way to cohesive instrumental responding after earning 40 to 60 rewards.

We also collected non-genetic or nuisance covariates such as age at the start of the study, body weight prior to food restriction (free feeding body weight), and, on a daily basis, percent body weight lost when instrumental learning sessions started (i.e., when mice were placed inside operant chambers), and when they finished, relative to free feeding weight. Averages of percent weight loss before and after sessions the three sessions were also calculated.

Our dataset exhibited heavy rightward skew, likely owed to the absence of any shaping aside from magazine training (i.e., all instrumental behavior was spontaneously acquired).

Consequently, in addition to acting as an omnibus test of instrumental behavior, the 'responder' phenotype also served as a cutoff: only mice that made 50 or more lever presses were included in analyses of quantitative instrumental learning traits. To further improve normality, all variables were Box-Cox transformed prior to genome wide association mapping and gene co-expression network analysis.

Response rates during periods of VR5 ratio of reinforcement were used as a measure of asymptotic behavior. Differences in asymptotic rates of responding were considered reflective of vigor or drive to obtain the sucrose reward, and therefore served as our primary measure of motivational aspects of reward seeking.

Finally, we characterized learning curves using the methods of Gallistel et al. (2004). In brief, this algorithm seeks to find the points in time where changes in behavior occur, by progressively iterating through successive behavioral responses, testing the distribution of response rates prior to a putative 'change point' and afterwards. A change point is detected when statistically significant differences in these response distributions are detected (threshold $p < 10^{-6}$). The algorithm then treats this point as its new starting point, and continues to iterate through the

data to find further change points relative to the newly identified change point. From this algorithm we extracted the time and number of rewards earned prior to the first change point being detected (i.e., when behavior first changed to a statistically significant degree, screening out single or small bursts of lever pressing that did not result in sustained increase in response rate). These values were considered reflective the latency and reinforcement required to induce the onset of learning.

Genome-wide association mapping

HMDP strains genomic data, acquired using Affymetrix Mouse Diversity arrays (Yang et al., 2009), were provided by the laboratory of Aldons Lusi. Nucleotide positional coordinates are GRCm37/mm9 genome assembly format.

Genome-wide association analysis was performed using Factored Spectrally Transformed Linear Mixed Models (Lippert et al., 2011), a computationally efficient algorithm which offers effective control for confounding introduced by non-uniform population substructure. FaST-LMM uses a linear mixed model of the form:

$$Y = X\beta + \sigma_g^2 K + \sigma_e^2 I,$$

where X is the matrix of fixed effects (SNPs and covariates) with weights β , σ_g^2 is the genetic variance component weighted by the genetic similarity matrix K , and σ_e^2 is the residual variance component multiplied by identity matrix I . Genetic similarity is estimated using the realized relationship method, based upon identity-by-state genome data. Restricted maximum likelihood is used to optimize estimation of δ , the ratio between the genetic variance component and the residual variance component. The algorithm achieves high computational efficiency by performing spectral decomposition of the genetic similarity matrix without explicitly computing the genetic similarity matrix, which is achievable using the realized relationship approach; this

spectral decomposition permits transformation of the data in a manner that corrects for unequal genetic relatedness. Consequently, it can achieve rapid run times, and this is the case even when variance components are estimated anew for each SNP. SNP effects are tested using F statistics, and heritability is estimated by calculating $\frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$.

Prior to submission to FaST-LMM, we performed bivariate correlations between behavioral data and the collected covariates. If a significant relationship was detected, the covariate was included in the mixed model.

Wrapper scripts were written in Matlab R2011b (Mathworks, Inc., Natick, MA) to perform the following functions: genomes were extracted from a master genome file and reassembled in Plink binary format (Purcell et al., 2007) into a set representative of the subjects in our sample. SNPs were discarded on the basis of minor allele frequency less than 5% and if missingness rate across subjects exceeded 10%; approximately 200,000 SNPs remained after filtration.

Inclusion of SNPs in the genetic similarity matrix that are in linkage disequilibrium (LD) with the SNP being tested is akin to including that SNP itself as a covariate in during the test of its association with a phenotype (Lippert et al., 2011; Listgarten et al., 2012). We used the leave-one-out strategy, which exploits the relatively low LD rate of SNPs on different chromosomes to limit this effect: SNPs were binned by chromosome, and successive calls were made to FaST-LMM with SNP association performed on one chromosome at a time, using SNP data from all other chromosomes for estimation of genetic similarity.

Genome scan results were visualized using Haploview (Barrett et al., 2005), and SNPs were considered significant if they met a false discovery rate (FDR) cutoff of ≤ 0.05 (Storey, 2002). QTL widths were defined by the LD block size that surrounding the most statistically significant SNP (in the case of clusters of SNPs with $\text{FDR} \leq 0.05$); LD blocks were previously defined (Ghazalpour et al., 2012) on the basis of $\text{SNP } r^2 \geq 0.8$; for SNPs that resided outside these

blocks, the window was fixed at ± 500 Kb (HMDP mapping resolution is on average 1 mB; Bennet et al. 2010). RefSeq genes within LD blocks were collected and visualized using the UCSC Genome Browser (Kent et al., 2002), available at <http://genome.ucsc.edu>.

Expression data analysis

Gene expression data was acquired from striatal tissue of HMDP strains using the Illumina Mouse-Ref 8 v2.0 Expression BeadChip microarrays (25,697 probes/genes); these data were collected by the laboratory of Dr. Desmond Smith (Park et al., 2011) and are available under Gene Expression Omnibus (GEO) accession number GSE26500 (<http://ncbi.nlm.nih.gov/geo>). Expression QTLs (eQTLs) were determined previously by Park et al. (2011), with significant SNPs being defined as ‘*cis*-acting’ eQTLs (*cis*-eQTL) if they resided within 2 Mb of the probe, and ‘*trans*-acting’ eQTLs if they did not.

We performed a weighted gene co-expression network analysis (WGCNA) of striatal samples using a suite of functions implemented in R (Zhang and Horvath, 2005; Langfelder and Horvath, 2008; Langfelder et al., 2008). This technique discerns patterns of correlated gene expression, assigning them into different modules on the basis of collective coexpression. By leveraging the finding that co-expressed genes tend to perform similar biological functions (Wolfe et al., 2005), inferences regarding poorly characterized genes can be made on the basis of known functions of co-expressed genes. The set of parameters for network construction and subsequent analyses were modeled after tutorials available at <http://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA>.

Of the strains phenotyped, 50 overlapped with the set of strains for which expression data were collected. Probe sets were first filtered for rates of high rates missingness, and hierarchical clustering of expression data by strain was used to filter outliers. An effective soft-thresholding parameter for calculation of co-expression via estimation of adjacency was determined

empirically on the basis of lowest power transformation required to achieve a high level of scale-free topological fit (in our case, 12). A single-block gene co-expression network was calculated on the basis of hierarchical clustering of unsigned gene topological overlap mapping (TOM), a quantitative metric of gene co-expression, based upon the number of 'nodes' to which a given pair of genes are both connected. The output is represented as a dendrogram (see results), with downward-deflected branches indicating increased TOM; branched regions depict genes segregated into modules (of genes with high TOM). Modules were denoted by different colors. Each was required to contain at least 30 genes, and modules that were correlated $r \geq 0.25$ were merged.

The first principle component of the resultant gene modules – the module 'eigengene,' i.e., the single vector (of elements equal to the number of strains) most representative of the module itself – was correlated with phenotypic data. Modules for which a significant correlation was found were further studied by calculation of Gene-trait Significance (GS), reflecting the degree to which the expression level of a gene correlates with an instrumental behavior trait (the absolute value of the r statistic value of this correlation). Secondly, Module Membership (MM) was calculated, which describes the magnitude of the association between a gene's expression level and the eigengene of the module it is in (also the absolute value of the r statistic value of this correlation). GS was correlated with MM, testing the whether the degree to which a gene correlated with the trait was also reflective of the degree to which it was correlated with the structure of the module (i.e., its eigengene). Modules were further advanced on the basis this correlation reaching statistical significance. We also calculated average GS across all genes within a module as a measure of strengths of the correlation between its genes and instrumental behavior traits. Selected modules were submitted to ontology analysis using DAVID tools, as were the sets of hub genes extracted from these modules (Huang da et al., 2009a, b).

Prioritization of candidate genes

Genes within identified QTLs windows were prioritized as candidates for future causal studies on the basis of systems genetics tools (Flint et al., 2011; Park et al., 2011; Ghazalpour et al., 2012). In particular, we examined what WGCNA co-expression module each genes resided in, whether the gene's expression correlated phenotypic variation, and amongst those that did, whether *cis*-eQTLs regulating its expression had been identified.

Results

HMDP strains exhibit a range of instrumental responding

We first tested whether sucrose intake during free access condition predicted overall instrumental behavior; no significant correlations were found (data not shown).

Figure 1 shows the total number lever presses made during instrumental learning sessions (summed across the three sessions), and within-strain variance (SEM). The inbred strains tested exhibited considerable variability in behavior: several strains consistently failed to engage in any instrumental responding, while other classical inbreds spontaneously acquired the response and engaged in high levels of sucrose seeking. On average, it appeared that variants of the 129 classical inbred lines, as well as BXA and AXB recombinant inbred strains were the poorest performers; other classical inbred strains responded at low, moderate, and high levels, and BXD strains tended to perform moderately. There were, however, notable exceptions: in striking contrast to other BXA strains, BXA13/PgnJ mice were the highest responders in the entire cohort, and BXD39/TyJ mice pulled away from other BXD lines by failing to respond entirely.

Genome-wide association scan reveals multiple QTL for instrumental sucrose-seeking

Prior to submission to QTL mapping, the relationship between individual covariates and total lever presses was examined. These data are presented as Supplemental Figures content. No

significant correlations with age ($r = 0.085$, $p = 0.23$), free-feeding body weight ($r = 0.035$, $p = 0.62$), or average percentage weight loss from free-feeding body weight measured directly before sessions started ($r = -0.096$, $p = 0.18$) were found; only average percent weight loss measured at the completion of sessions (likely the consequence of variation in instrument performance; see Supplemental Figure 5) was negatively associated with lever presses ($r = -0.3$, $p = 1.8 \times 10^{-5}$). Consequently, the genome association scan was performed without inclusion any covariate data.

We first performed an association scan on performed an association scan on the binary 'responder' trait. Of 300 mice tested, approximately 100 failed to performed a nominal level of instrumental behavior (≥ 50 presses). Thus, we used this phenotype to seek genetic determinants of the basic tendency to exploit the environment by engaging in an appetitive instrumental behavior. As shown in Figure 2, several SNPs on chromosome 15 were suggestive, but not significant ($FDR = 0.060$). Narrow sense heritability for engaging in instrumental responding for sucrose was estimated as 29.76%.

We then analyzed quantitative variation in instrumental responding for sucrose, constraining the sample to mice designated as responders. These results are plotted in Figure 3. Six QTL with $FDR \leq 0.05$ were uncovered, mapping to chromosomes 3, 10, 11, 12,, and 19; SNPs on chromosome 4 and 16 were near-significant as well ($FDR = 0.051$, $FDR = 0.063$, respectively). The Narrow-sense heritability estimate for variation in instrumental behavior was 28.16%.

Genes from LD blocks defined by the most significant SNP per QTL, or within a 1 Mb window for SNPs which were found between LD blocks are shown in Table 2 (note: this table includes SNPs from suggestive QTL on chromosome 4). The LD window surrounding the chromosome 16 suggestive QTL (a single SNP reached statistical significance) did not map to any known genes.

The genomic regions that the identified QTLs for instrumental behavior span are depicted in detail in Supplemental Figure 1. The genomic area surrounding suggestive signal from the responder GWAS is also shown in Supplemental Figure 2.

Weighted gene co-expression analysis reveals gene modules associated with instrumental behavior

Prior to formation of the gene co-expression modules, clustering algorithms were used to screen for outliers gene expression in the gene expression data (3 strains were removed; Figure 4). Data were further screened for systematic patterns of highly divergent or skewed gene expression across the quantitative range of phenotype measured that would make the phenotype unsuitable for parametric analyses with expression data (Figure 5); no patterns to this effect were detected. Subsequently, a striatal gene co-expression network was constructed, yielding 15 modules, containing between 35 to 4,243 genes each. Module structure is depicted in Figure 6; branches indicate regions of increased gene expression similarity, and colors correspond to names used to identify modules (genes not assigned to modules assigned grey).

Each module's eigengene – a vector most descriptive of the co-expression structure of the module (its first principle component) – was related to our instrumental learning traits, as shown in Figure 7, in order to ascertain which modules' co-expression patterns were associated with variation in instrumental behavior. Clear patterns of association with instrumental behavior were found: total lever presses was significantly correlated with six of the 15 modules, including the turquoise module, which over 5 times as many genes as the next most populated module.

We submitted two modules – the turquoise module and the green module – to further analysis, on basis of their strong eigengene association with lever pressing behavior and the number of genes within the module. These data are shown in Figure 8. Both modules exhibited

prominent correlations between green significance and module membership (turquoise: $r = 0.56$, $p < 10^{-200}$; green: $r = 0.18$, $p = 0.011$). That is, the degree to which a gene's expression level correlated with the lever pressing trait was also related to the degree to which it was similar to the overall structure of the module itself (i.e., the module eigengene). These are the properties indicated a cohesive trait – gene expression structure within the green and turquoise module. Moreover, plotting the correlation between strain-level lever pressing and module eigengene for these modules indicated that though there was some degree of skew, it was not dramatic, and the correlations were not driven by outliers.

Figure 9 depicts the average correlation statistic between gene expression and lever pressing across all genes within a module. Turquoise and green modules are amongst the highest; though salmon and greenyellow modules show high mean significance, we discounted these modules on the basis that they did not exhibit significant gene significance – module membership correlations, indicating that the co-expression structure of these modules was poorly related lever pressing behavior.

Module ontology analysis

Having demonstrated a compelling relationship to phenotypic variation in instrumental responding for sucrose, the turquoise and green modules were then submitted to ontology analysis. Because modules were comprised of large numbers of genes, clustering algorithms were used, and the resultant annotation groups are presented in Tables 3 and 4. Terms prominent in the turquoise module include intracellular protein transport and localization, nucleotide and ATP binding, and vesicular (synaptic and cytoplasmic), mitochondrial, and Golgi-related processes. The green module was highly enriched in proteasome and mitochondrion-related processes, as well as cellular catabolic processes.

Genome and transcription data reveal escalate 3 genes as candidates for controlling variation in instrumental behavior

Table 2 all genes within QTL identified for quantitative variation in instrumental behavior. Gene co-expression modules that these genes reside in are also shown, and where statistically significant relationships between a gene's expression and instrumental behavior were found, statistics r and p for this gene-trait correlation. Finally, among the genes that have significant correlations, whether eQTLs that have been identified (Park et al., 2011) are *cis*-acting or *trans*-acting is denoted.

By virtue of residing within co-expression modules that relate to lever pressing behavior (3 of 4 in the turquoise module, 1 in the yellow module), exhibiting statistically significant correlations between expression levels and lever pressing, *Gns*, *Bzrap*, *Tmem44*, and *Ppp1R2* are three top candidates, prioritizing them as genes that potentially harbor polymorphisms in control of individual variation in reward-driven instrumental behavior.

Phenotype relationships

Interestingly, there was a unique pattern in the relationships between change point measures and module eigengenes, and motivational measures and eigengenes: where motivational measures – last session, asymptotic response rate under VR5 schedule of reinforcement – correlated positively with a module eigengene, the change point measure of number of reinforcement events prior to the onset of instrumental learning also correlated, with similar directionality and magnitude. Indeed, 5 out of the 5 modules with eigengene-trait correlations for final VR5 response rate that had positive r values above 0.1 also had positive r values above 0.1 for reinforcers prior to onset of learning, and the same correspondence was found for negative r values, with all 4 of the modules with values < 0.1 for VR5 response rate also having negative r values < 0.1 for reinforcers prior to learning. This was specific to this aspect of onset

of learning, however: latency until the onset of learning was unrelated to most modules, and no discernable pattern amongst it and other measures of instrumental behavior were seen.

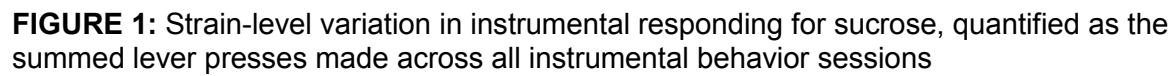
This result is particularly unexpected, as measures of learning and asymptotic behavior are frequently considered dissociable (Pubols, 1960; Pecina et al., 2003; Gallistel et al., 2004; Cagniard et al., 2006; Papachristos and Gallistel, 2006; Yin et al., 2006b). Consequently, we sought to determine whether this overlap in transcriptomics was found with respect to genetics as well. We performed bivariate non-parametric correlations, relating latency until onset of learning, reinforcement events until onset of learning, and response rates under VR5 schedule of reinforcement during the final instrumental behavior: while no relationship between latency until onset of learning and final VR5 response rate was found (Spearman's $\rho = -0.161$, $p = 0.177$), strain-level variation in number of reinforcement events required to elicit the onset of learning was significantly associated with strain-level variation in final VR5 response rates (Spearman's $\rho = -0.244$, $p = 0.039$). Moreover, the geography of GWAS results for final session VR5 response rate and number of reinforcers prior to onset of instrumental learning (but not latency until onset of learning) were highly similar: where low p value SNPs were observed for one phenotype, they were found for the other (data not shown).

Conclusion

Here, we provide evidence of genomic regions that determine whether an animal engages in instrumental behavior, and a set of genomic regions related to quantitative variation in appetitive instrumental behavior. Additionally, we show that this behavior has strong associations with large modules of co-expressed genes. Further work will be needed to extract from this dataset the underlying biological processes that link together these co-expressed gene modules, the most prominent genes within the modules, and the QTL we have identified here, in order to provide detailed characterization of the genetic landscape of appetitive instrumental behavior.

We also show that at both a genomic level (inter-strain differences) and transcriptomic level (gene co-expression module overlap), differences in asymptotic response rates relate specifically to number of reinforcement events needed to elicit learning, but not time until learning begins. The relationship between learning and asymptotic behavior is indeed unexpected, and will be discussed further in the conclusions chapter.

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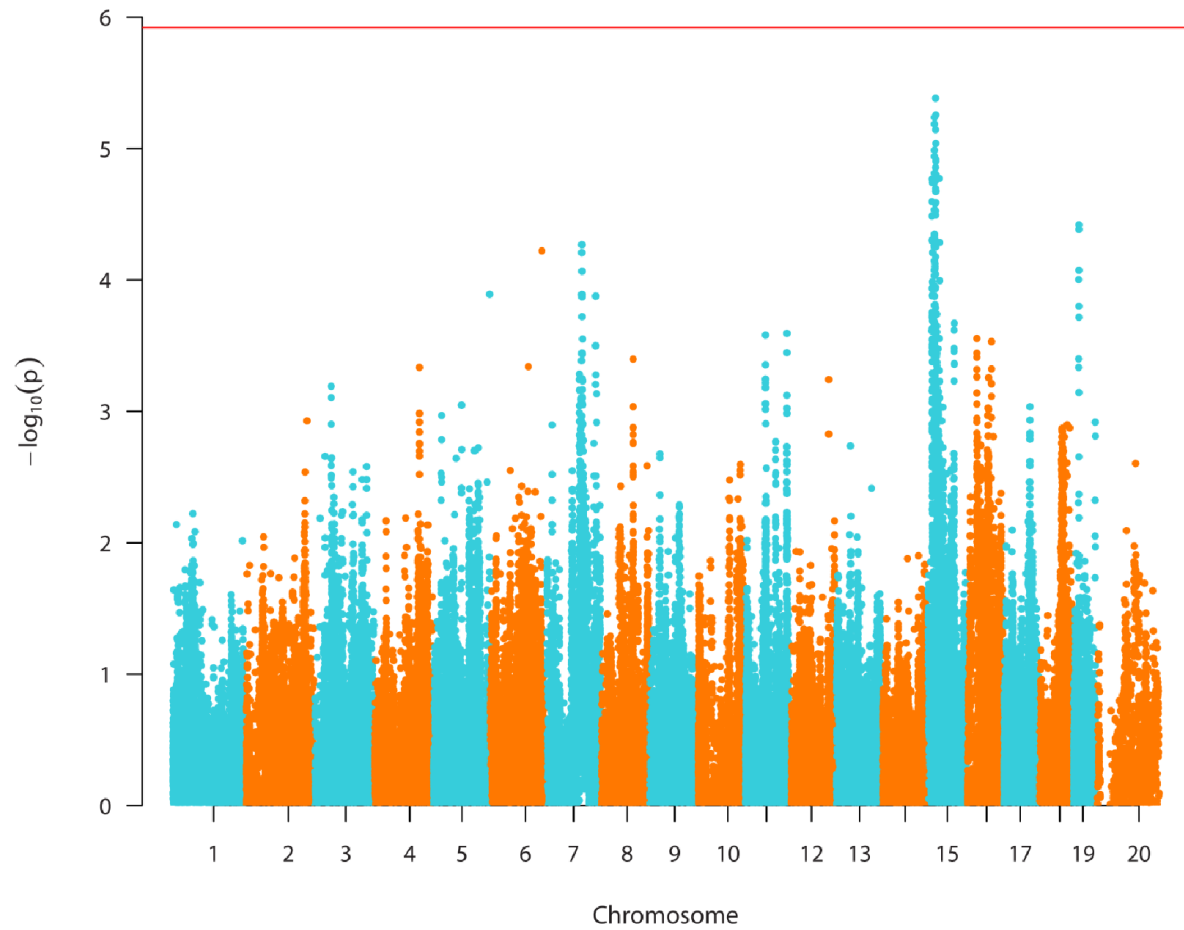


FIGURE 2: Genome-wide association scan for 'responder' status yields suggestive QTL on chromosome 15. A single gene, *Fam173b*, maps to this locus.

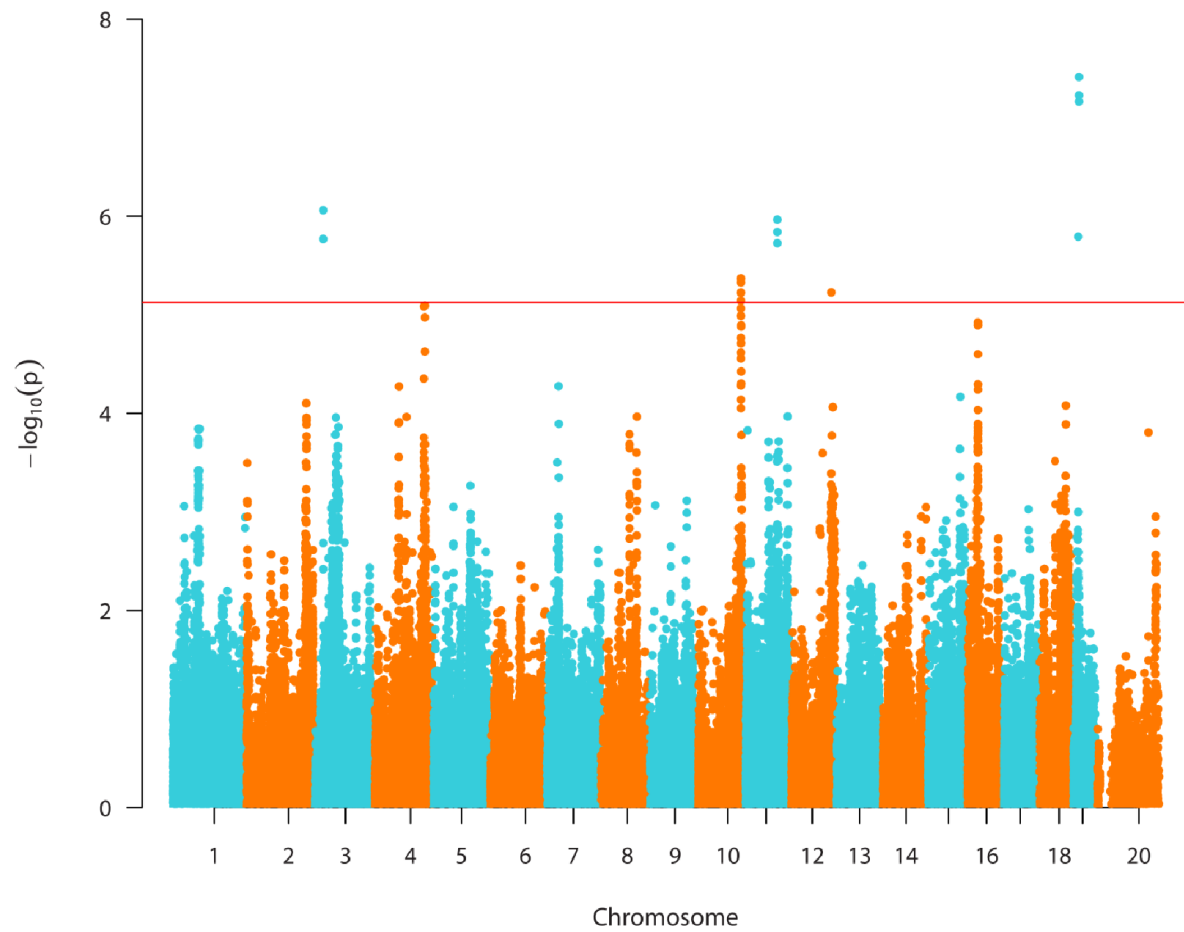


FIGURE 3: Genome-wide association scan for quantitative variation in instrumental behavior reveals 8 statistically significant QTL across chromosomes 3, 4, 10, 11, 12, 16, and 19.

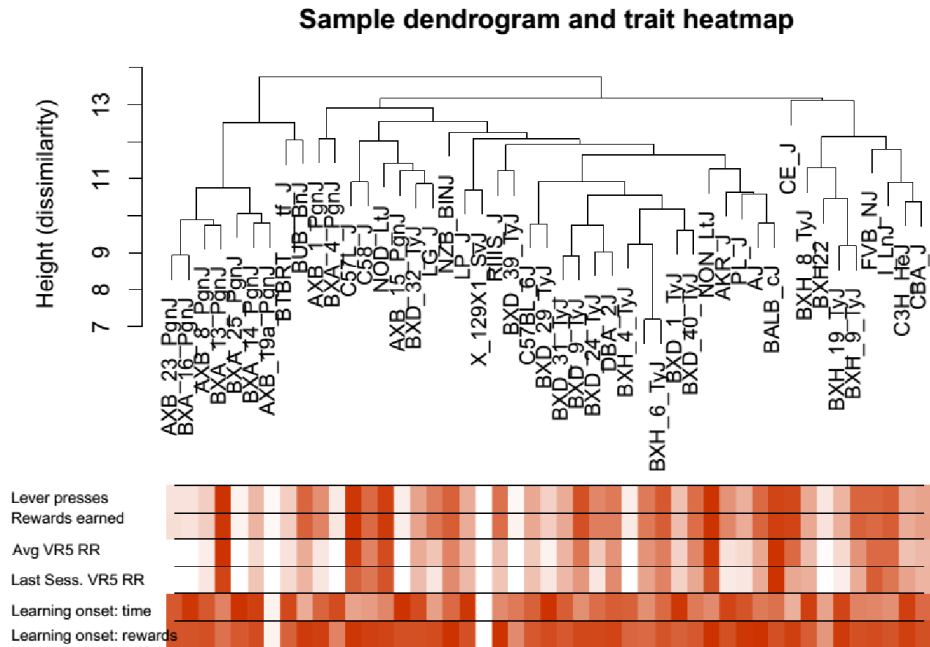


Figure 5: Dendrogram relating strains gene expression to heatmap of phenotype data. Colors scaled from white to dark red indicate low to high value phenotype values for the strain positioned in the dendrogram above the heatmap. Because branches of the dendrogram (created based upon clustering of gene expression data) do not systematically correspond to large phenotype differences (i.e., branches are not placed at regions of white to red transitions for phenotypes analyzed), expression data is likely not systematically biased by phenotype values. That is, expression data is likely relatively evenly distributed across phenotypic ranges.

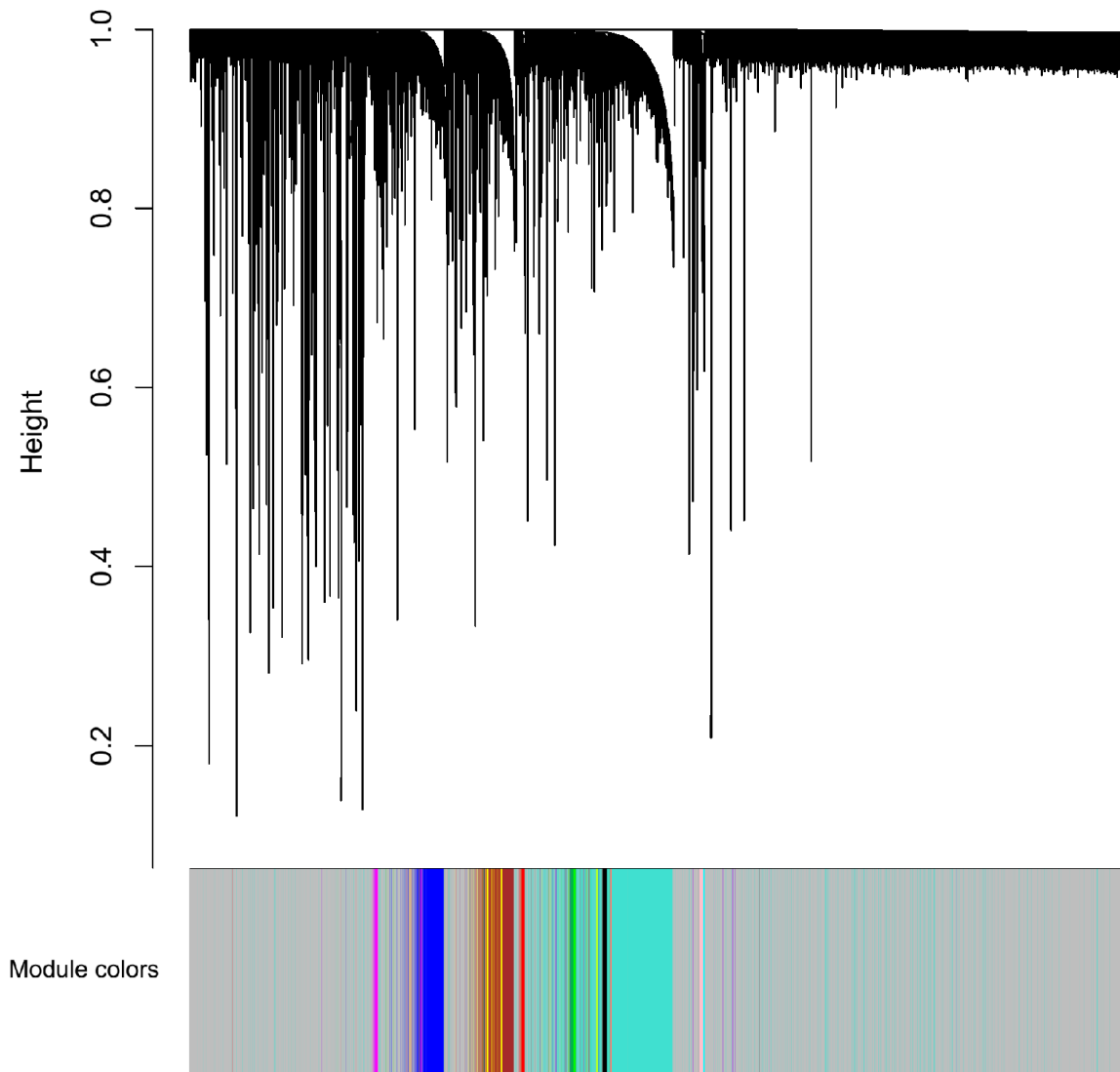


Figure 6: Weighted gene co-expression network analysis formation. Genes demonstrating significant co-expression – quantified by topological overlap mapping, a measure of the degree to which two genes are connected to the same nodes – are mapped into modules, designated by colors. Each downward deflected line represents a gene, and the degree of deflection corresponds to the departure from dissimilarity (i.e., greater co-expression).

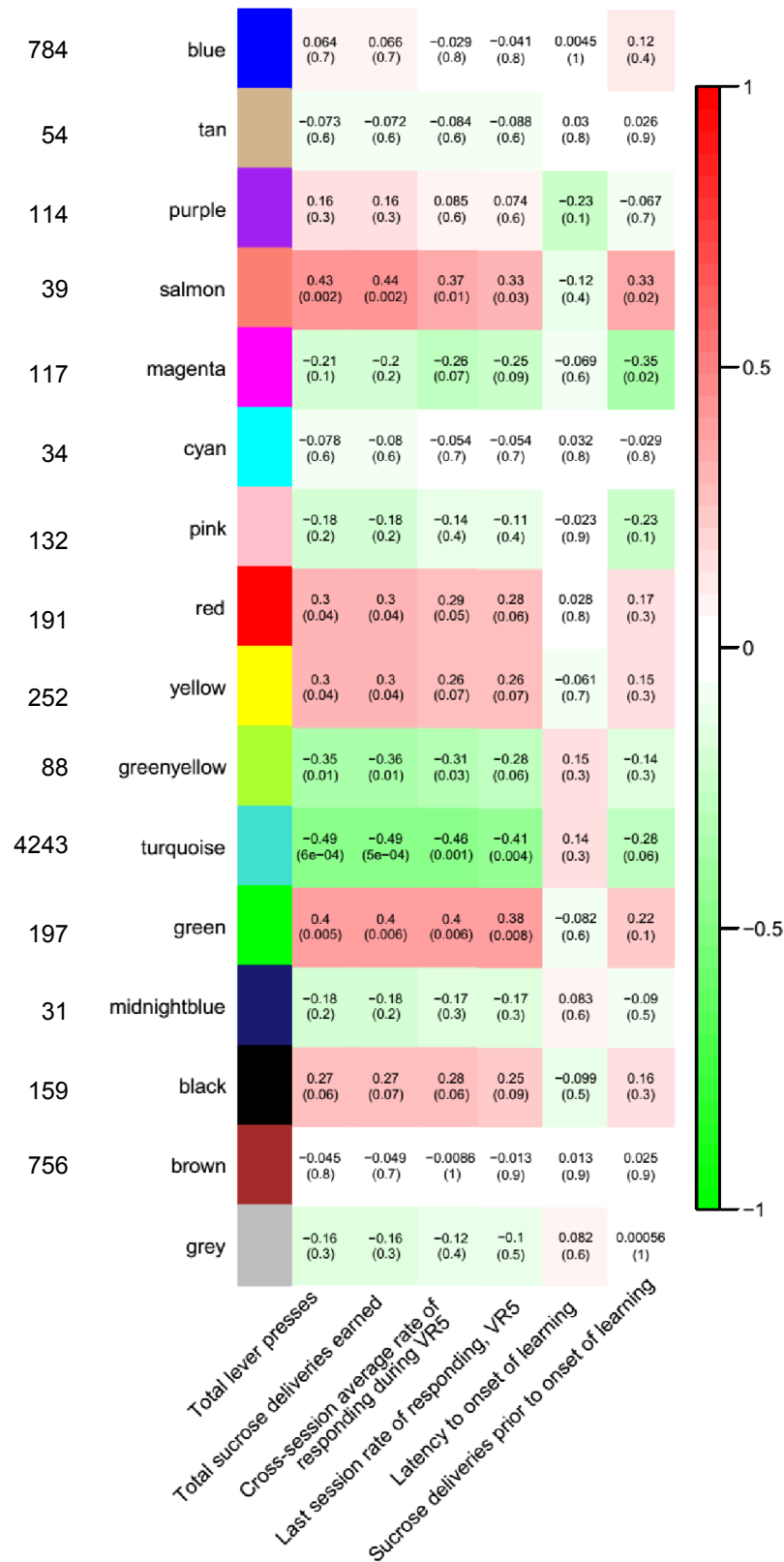


Figure 7: Relationship between module eigengenes (first principle component) and instrumental behavior traits. Numbers to the left are numbers of genes within modules; in the heatmap, data are presented as:

Correlation statistic r
(correlation p value)

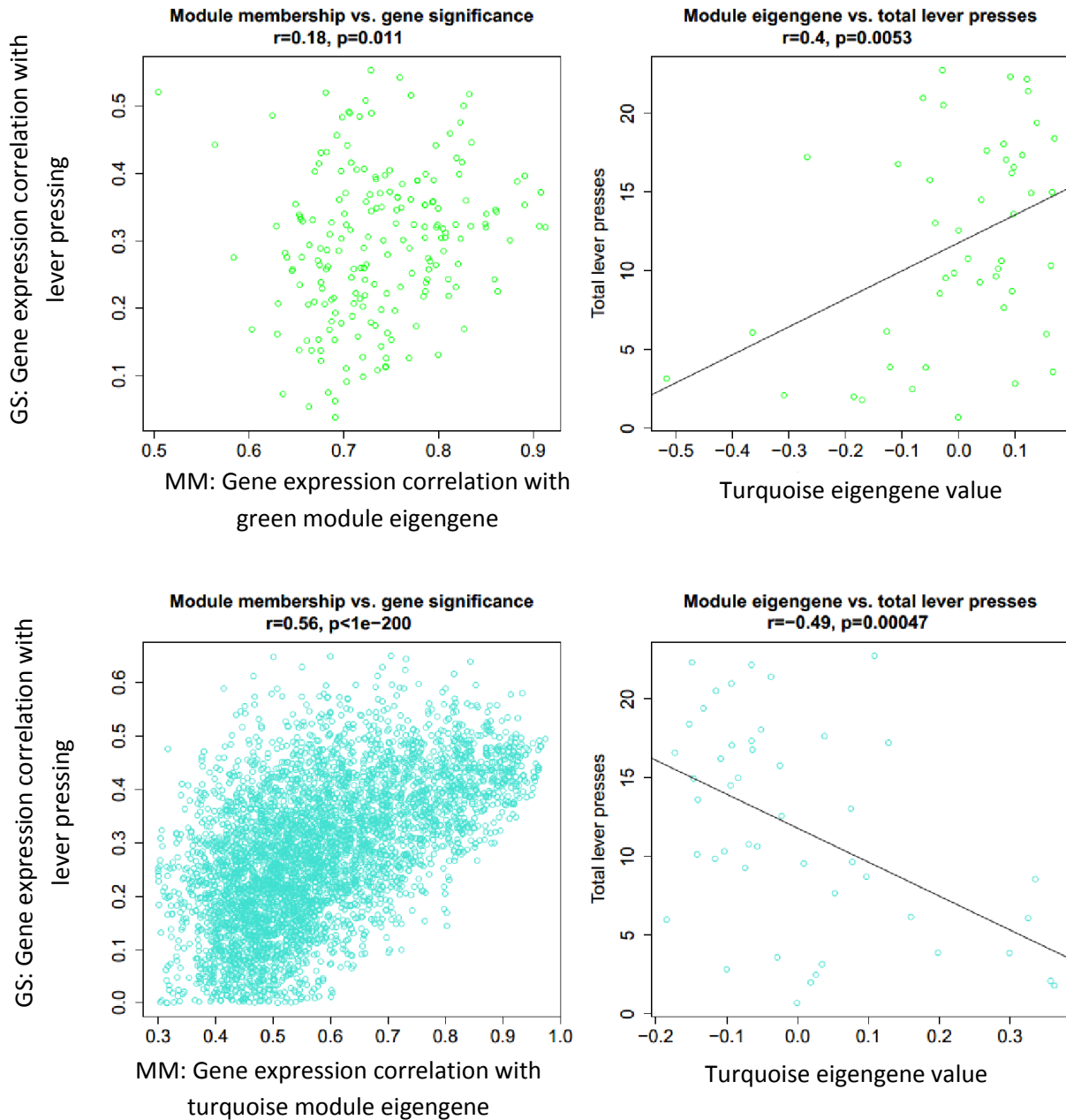


Figure 8: Diagnostic measures for selected modules (turquoise and green).

Left: Gene module membership (correlation of a gene's expression with module eigengene) related to gene significance (correlation of a gene's expression and lever pressing). Positive correlation indicates that the structure of the module relates cohesively to the expression of the lever pressing trait itself.

Right side: strains' module eigengene values plotted against strain means of lever pressing behavior. Well distributed properties of the correlation considered indicate non-spurious association (i.e., not driven by outliers), and lend support to the notion of relationship between the module (and the genes that comprise it) and phenotypic variation in lever pressing for sucrose.

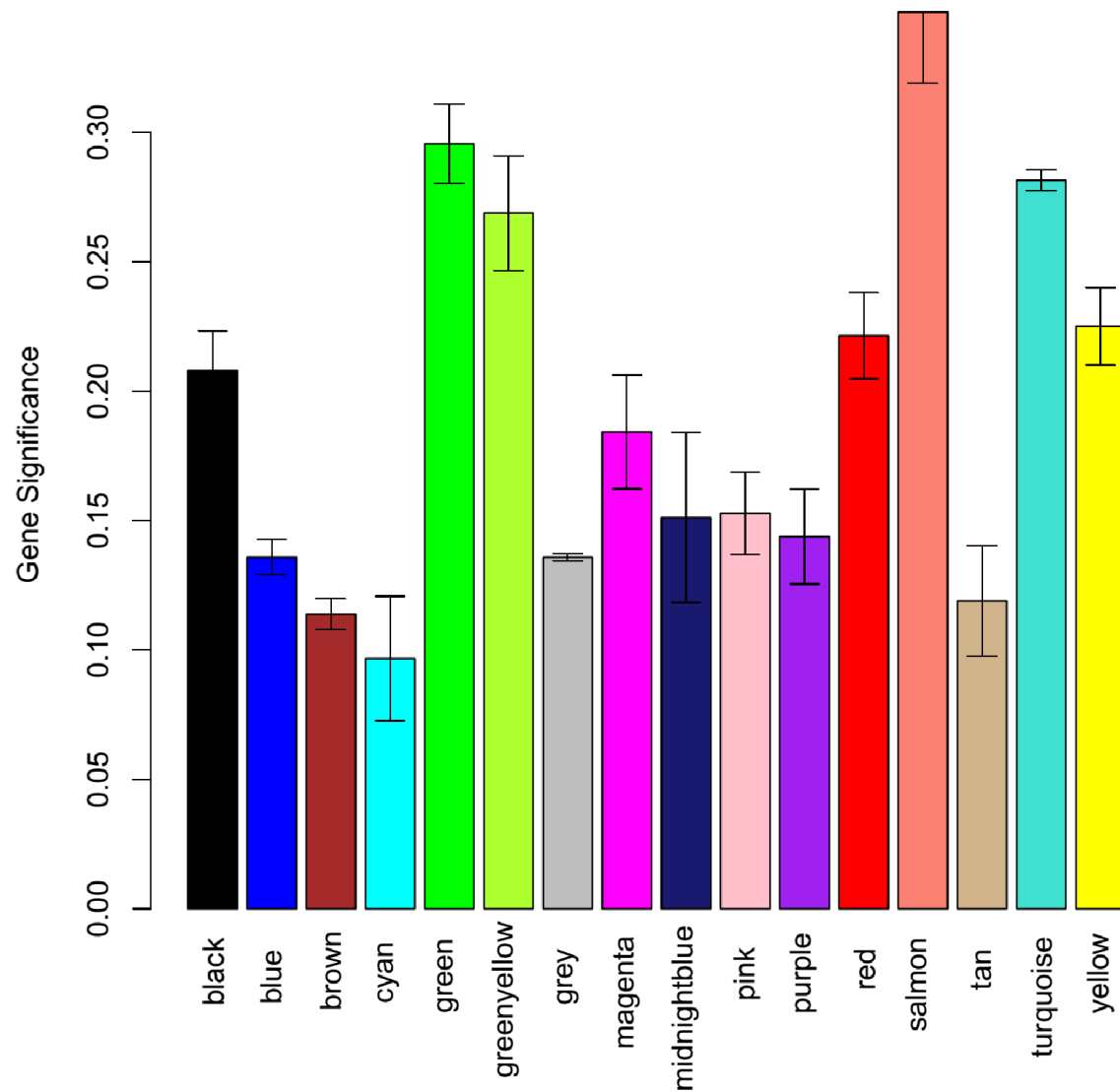


Figure 9: Average gene significance. Mean correlations (absolute value of r statistics) of a gene's expression with lever pressing – across all genes within a given module.

Sample demographics and strain covariate averages				
	N per strain	Age	Free feeding body weight	Pre-session weight loss
129S1/SvImJ	2	69.0	22.6	16.0%
129X1/SvJ	5	96.8	24.7	10.2%
A/J	5	95.4	23.8	11.0%
AKR/J	2	80.0	27.6	14.0%
AXB1/PgnJ	3	62.0	21.4	10.7%
AXB15/PgnJ	2	65.0	23.9	11.5%
AXB19a/PgnJ	2	74.0	23.9	13.0%
AXB23/PgnJ	2	55.0	21.2	11.0%
AXB8/PgnJ	3	79.0	21.9	.
BALB/cByJ	6	89.0	25.5	13.2%
BALB/cJ	7	90.7	27.6	12.7%
BPL/1J	5	64.8	18.8	9.4%
BTBR T<+> tf/J	4	94.5	31.5	10.0%
BUB/BnJ	2	78.0	31.4	11.5%
BXA13/PgnJ	2	70.0	24.5	10.5%
BXA14/PgnJ	5	56.4	25.3	12.0%
BXA16/PgnJ	4	70.5	21.1	10.3%
BXA25/PgnJ	4	64.5	24.1	11.3%
BXA4/PgnJ	1	74.0	25.4	13.0%
BXD1/TyJ	3	72.0	22.3	10.0%
BXD100/RwwJ	3	72.0	25.1	12.7%
BXD24/TyJ	4	115	31.4	14.3%
BXD29/TyJ	4	98.0	26.0	11.8%
BXD31/TyJ	6	100	25.9	10.3%
BXD32/TyJ	2	163	28.6	11.5%
BXD39/TyJ	5	118	35.4	13.2%
BXD40/TyJ	4	134	22.6	12.0%
BXD42/TyJ	11	75.5	22.0	10.7%
BXD48/RwwJ	5	96.8	28.1	12.2%
BXD51/RwwJ	3	92.0	21.9	12.7%
BXD60/RwwJ	7	107	31.1	12.1%
BXD62/RwwJ	3	104	24.7	12.7%
BXD65/RwwJ	6	113	31.6	13.5%
BXD66/RwwJ	3	83.0	23.1	10.7%
BXD68/RwwJ	5	92.6	23.5	12.8%
BXD75/RwwJ	6	65.0	22.6	10.8%
BXD77/RwwJ	3	133	26.1	9.7%
BXD84/RwwJ	2	76.0	21.5	11.5%
BXD9/TyJ	5	116	33.5	13.2%

Table 1: Sample demographics. Strain names, number of subjects per strain, average age by strain are shown, as well as degree of weight reduction induced by caloric restriction prior to the start of instrumental learning.

Chrom.	Symbol	Gene name	Module	r value	p val	eQTL
3	<i>Nlgn1</i>	neuroligin 1	blue			
4	<i>Cd164L2</i>	CD164 sialomucin-like 2				
4	<i>Gpr3</i>	G-protein coupled receptor 3	grey			
4	<i>Map3K6</i>	mitogen-activated protein kinase kinase kinase 6	grey			
4	<i>Sytl1</i>	synaptotagmin-like 1				
4	<i>Tmem22</i>	similar to D4Ert196e protein				
4	<i>Wasf2</i>	WAS protein family, member 2	turquoise			
10	<i>4921513I</i>	RIKEN cDNA 4921513I03 gene				
10	<i>D930020</i>	RIKEN cDNA D930020B18 gene				
10	Gns	glucosamine (N-acetyl)-6-sulfatase	turquoise	-0.58	1.29E-05	trans
10	<i>Lemd3</i>	LEM domain containing 3				
10	<i>MsrB3</i>	methionine sulfoxide reductase B3	grey			
10	<i>Rassf3</i>	Ras association (RalGDS/AF-6) family member 3	grey			
10	<i>Tbc1D30</i>	TBC1 domain family, member 30				
10	<i>Tbk1</i>	TANK-binding kinase 1	grey			
10	<i>Wif1</i>	Wnt inhibitory factor 1	grey			
10	<i>Xpot</i>	exportin, tRNA (nuclear export receptor for tRNAs)				
11	<i>1110028I</i>	RIKEN cDNA 1110028F11 gene				
11	Bzrap1	benzodiazapine receptor assoc. protein 1	turquoise	-0.458	0.001	trans
11	<i>Epx</i>	eosinophil peroxidase	grey			
11	<i>Gdpd1</i>	glycerophosphodiester phosphodiesterase	grey			
11	<i>Hsf5</i>	heat shock transcription factor family member 5	grey			
11	<i>Lpo</i>	lactoperoxidase	grey			
11	<i>Mir301</i>	microRNA 301				
11	<i>Mks1</i>	Meckel syndrome, type 1				
11	<i>Mpo</i>	myeloperoxidase	grey			
11	<i>Mtmr4</i>	myotubularin related protein 4	grey			
11	<i>Olf462</i>	olfactory receptor 462	grey			
11	<i>Olf463</i>	olfactory receptor 463	grey			
11	<i>Olf464</i>	olfactory receptor 464	grey			
11	<i>Ppm1E</i>	protein phosphatase 1E (PP2C domain)	brown			
11	<i>Prr11</i>	proline rich 11	grey			
11	<i>Rad51C</i>	RAD51 homolog c (S. cerevisiae)	grey			
11	<i>Rnf43</i>	ring finger protein 43	grey			
11	<i>4-Sep</i>	septin 4				
11	<i>Supt4h1</i>	predicted gene 3258;	grey			
11	<i>Tex14</i>	testis expressed gene 14	grey			
11	<i>Trim37</i>	tripartite motif-containing 37	blue			
11	<i>Ypel2</i>	yippee-like 2 (Drosophila)	grey			
11	<i>Smg8</i>	smg-8 homolog, nonsense mediated mRNA decay factor				
11	<i>Ska2</i>	spindle and kinetochore associated complex subunit 2				
16	<i>Acap2</i>	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2				
16	<i>Apod</i>	apolipoprotein D				
16	<i>Atp13A3</i>	ATPase type 13A3				

16	<i>Bdh1</i>	3-hydroxybutyrate dehydrogenase, type 1	grey			
16	<i>Cpn2</i>	carboxypeptidase N, polypeptide 2	grey			
16	<i>Fam43A</i>	family with sequence similarity 43, member A				
16	<i>Gp5</i>	glycoprotein 5 (platelet)	grey			
16	<i>Lrrc15</i>	leucine rich repeat containing 15	grey			
16	<i>Lsg1</i>	large subunit GTPase 1 homolog (S. cerevisiae)	grey			
16	<i>Ppp1R2</i>	protein phosphatase 1, regulatory (inhibitor) subunit	yellow	0.38	0.008	trans
16	<i>Tmem44</i>	transmembrane protein 44	turquoise	0.32	0.028	trans
19	<i>C130060</i>	RIKEN cDNA C130060C02 gene				
19	<i>Cep78</i>	centrosomal protein 78	grey			
19	<i>Foxb2</i>	forkhead box B2	grey	-0.3	0.023	
19	<i>Gcnt1</i>	glucosaminyl (N-acetyl) transferase 1, core 2	grey			
19	<i>Pcsk5</i>	proprotein convertase subtilisin/kexin type 5				
19	<i>Prune2</i>	RIKEN cDNA A230083H22 gene				
19	<i>Psat1</i>	similar to Phosphoserine aminotransferase 1	grey			
19	<i>Rfk</i>	riboflavin kinase	grey			

Table 2: Genes underlying QTL for total lever pressing for sucrose, integrated with transcriptomics data. Corresponding module name from WGCNA, and, where statistically significant, correlations between the gene expression and the lever pressing. Presence of *cis*- vs *trans*-eQTLs shown for genes with significant trait correlation. Prioritized genes are in bold-face type.

Annotation Cluster 1		11.685			
Term	Count	%	PValue	FDR	
protein localization	177	7.409	2.610E-17	4.820E-14	
establishment of protein localization	159	6.656	7.140E-17	2.000E-13	
protein transport	158	6.614	1.900E-16	4.110E-13	
protein transport	115	4.814	5.900E-13	8.590E-10	
transport	289	12.097	8.970E-12	1.310E-08	
intracellular transport	104	4.353	5.310E-11	9.790E-08	
intracellular protein transport	71	2.972	5.090E-09	9.390E-06	
cellular protein localization	73	3.056	3.170E-08	5.850E-05	
cellular macromolecule localization	73	3.056	4.240E-08	7.820E-05	

Annotation Cluster 2		9.4603			
Term	Count	%	PValue	FDR	
nucleotide binding	393	16.450	1.770E-16	3.550E-13	
nucleotide-binding	311	13.018	1.050E-14	1.540E-11	
purine nucleotide binding	333	13.939	4.010E-13	6.500E-10	
ribonucleotide binding	319	13.353	1.950E-12	3.150E-09	
purine ribonucleotide binding	319	13.353	1.950E-12	3.150E-09	
atp-binding		243	10.172	4.110E-11	5.980E-08
nucleoside binding		276	11.553	1.260E-10	2.050E-07
purine nucleoside binding		274	11.469	1.640E-10	2.660E-07
adenyl nucleotide binding		272	11.386	1.740E-10	2.820E-07
ATP binding		257	10.758	3.820E-10	6.180E-07
adenyl ribonucleotide binding		258	10.799	7.820E-10	1.270E-06
nucleotide phosphate-binding region:ATP		159	6.656	5.360E-04	1.011E+00
kinase		120	5.023	6.090E-04	8.825E-01
binding site:ATP		95	3.977	8.855E-03	1.552E+01

Annotation Cluster 3		8.002			
Term	Count	%	PValue	FDR	
mitochondrion	251	10.506	4.940E-12	7.240E-09	
mitochondrion	165	6.907	4.110E-11	5.980E-08	
mitochondrial part	113	4.730	9.160E-09	1.340E-05	
transit peptide	95	3.977	9.790E-07	1.426E-03	
transit peptide:Mitochondrion	92	3.851	5.340E-05	1.012E-01	

Annotation Cluster 4		7.748			
Term	Count	%	PValue	FDR	
organelle membrane	176	7.367	1.340E-13	1.970E-10	

mitochondrion	165	6.907	4.110E-11	5.980E-08
envelope	121	5.065	2.760E-10	4.040E-07
organelle envelope	120	5.023	4.460E-10	6.530E-07
mitochondrial part	113	4.730	9.160E-09	1.340E-05
mitochondrial envelope	88	3.684	6.690E-08	9.810E-05
mitochondrial membrane	81	3.391	6.200E-07	9.090E-04
organelle inner membrane	68	2.846	7.790E-06	1.141E-02
mitochondrial inner membrane	65	2.721	9.830E-06	1.440E-02
mitochondrion inner membrane	42	1.758	1.660E-04	2.421E-01

Annotation Cluster 5		6.959		
Term	Count	%	PValue	FDR
Golgi apparatus	137	5.735	2.360E-08	3.450E-05
golgi apparatus	114	4.772	1.150E-07	1.670E-04
Golgi apparatus part	57	2.386	4.900E-07	7.180E-04

Annotation Cluster 6		6.9072		
Term	Count	%	PValue	FDR
vesicle-mediated transport	115	4.814	9.260E-13	1.710E-09
membrane organization	69	2.888	1.570E-08	2.900E-05
endocytosis	50	2.093	4.180E-07	7.710E-04
membrane invagination	50	2.093	4.180E-07	7.710E-04
Endocytosis	21	0.879	1.143E-02	1.541E+01

Annotation Cluster 7		5.2085		
Term	Count	%	PValue	FDR
membrane-bounded vesicle	90	3.767	4.770E-07	6.990E-04
vesicle	105	4.395	8.910E-07	1.306E-03
cytoplasmic membrane-bounded vesicle	88	3.684	9.240E-07	1.354E-03
coated vesicle	37	1.549	9.590E-07	1.405E-03
cytoplasmic vesicle	103	4.311	1.050E-06	1.544E-03
clathrin-coated vesicle	32	1.339	2.910E-06	4.265E-03
cytoplasmic vesicle	50	2.093	1.910E-04	2.770E-01
synaptic vesicle	21	0.879	1.980E-04	2.905E-01
synapse part	46	1.925	3.040E-04	4.454E-01

Annotation Cluster 8		5.0822		
Term	Count	%	PValue	FDR
protein catabolic process	113	4.730	2.270E-07	4.190E-04
cellular macromolecule catabolic process	121	5.065	2.940E-07	5.420E-04
macromolecule catabolic process	128	5.358	3.030E-07	5.590E-04

cellular protein catabolic process	108	4.521	7.530E-07	1.388E-03
proteolysis involved in protein catabolic process	106	4.437	1.820E-06	3.357E-03
modification-dependent protein catabolic process	98	4.102	1.600E-05	2.945E-02
modification-dependent macromolecule catabolic	98	4.102	1.600E-05	2.945E-02
ubl conjugation pathway	88	3.684	4.030E-04	5.853E-01
proteolysis	148	6.195	6.382E-02	7.037E+01

Table 3: Gene ontology cluster analysis for turquoise module. Data are clustered into related annotation groups; values presented in the top row for each cluster are enrichment scores, representing the fold enrichment of the genes in the cluster relative to background (i.e., all other genes in the mouse genome). Number of genes, percent of module, p and FDR statistics are shown for each ontology term.

Annotation Cluster 1		2.5337			
Term	Count	%	PValue	FDR	
proteasome	5	0.299	1.355E-03	1.667E+00	
proteasome complex	5	0.299	1.843E-03	2.332E+00	
mmu03050:Proteasome	4	0.239	1.003E-02	9.737E+00	

Annotation Cluster 2		2.483			
Term	Count	%	PValue	FDR	
mitochondrion	26	1.554	2.550E-04	3.258E-01	
mitochondrion	17	1.016	1.858E-03	2.279E+00	
mitochondrial part	12	0.717	7.737E-03	9.459E+00	
transit peptide	11	0.658	7.910E-03	9.375E+00	
transit peptide:Mitochondrion	11	0.658	1.311E-02	1.727E+01	

Annotation Cluster 3		2.197			
Term	Count	%	PValue	FDR	
mmu04120:Ubiquitin mediated proteolysis	9	0.538	3.858E-05	3.919E-02	
protein catabolic process	15	0.897	3.552E-04	5.402E-01	
ubl conjugation pathway	13	0.777	1.340E-03	1.648E+00	
modification-dependent macromolecule catabolic process	13	0.777	1.645E-03	2.478E+00	
modification-dependent protein catabolic process	13	0.777	1.645E-03	2.478E+00	
macromolecule catabolic process	15	0.897	1.720E-03	2.590E+00	
proteolysis involved in cellular protein catabolic process	13	0.777	2.490E-03	3.730E+00	
cellular protein catabolic process	13	0.777	2.607E-03	3.902E+00	
small conjugating protein ligase activity	6	0.359	4.579E-03	5.812E+00	
cellular macromolecule catabolic process	13	0.777	7.064E-03	1.024E+01	
ubiquitin-protein ligase activity	5	0.299	1.262E-02	1.527E+01	
acid-amino acid ligase activity	6	0.359	1.462E-02	1.748E+01	
ligase activity, forming carbon-nitrogen bonds	6	0.359	2.613E-02	2.921E+01	
ubiquitin-dependent protein catabolic process	5	0.299	3.533E-02	4.221E+01	
proteolysis	16	0.956	3.627E-02	4.306E+01	
protein modification by small protein conjugation or removal	4	0.239	6.315E-02	6.301E+01	
protein modification by small protein conjugation	3	0.179	1.522E-01	9.193E+01	
ligase	5	0.299	2.590E-01	9.757E+01	

Annotation Cluster 4		1.906			
Term	Count	%	PValue	FDR	

organelle membrane	17	1.016	2.446E-03	3.085E+00
organelle inner membrane	9	0.538	7.303E-03	8.951E+00
mitochondrial part	12	0.717	7.737E-03	9.459E+00
organelle envelope	12	0.717	9.582E-03	1.159E+01
envelope	12	0.717	9.835E-03	1.188E+01
mitochondrial inner membrane	8	0.478	1.780E-02	2.053E+01
mitochondrion inner membrane	6	0.359	2.403E-02	2.603E+01
mitochondrial envelope	9	0.538	2.529E-02	2.795E+01
mitochondrial membrane	8	0.478	4.919E-02	4.755E+01

Annotation Cluster 5		1.7503		
Term	Count	%	PValue	FDR
IPR011333:BTB/POZ fold	7	0.418	5.015E-03	6.645E+00
IPR000210:BTB/POZ-like	6	0.359	2.089E-02	2.508E+01
domain:BTB	5	0.299	2.510E-02	3.058E+01
IPR013069:BTB/POZ	5	0.299	2.587E-02	3.013E+01
SM00225:BTB	6	0.359	2.603E-02	2.508E+01

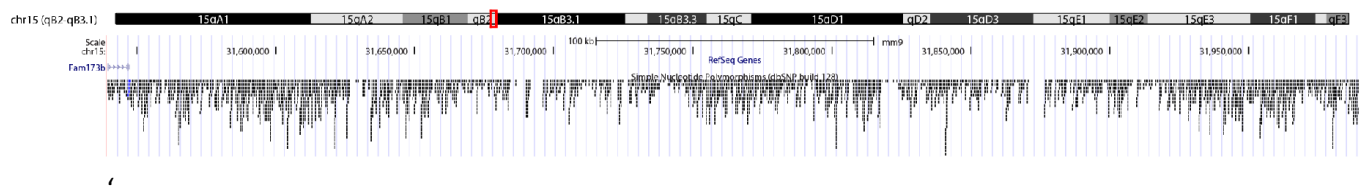
Annotation Cluster 6		1.573		
Term	Count	%	PValue	FDR
biopolymer methylation	5	0.299	3.459E-03	5.146E+00
methylation	5	0.299	5.070E-03	7.458E+00
one-carbon metabolic process	5	0.299	1.944E-02	2.587E+01
protein amino acid methylation	3	0.179	5.441E-02	5.739E+01
protein amino acid alkylation	3	0.179	5.441E-02	5.739E+01
methyltransferase	3	0.179	3.610E-01	9.961E+01

Annotation Cluster 7		1.5077		
Term	Count	%	PValue	FDR
membrane-enclosed lumen	21	1.255	4.019E-03	5.022E+00
intracellular organelle lumen	20	1.195	6.009E-03	7.421E+00
organelle lumen	20	1.195	6.179E-03	7.623E+00
nuclear lumen	16	0.956	1.307E-02	1.549E+01
nucleolus	8	0.478	2.229E-02	2.505E+01
nucleoplasm part	8	0.478	1.820E-01	9.235E+01
nucleoplasm	8	0.478	2.976E-01	9.891E+01
nuclear body	3	0.179	3.683E-01	9.972E+01

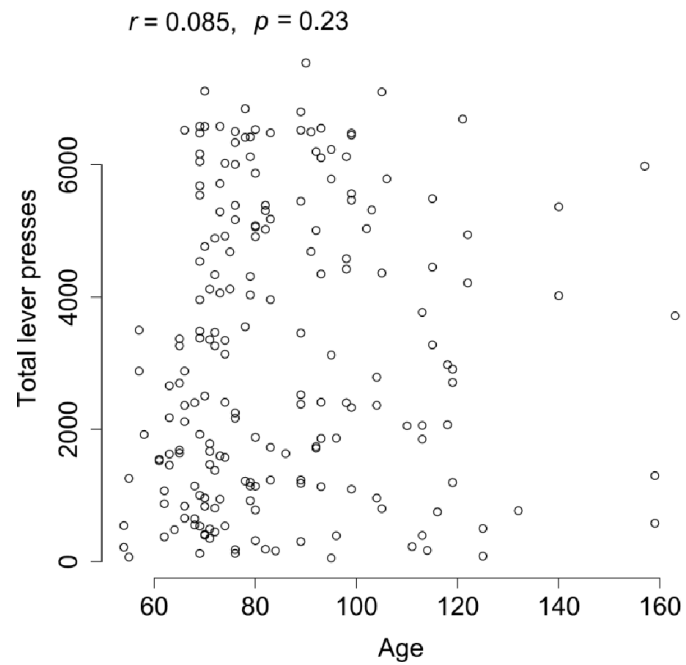
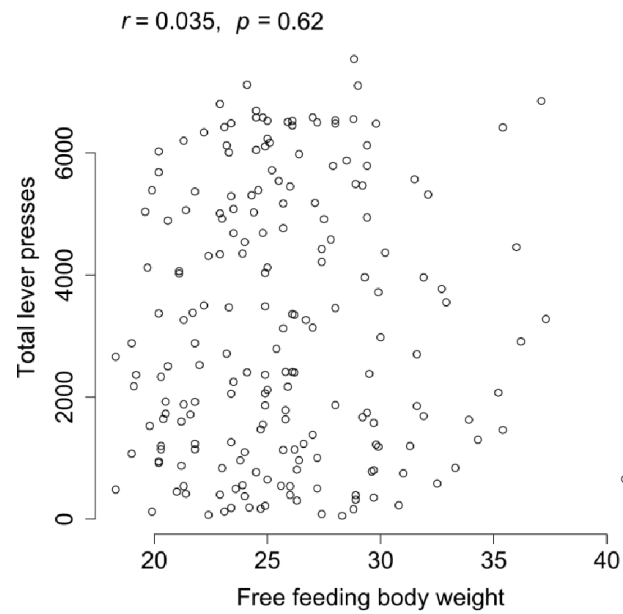
Annotation Cluster 8		1.472		
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Term	Count	%	PValue	FDR
PIRSF002337:Actin	3	0.179	5.988E-03	6.229E+00
IPR004000:Actin/actin-like	3	0.179	2.144E-02	2.565E+01
SM00268:ACTIN	3	0.179	2.408E-02	2.342E+01
cytoskeleton	7	0.418	4.184E-01	9.988E+01

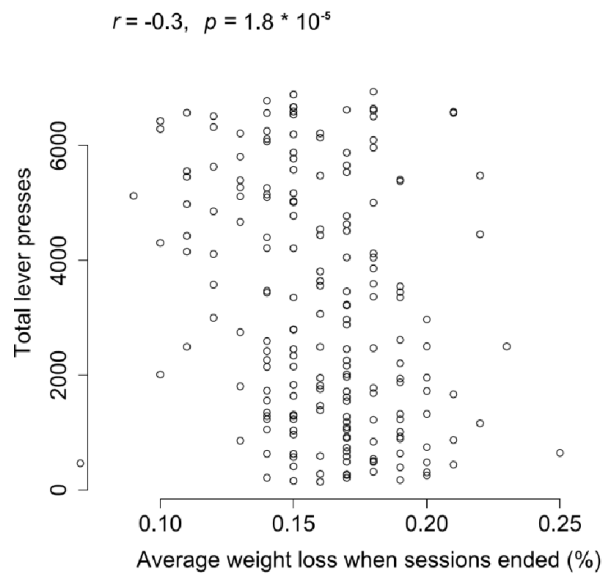
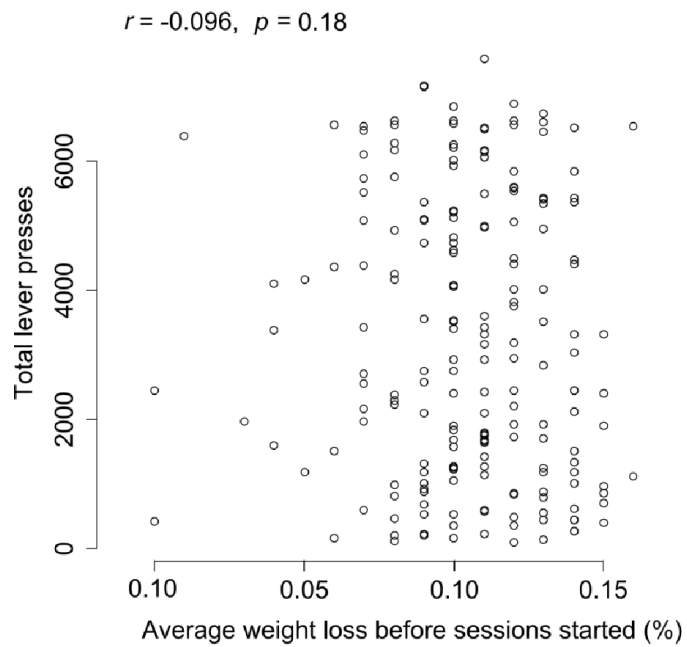
Table 4: Gene ontology cluster analysis for green module. Data presented as in format described in table 3.



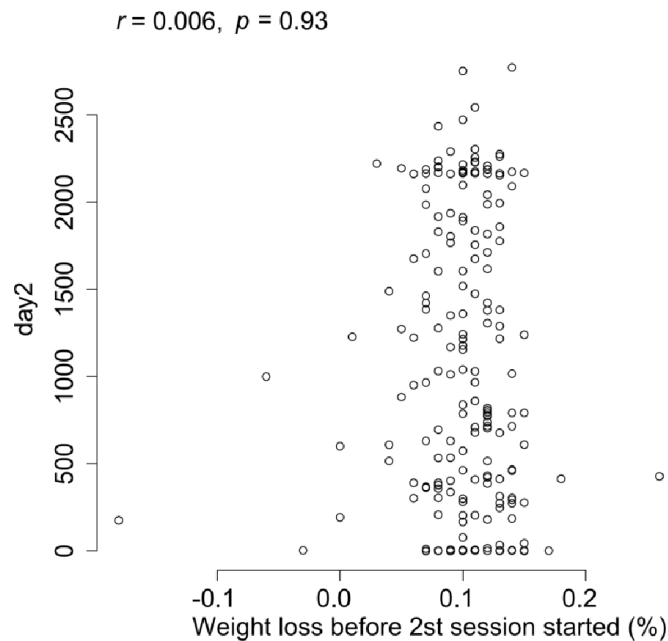
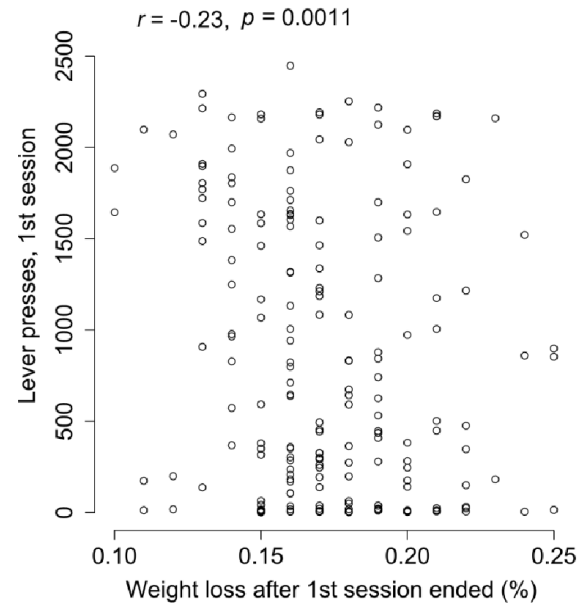
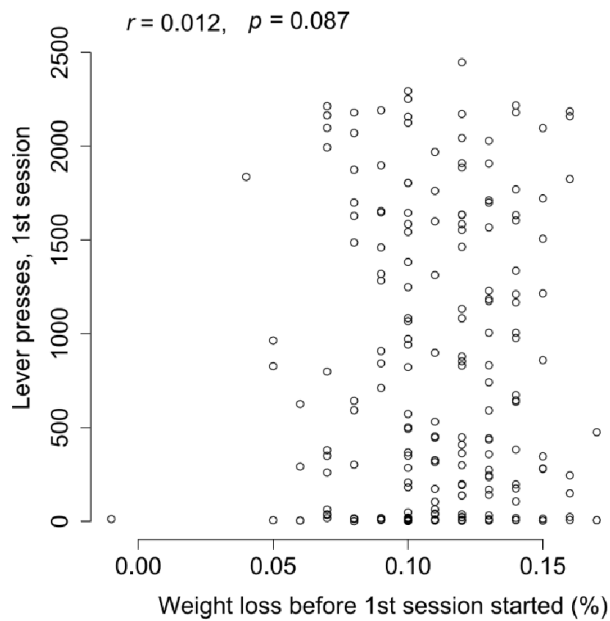
Supplemental Figure 2: Detailed genomic landscape surrounding suggestive SNPs for 'responder' status



Supplemental Figure 3: Scatterplots of age and free-feeding body weight with total lever presses. No significant relationships were found.



Supplemental Figure 4: Scatterplots of average weight reduction before and after sessions with total lever presses. While average weight loss (relative to free-feeding body weight) before the 3 sessions of instrumental learning was not predictive of performance, body weight loss after sessions was significantly associated.



Supplemental Figure 5: Patterns of correlation of weight reduction with lever pressing before and after individual session. The pattern of absence of correlation before session 1, a significant correlation after session 1, and again no association prior to session 2, indicated that the correlations observed relating total lever pressing to post-session weight were likely the result of performance in the operant chamber rather, and consequently GWA results did not incorporate these covariates.

CHAPTER 4

CONCLUSIONS

Because Pavlovian and instrumental processes enabling the prediction and pursuit of desired rewards, as well as the avoidance of dangers and noxious stimuli, they are integral to a wide range of behaviors that we exhibit. The first series of studies sought to disentangle the complex role of NMDA-mediated dopamine cell burst firing in reward-related learning, and to test causally, for the first time, predictions made by the incentive salience account of phasic dopamine function. While instrumental learning was impaired in our genetic model of attenuated phasic dopamine release, Pavlovian magazine approach was not, and importantly, we found no evidence to support a selective dependency of incentive salience attribution, but not prediction error learning, on phasic dopamine release. The second study used methods that are in many ways opposite of those used in the first – forwards genetics versus reverse genetics, hypothesis-free versus hypothesis-based – but was linked by a common behavioral paradigm of critical significance to human behavior. Association-level genome-wide scans yielded several QTL related to quantitative variation in instrumental responding for sucrose; transcriptomics data provided an empirical basis for prioritizing QTL genes for subsequent causal studies, and offered a resource for the development of new hypotheses regarding the biology that links genes to behavior in the context of reward-related learning.

Insights into the role of phasic dopamine release in reward-related learning

Our pattern of findings in Chapter 2 indicate that the NMDA receptors in dopamine neurons, and by extension glutamatergic plasticity and/or phasic firing patterns, regulate the strength of instrumental responding, but are not required for discriminative Pavlovian approach learning.

The mechanisms by which attenuation of phasic dopamine release could impact instrumental learning while leaving Pavlovian approach conditioning unaffected are many.

One potential explanation is that – during instrumental behavior – attenuated incentive salience attribution to instrumental outcomes associated with diminished capacity for phasic dopamine release results in lower levels of vigorous reward-seeking; on the other hand, the Pavlovian approach paradigm used may have emphasized contingency learning rather than incentive salience attribution. Alternatively, it may be that the failure to detect effects on Pavlovian approach behavior is a consequence of residual phasic dopamine release: though burst firing is strongly attenuated in our model system, previous reports indicate that in vivo phasic dopamine release in DAT^{cre};NR1 mice is approximately 30% that of NR1 heterozygotes, and CSs are capable of eliciting this remaining capacity (Zweifel et al., 2009; Parker et al., 2010) . What is left may provide sufficient signal to noise for acquisition and representation of Pavlovian delay conditioning, especially when associative contingencies are binary and deterministic (i.e., $P(US|CS)=1$, $P(US|\sim CS)=0$).

Nevertheless, the incomplete loss of phasic dopamine release in this genetic model offers a unique opportunity to distinguish between the prediction-error and the incentive salience perspectives of dopamine in learning. In many cases, learned behavioral phenomena can be equally explained by the acquisition of the contingencies between stimuli using a phasic dopamine prediction error signal, or by the combination of non-dopaminergic contingency learning with attribution of incentive properties to associative stimuli via phasic dopamine release. Certainly acquisition of Pavlovian and instrumental behaviors would suffer in a relatively similar manner if phasic dopamine release was impeded; even Pavlovian-to-instrumental transfer, considered a canonical manifestation of incentive salience attribution (Robinson and Berridge, 2008), could perhaps be conceptualized as an positive prediction error arising from an unexpected CS presentation.

Autoshaping of Pavlovian conditional approach, however, offers a scenario in which contingency learning and incentive salience learning can putatively be dissociated (Robinson and Flagel, 2009). Rats that exhibit a sign-tracking (directed to the CS) response during conditioning, argued to be demonstrative of attribution of incentive salience to reward-predictive cues, show greater changes in dopamine concentration in response to reward-predictive CSs than those that exhibit a goal-tracking (food magazine approach) response (Robinson and Flagel, 2009; Flagel et al., 2011). If this is a causal relationship, then a quantitative reduction in phasic dopamine release amplitude should diminish the probability of displaying sign-tracking conditional responses.

No such relationship, however, was found here. While it remains possible that the sign-tracker phenotype is under dopaminergic regulation (Flagel et al., 2011), we did not yield any data in support its emergence as a *consequence* of quantitative variation in stimulus-evoked dopamine transients.

Intricacies of goal-tracking and sign-tracking behaviors: a valid model of individual variation in incentive salience attribution?

Whether Pavlovian magazine approach behavior is under the control of Pavlovian processes, rather than instrumental processes, has historically been a contentious issue. At once, the behavior is CS-elicited, insofar as behaviors come to be elicited selectively during presentation of the cue; however, the magazine approach itself is a response that is required to experience the CS-US association. Moreover, while the head-entry measure of conditional approach learning does come to be evoked by the CS as training proceeds, it is nevertheless the same response that is elicited by the US, which renders conclusions regarding conditioning less clear than in paradigms where the unconditional response and conditional response differ (e.g., fear conditioning). It has been suggested that the magazine approach behavior may also represent

non-specific, 'superstitious' instrumental behavior directed towards an area where the animal has experienced reinforcement.

Experiments of approach behavior under an omission schedule of reinforcement (i.e. an instrumental response suppressive paradigm) indicated that approach behavior emerges, suggesting it is Pavlovian (Holland, 1979). However, at the same time, the rate of approach responding under an omission schedule is significantly lower than responding without omission contingencies, an indication that normal Pavlovian approach behavior may be comprised of instrumental and Pavlovian components, and that the omission schedule precludes the expression of the former.

It has been argued, therefore, that behavior under omission schedule is the purely Pavlovian component – invoked reflexively by the CS despite its negative consequences. A recent experiment attempted to argue that this is not the case. A series of experiments contrasting omission-schedule cues and normal Pavlovian cues within-subjects indicated that the lower rate of responding under omission schedule was the consequence of suppression of behavior by reinforcement of response competition, rather than an isolation of the Pavlovian component of an approach response that both instrumental and Pavlovian at once (Harris et al., 2013).

However, this conclusion may have been derived more from a particular interpretation of the data rather than raw, causal findings. The distinction does not appear to have yet been conclusively resolved, and its debate will likely continue. What can be concluded, however, is that in the experiments shown here, is that NMDA-mediated phasic dopamine release is not required to adaptively respond to the additional Pavlovian component in the Pavlovian approach paradigm. However, it must be acknowledged that order effects (i.e., the instrumental learning prior to the Pavlovian approach) may have influenced this result.

Stemming from the complications of instrumental components potentially residing within Pavlovian approach responses is the possibility possible that the phenotypic difference of the sign-tracking and goal-tracking conditional response does not reflect differential incentive salience attribution, but is there result of misattribution of instrumental contingences; of course, one can argue that a mistaken instrumental behavior *is* a form of incentive salience attribution, a more powerful form of 'wanting' that leads an animal to seek out instrumental responses with such a desperate vigor that it acquires incorrect instrumental responses. An argument of this nature, however, leans dangerously in the direction of non-falsifiability.

As reviewed in Meyer et al. (2012), sign-tracking behavior can be interpreted as maladaptive, as it directs behavior away from the primary reward. It may even cost the animal the experience of the reward entirely. For example, sign-tracking readily develops towards predictive cues that are spatially distant from briefly presented rewards, and when contacts with the CS lead to reward omission (Williams and Williams, 1969; Hearst and Jenkins, 1974; Killeen, 2003). In most sign-tracking experiments, however, behavior is observably redirected away from the CS and towards the US when the US is actually presented (though the redirection may be unsuccessful). An example to the contrary is that of quails that developed copulatory behaviors towards a cue predictive of access to opposite sex conspecifics. In a subset of these animals, the CS-directed copulation continued after access to the mate was granted (Koksal et al., 2004).

Sign tracking has other unique qualities: while it is not uncommon for the type of CS to determine the particular form of the conditional response expressed (Holland, 1977; Holland, 1980), the degree to which this is the case for sign-tracking behavior is particularly striking: animals readily interact with a visual or tactile cue such as a lever, but sign-tracking never develops to auditory cues (Meyer et al., 2012). And perhaps of greatest significance here, sign-tracking responses are less readily impacted by omission schedules than are goal-tracking responses: orienting responses to a predictive stimulus are unaffected but magazine

approaches are suppressed (Holland, 1977; Holland, 1979). Because sign-tracking is particularly sensitive to the CS – a property of Pavlovian responses – and because sign-tracking responses are not affected by omission schedules, a parsimonious conclusion might be that goal-tracking is partly instrumental, while sign-tracking is purely Pavlovian. Evidence indicating sign-tracking is an incentive salience attribution response is largely derived from its relationship with dopamine, its correlation with other putatively incentive salience attribution-related behaviors, and its presentation as a vigorous, compulsive cue-directed response. However, the first is a reverse inference, the second circumstantial and dependent upon assumptions about several other behaviors, and the third is face validity only. Consequently, the basic behavioral results of Holland – which rely upon few assumptions – could be considered at least as compelling as the incentive salience perspective of sign-tracking and goal-tracking behavior.

Implications for other processes previously thought to be dependent upon NMDA receptors in dopamine neurons

In addition to Pavlovian approach, other phenotypes that were thought of as requiring NMDA receptors in dopamine neurons, such as sensitization to psychostimulants (Kalivas and Alesdatter, 1993; Wolf et al., 1994; Wolf, 1998; Vanderschuren and Kalivas, 2000), have also turned out to be possible in their absence (Zweifel et al., 2008; Beutler et al., 2011; Luo et al., 2011). Given that the degree of NMDA receptor dependent plasticity (i.e., AMPA/NMDA ratio) induced by drugs of abuse correlates with the magnitude behavioral sensitization observed (Ungless et al., 2001; Borgland et al., 2004), and that it is expressed selectively during periods of active learning in Pavlovian conditioning (Stuber et al., 2008), these results are especially confounding.

Though these phenomena are associated with heightened dopamine outflow (Kalivas and Duffy, 1993; Stewart and Badiani, 1993; Harmer and Phillips, 1999; Venton et al., 2006; Stuber et al.,

2008; Addy et al., 2010), cellular recordings have frequently used electrophysiological profiles or immunocytochemistry to identify dopamine neurons, and the reliability of both methods has been the subject of significant controversy (Borgland et al., 2006; Margolis et al., 2006; Margolis et al., 2010; Zhang et al., 2010). However, genetics methods have made it clear that this form of plasticity does, in fact, occur in mesencephalon dopamine neurons: it can be induced by selective activation of dopamine neurons (Brown et al., 2010) and when NMDA receptors on dopamine cells are eliminated (Engblom et al., 2008; Zweifel et al., 2008; Luo et al., 2011). Consequently, failure to detect anticipated behavioral effects in the DAT^{cre};NR1 mouse is unlikely to be explained by misidentified neurons in prior studies.

It is indeed possible that NMDA-related synaptic plasticity in dopamine neurons plays no role in Pavlovian learning and sensitization, or that its role is quickly compensated for after its loss. Both GABAergic and glutamatergic neurons are found within the VTA and substantia nigra pars compacta (Mugnaini and W.H., 1985; Lacey et al., 1989; Olson and Nestler, 2007; Yamaguchi et al., 2007); the finding that an NMDA antagonist delivered to the VTA of the DAT^{cre} mouse inhibits sensitization supports their involvement in functions previously ascribed to dopamine neurons (Luo et al., 2011). Alternatively, there is evidence that NMDA receptors in more distal nuclei carry out these functions: NMDA receptor activity in dopamine receptor expressing medium spiny neurons of the striatum and in VTA afferents arising from the prefrontal cortex have been implicated in sensitization and Pavlovian approach conditioning as well (Beutler et al., 2011; Parker et al., 2011a)

Design of cre-loxP and other transgenic mouse studies

'Because we included animals with and without cre recombinase and with one or two floxed alleles, we were able to observe statistical interactions between cre genotype and floxed genotype. This strategy lends increased confidence that the results presented here are the

result of dopamine neuron NR1 gene excision, rather than a non-specific flanking gene or background artifact of mouse transgenic techniques (Wolfer et al., 2002; Crusio, 2004a). Notably, these factors are especially significant for studies utilizing the NR1^{flox/flox} mouse, which was created using homologous recombination in 129S4/SvJae strain embryonic stem cells (Tsien et al., 1996b; Tsien et al., 1996a), followed by a moderate level of backcrossing to a C57Bl/6N background. Further, because the NR1^{flox/flox} mouse is often maintained by homozygote-to-homozygote breeding (Jackson Laboratories), its background remains diverged from the traditional C57Bl/6J reference background. And this background difference is quite significant: C57Bl/6J and C57Bl/6N mice, though highly related, have shown marked phenotypic differences. For example, a study of between 700 and 2,200 mice found significant differences in locomotor behavior, elevated plus maze behavior, social interaction, pre-pulse inhibition, forced swim, and spatial working memory (Matsuo et al., 2010); C57Bl/6J mice are the canonical 'alcohol preferring' strain, but C57Bl/6N mice do not express this trait (Blum et al., 1982), and they differ in susceptibility to diet-induced obesity (Nicholson et al., 2010), behavioral response to phencyclidine (Mouri et al., 2012). A recent study carried out by four centers that comprise the European Mouse Disease Clinic found so many phenotypic differences that they cannot be listed here (Simon et al., 2013). These important factors render transgenic mouse studies – and in particular, behavioral studies – less conclusive than they might otherwise be.

Furthermore, confounds of linked genes flanking the mutated allele and interactions with background are, in fact, quite significant: examples of both effects are found throughout the literature (Wade, 1987; Le Roy et al., 2000; Spyropoulos et al., 2003; Chen et al., 2004; Pekarik and Izpisua Belmonte, 2008). Phenotypic differences between donor and host strains are large and many (Belknap et al., 1992; Hitzemann et al., 1998; Reed et al., 2003; Ghazalpour et al., 2012). And even after 12 generations, 1% of the donor strain's genes remain linked (Gerlai, 1996) – such a significant proportion of the genome that knockout mice themselves have been

used for identification of quantitative trait loci (Bolivar et al., 2001; Crusio, 2004a). In fact, examples have been identified wherein a single additional backcross completely eliminated a knockout mouse phenotype completely (Geurts et al., 2011). As such, wherever possible, reducing the likelihood of these confounding factors influencing results – whether by using different control strains or multiple backgrounds (Wolfer et al., 2002), using embryonic stem cells from the anticipated host strain (Backman et al., 2006), various inducible methods (Baron and Bujard, 2000). In that sense, the 2 x 2 cre/flox genotype design employed here is especially significant.

Mouse systems genetics as a tool for understanding reward-related learning

We are aware of only three studies that have performed a genome-wide scan for appetitive instrumental conditioning. One used recombinant inbred mice to locate a QTL for response-inhibition components of flexible instrumental responding under reversal learning conditions (Laughlin et al., 2011), and another identified a QTL for appetitively-reinforced sustained attentional performance (Loos et al., 2012). The third, also involving recombinant inbred mice, focused on different components of acquisition of an instrumental response; this study found suggestive QTLs for food magazine-directed exploratory behavior and for a chained instrumental response (Malkki et al., 2010).

Clearly, more work is needed to extract from the very dense dataset acquired here as much insight into the genetic basis of appetitive instrumental learning – both the learning and motivational aspects of sucrose seeking – but we have laid out the groundwork for these analyses. In doing so, we have shown that variation in whether an animal engages in instrumental behavior, and variation in the degree to which it performs an instrumental responding for sucrose response – that is, quantitative variation in instrumental behavior – have heritable components and identified genetic loci that underlie them.

Systems genetics tools used in this study were effectively prioritized genes within identified QTL for future causal studies. In particular, weighted gene co-expression network analysis yielded significant insights. This technique parses patterns of correlated gene expression, assigning them into different modules on the basis of collective coexpression. By leveraging the finding that co-expressed genes tend to perform similar biological functions (Wolfe et al., 2005), this ‘guilt-by-association’ phenomenon aids making inferences regarding poorly characterized genes. That is, on the basis of what is known about genes with which it is co-expressed, a functional role for a poorly characterized gene can be surmised.

This, in combination with integrating trait data and gene ontology tools into the network analysis, and a can provide insight into the functions of genes that relate to a phenotype of interest and yield inferences regarding the mechanisms by which particular genes or sets of genes impact the phenotype, and offer an empirical mechanism for escalate genes within a QTL for causal studies. The analysis offers a particularly compelling balance of hypothesis-free approach and literature-based ontology approaches. For example, by investigating how the network analysis treats genes which are known to relate to your trait, it can lend confidence to your phenotyping effort. Meanwhile, the wealth of information produced can act as a hypothesis generating tool for new avenues of research.

Much more work is needed to fully integrate our gene co-expression network data with our GWAS data. Moreover, the gene modules themselves – those that relate strongly to instrumental behavior – deserve further investigation. Among the over 4,000 genes within the turquoise module for which there exists a significant correlation between the gene’s expression and instrumental behavior, are a set of genes well known to most neuroscientists, many of which have been directly implicated in learning and memory, along with motivation and reward seeking:

- Gria2, Gria 3, Gria 5
- Kainate receptor 5
- Glycine receptor
- GABAA-beta1
- GABA-B, 1
- HDAC4, HDAC6
- 5HT1B receptor
- Adenosine kinase
- alpha-synuclein
- beta arrestin 1
- Many prominent protein phosphatases and phosphodiesterases
- Many map-kinase genes
- Many potassium channel genes, shaker and otherwise
- Gpr6
- Pdyn
- Adenosine 2a receptor
- ACh vesicle transporter
- N-acetyltransferase
- Many PKC pathway genes - PLC, phosphatidylinositol transfer genes,
- pleckstrin homology domain genes
- Many DNA and RNA polymerase genes
- synaptotagmin
- RasGEF, RhoGEF
- DRD4
- NF1
- Dysbindin-related genes, 4 other major schizophrenia risk genes

Their documented role in reward-related learning and memory (Handelmann et al., 1989; Nakagawa et al., 1993; Silva et al., 1997; Selcher et al., 1999; Selcher et al., 2001; Chen et al., 2002; Mazzucchelli et al., 2002; Keith et al., 2003; Mead and Stephens, 2003; James et al., 2007; Lobo et al., 2007; Deng et al., 2009; Fontinha et al., 2009; Jentsch et al., 2009; Lasarge

et al., 2009; Wiltgen et al., 2009; Zyablitseva et al., 2009; Keri et al., 2010; Shilyansky et al., 2010; Karlsgodt et al., 2011; Ujike et al., 2011; Wei et al., 2011; Facciolo et al., 2012; Miszkiet et al., 2012; Singer et al., 2013; Glen et al., 2014) at once lends credibility to the modules identified, and bridges the data gathered using the Hybrid Mouse Diversity Panel to the data gathered with the DATcre mouse; see, for example, Parker et al. (2011b).

And perhaps of particular interest to behavioral neuroscientists, gene co-expression analysis can offer insight into the relationship between different dependent measures acquired from behavioral assays. By logical extension of the guilt-by-association principle to the level of behavior, there is a significant opportunity to gather new insight these behaviors, arising from a new level of analysis. For example, behaviors which relate to overlapping gene co-expression modules might be considered components of a singular (or similar) biobehavioral construct (constructs), while those that are not are conferred some degree of orthogonality. These inferences flow naturally from the principle components methodology that extracts module eigengenes, capturing maximal variance by harnessing the mathematics of orthogonal constraints.

By permitting the discovery of the genes and modules of co-expressed genes that underlie two behaviors, these techniques offer the possibility of significant insight into what neural processes link them. Moreover, the impact of research coming from multiple sources could be maximized. For example, one might study an appetitive incentive motivational process, someone else a measure of impulsivity, a third group stimulus-evoked dopamine transients; because the data are gathered from isogenic, inbred mice, they can be pooled, and together used to understand the overlapping and non-overlapping components of the transcriptome. These data might then be utilized by an investigator who studies variation in self-administration of drugs of abuse, in order to establish, empirically, the behavioral constructs that do relate to propensity for drug addiction, and distinguish them from the correlations that are only spurious. The result may be

a diminished reliance upon face validity of pre-clinical measures, and importantly, significant insight into the underlying neurobiology – the gene networks – shared between drug self-administration and the construct-valid behavioral processes that predict drug taking.

Genomics as phenotype construct interrogation tool

The segregating, categorizing, and clustering of different forms of learning and motivational processes has long been a cornerstone of research in the field of behaviorism (Thorndike, 1911; Pavlov and Anrep, 1927; Hull, 1943; Estes, 1948; Rescorla and Solomon, 1967; Bindra, 1974; Bolles, 1975; Dickinson, 1980; Holland, 1984; Toates, 1986). An important framework of conceptually distinct behavioral processes (e.g., associative and non-associative learning, Pavlovian responses and instrumental responses) emerged from this work, which in many ways, set the stage for the wave of neuroscience studies of behavior that followed. That is to say, many of the most important insights offered by behavioral neuroscience are discoveries of the neural bases of the constructs outlined by behaviorists, or variants thereof, even as methodology sophistication grows exponentially (Lashley, 1950; Kandel, 1976; McCormick and Thompson, 1984; Tsien et al., 1996a; Schultz et al., 1997; Silva et al., 1998; Fendt and Fanselow, 1999; Bi and Poo, 2001; Cardinal et al., 2002; Kelley, 2004; Malenka and Bear, 2004; Yin et al., 2004; Steinberg et al., 2013).

There are multiple avenues towards establishing construct validity of behavioral measures, such as meta-analytic principle components analysis, which orthogonalizes putative construct components or factor analysis to extract latent constructs; both can facilitate the discernment of phenotypes that are measures of the same construct and phenotypes that are measures of distinct constructs (Clark and Watson, 1995; Sharma et al., 2014). Amongst the varied sources

of evidence for establishing construct validity is that of gathering systems-biological evidence that there indeed exists a biological basis for the construct.

Stemming from melding systems genetics tools with combined behavioral traits data is the possibility of a significant new mechanism for the generation of multi-tiered, convergent evidence in behavioral neuroscience research. Therefore, further analysis of the genomics, transcriptomics, and behavioral data acquire will be undertaken, in an effort to yield a more nuanced understanding of the genetic landscape of reward-seeking, in addition to its exploratory, learning, and motivational subcomponents. Often, these components have been considered orthogonal: learning and motivation measures uncorrelated, as in Gallistel et al 2004, or neurotransmitters are thought of as being related to one but not the other, as in perspectives on dopamine activity during prediction error learning versus incentive motivational processes. The degree to which they do and or do not overlap, with respect to genome QTL and to gene co-expression modules, will offer a new basis for assessing the construct validity of processes that at times have been considered orthogonal -- at a construct level -- but do clearly interact in the ultimate presentation of reward-seeking behavior.

Here, we utilized systems genetics to implement this strategy, by highlighting the relationship between a measure of learning and a measure of asymptotic behavior. These are commonly thought of as being distinct; the slope of the learning curve has been considered independent of the final rate of behavioral output, and their independence has been explicit conclusion of a review of appetitive learning in rodents (Pobols, 1960). Asymptotic rate of behavior is manipulable by changing the incentive value of the unconditional stimulus: the degree to which the US supports responding is considered related to its magnitude. Indeed, level of conditioning supported by a US is an entirely separate term from learning rate parameters in classical models of learning (Rescorla and Wagner, 1972).

In the context of the Hybrid Mouse Diversity Panel experiment, because all animals received the same US magnitude, strain differences in asymptotic responding may be better thought of as differences in incentive motivation instilled by the reward that are driven by genetic differences. In contrast to commonly held perspectives regarding learning and motivation (or level of conditioning supportable by the US), here we provide genetic and transcriptome-based evidence that these processes may reflect a common construct.

Certainly, this line of argumentation is not meant to indicate that they are the same thing; many behavioral experiments elegantly dissociate reward- or punishment-based learning rate from asymptotic conditional responding. However, we do make the argument that they may have a common neurobiological basis – at the level of the genome and the level of gene transcription.

Interestingly, this brings the dissertation full circle: the debate surrounding dopamine's role in reward-related learning is exactly this. That is, learning rate and asymptotic motivational processes effectively map on to the prediction error and incentive salience perspectives of dopamine's role in learning. Some have argued it has no effect on learning – that it only scales motivational processes – as evidenced by rates of behavior in DAT knockout mice, for example (Pecina et al., 2003; Cagniard et al., 2006; Yin et al., 2006b). Others have convincingly shown dopamine signaling reflects a prediction error process in learning, and does have a causal role in learning itself (Schultz et al., 1997; Adamantidis et al., 2011b; Witten et al., 2011b; Kim et al., 2012; Steinberg et al., 2013). And ultimately, the data from the DAT^{cre} experiments are most consistent with a conclusion somewhere in the middle: phasic dopamine release may not exclusively regulate incentive salience attribution, but its quantitative output does not always impact learning rate either. The mixing of learning and motivation in the DAT^{cre} and Hybrid Mouse Diversity Panel experiments is something that certainly deserves further study, but here I offer a piece of conjecture: it may be that at a behavioral level, one can dissociate learning processes from motivational or asymptotic conditioning supportable by manipulating parameters

such as US magnitude. But the underlying neurobiology, the underlying genetics, the underlying gene transcription, may not in fact be as dissociable as these experiments would imply.

Combined hypothesis-free and hypothesis-based research as an effective research strategy

Hypothesis-free research, in a manner demonstrated here, can complement hypothesis-based research in behavioral neuroscience. The example of the relationship between the behavioral, transcriptional, and genomic results of the HMDP chapter and the DAT^{cre};NR1 chapter is a simple example of their interaction. But in a broader sense, their direct combination has tremendous potential. By understanding in greater depth what we do know about behavior and its implementation in the brain, hypothesis-based work yields strong conclusions about the role particular genes play in behavior. Meanwhile, hypothesis-free research allows the flow of new data – genes, proteins, transcripts, intracellular processes, signaling pathways, and on and on – that would otherwise never have been considered in the study of a particular behavioral process to come to the forefront. This influx of data acts as a hypothesis generating mechanism for further hypothesis-based research. These approaches, then, work in harmony, and their synergy – if implemented effectively – has the power to yield tremendous gains in the field of neuroscience.

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