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In vivo neurochemical profile of selectively bred high-responder and low-responder rats reveals baseline, cocaine- and novelty-evoked differences in monoaminergic systems

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Abstract

Relative to bred low-responder (bLR) rats, bred high-responder (bHR) rats have an exaggerated locomotor response to a novel environment, are more risk-taking, more impulsive, and more likely to exhibit compulsive drug-seeking behaviors. These phenotypic differences in addiction-related behaviors and temperament have previously been associated with differences in neurotransmitter signaling, including the mesolimbic dopamine system. In the current study, we applied advanced in vivo microdialysis sampling in the nucleus accumbens of bHRs/bLRs to assess differences in basal and stimulated neurochemical efflux more broadly. We used liquid-chromatography-mass spectrometry measurements of dialysate samples to quantify a panel of 17 neurochemicals including dopamine, norepinephrine, serotonin, histamine, glutamate, GABA, acetylcholine, adenosine, DOPAC, 3-MT, HVA, 5-HIAA, normetanephrine, taurine, serine, aspartate and glycine. We also applied a stable isotope labeling technique to assess absolute baseline concentrations of dopamine and norepinephrine in the nucleus accumbens. Finally, we investigated the role of norepinephrine tone in the nucleus accumbens on the bHR phenotype. Our findings show that bHRs have elevated basal and cocaine-evoked dopamine and norepinephrine levels in the nucleus accumbens compared to bLRs. Furthermore, norepinephrine signaling in the nucleus accumbens appeared to be an important contributor to the bHR phenotype since bilateral perfusion of the $\alpha 1$

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Author Contributions

OSM, SBF, RTK and HA designed the study. OSM, JLH and JMTW performed the *in vivo* microdialysis and behavior experiments. OSM and JMTW analyzed microdialysis samples with HPLC-MS/MS. OSM, SBF, RTK and HA analyzed the data and interpreted the findings. OSM and SBF drafted the manuscript. OSM, SBF, RTK and HA critically revised the manuscript. All authors critically reviewed content and approved final version for publication.

Supporting Information

To accompany the heat map illustration of neurochemical changes, we have included traditional microdialysis traces in the Supporting Information section (Fig S1).

Probe placements were visually inspected after brains were dissected. Probe tracts were visible in the nucleus accumbens and were then recorded on a rat brain atlas printout (Fig 2).

adrenergic receptor antagonist terazosin (10 μ M) into the nucleus accumbens abolished the bHR response to novelty. These findings are the first to demonstrate a role for norepinephrine in the bHR phenotype. They reveal a positive relationship between dopamine and norepinephrine signaling in the nucleus accumbens in mediating the exaggerated response to novelty, and point to norepinephrine signaling as a potential target in the treatment of impulse control disorders.

TOC image

Keywords

Neurochemistry; nucleus accumbens; high-responders; mass spectrometry; norepinephrine; dopamine

Introduction

The way an individual responds to its surrounding environment may be a key determinant of psychopathology. Previous work, using a unique genetic animal model, indicates that individual differences in environmental exploration are associated with differences in stress response, risk-taking behavior, impulsivity and compulsive drug-taking behavior (1, 2, for review see 3). In the current study, we exploit this established animal model—rats that have been selectively bred based on locomotor response to novelty—to examine the neurochemical profile that might be associated with this so-called “sensation-seeking trait” (4–5).

Over a decade ago, the selectively bred high-responder (bHR) and low-responder (bLR) rat lines were generated (6), modeling those that explore the most and the least, respectively, in a novel environment (7). This model provides an *a priori* way of predicting which rats are destined to become high- vs. low-responders, allowing us to identify the antecedent variables that might determine these traits. Locomotor response to an inescapable novel environment has previously been associated with anxiety-like behaviors and the propensity to self-administer drugs of abuse in outbred rats (7–8). It is important to note, however, that the selectively bred lines exhibit a number of other addiction-related traits (1–3) that are not necessarily apparent in outbred high- and low-responder rats (e.g. 9–10). In addition to increased exploration in a novel environment—the trait for which they were bred—bHR rats are also more aggressive, more impulsive on tests of impulsive action, and exhibit maladaptive and persistent responses to food- and cocaine-associated cues (1, 3). bHRs are also more likely to exhibit compulsive drug-seeking behavior following a prolonged cocaine

self-administration paradigm and an enhanced propensity for reinstatement of drug-seeking behavior, or relapse, following abstinence (2). Taken together, relative to bLR rats, bHRs appear to be more behaviorally disinhibited, with increased susceptibility to addiction-related behaviors.

To date, much of the work characterizing the neurobiological antecedents of the addiction-related behaviors exhibited by bHRs and bLRs has focused on the dopaminergic system, which has previously been implicated in a number of these traits (e.g. 11–16). Namely, relative to bLRs, bHRs have lower levels of dopamine D2 receptor mRNA in the striatum (1–2), but a greater proportion of dopamine D2^{high}, the functionally active form of the receptor; despite comparable levels of total D2 binding (1). In addition, relative to bLRs, bHR rats have more spontaneous dopamine ‘release events’ as measured using fast-scan cyclic voltammetry in the core of the nucleus accumbens (NAc) (1). In agreement with these reported differences in dopaminergic “tone”, bHRs exhibit increased psychomotor response to dopaminergic drugs, including quinpirole (1) and cocaine (17–18).

In the current study, we focused our analyses on the NAc to more thoroughly examine the neurochemical profile in bHR and bLR rats. Since bHRs display exaggerated behavioral responses to novelty and drugs of abuse compared to bLRs (1–2), we hypothesized that bHRs and bLRs would exhibit differential neurochemical release patterns in the NAc under both basal and stimulated conditions. To test this hypothesis, we used *in vivo* microdialysis to sample the NAc in both bHRs and bLRs then used benzoyl chloride derivatization and liquid chromatography-mass spectrometry (LC-MS) to assess concentrations differences in a total of 17 neurochemicals, including neurotransmitters, neuromodulators and metabolites (19). We evaluated these neurochemical profiles at rest (Experiment 1), in response to an acute injection of cocaine (Experiment 2), and in response to a novel environment (Experiment 3). Because of the broad analytical nature of this technique, it is well-suited for determining differences between different strains or genotypes. To more accurately quantify extracellular dopamine and norepinephrine (NE) in the NAc under basal conditions we employed a novel stable isotope labeling (SIL) procedure which corrects for variance in microdialysis probe recovery (20).

Our results demonstrate distinct neurochemical signaling patterns between bHRs and bLRs under basal conditions, in response to cocaine, and upon exposure to a novel environment. These findings identify a link between the bHR phenotype and dopamine-NE interactions in the NAc, and shed light on the neurobiological substrates underlying the behavioral traits exhibited by these selectively bred rat lines.

Results and Discussion

The current study aimed to test the hypothesis that bHR and bLR rats may differ in their neurochemical signaling profiles in the NAc, a brain area associated with locomotor activity and motivated behaviors including, reward-seeking and response to drugs of abuse (21–23). To achieve this, the study applied advanced LC-MS analytical techniques and video capture behavioral analysis to identify baseline and stimulus-induced neurochemical correlates of the bHR/bLR phenotypes.

bHRs show exaggerated behavioral responses to cocaine including, increased rates of acquisition of self-administration (24) and greater psychomotor response to acute and repeated cocaine treatment (17–18). Likewise, outbred HR rats have hyperdopaminergic responses in the NAc in response to psychostimulants like cocaine (25). Upon exposure to a reward-paired cue, bHR rats, who tend to sign-track to such cues, have exaggerated cue-evoked dopamine events in the NAc relative to bLRs, who tend to goal-track (26). Taken together, bHRs appear to be more sensitive to stimuli that drive ventral striatal dopamine release. Despite these lines of evidence for stimulus-induced differences, basal differences in dopamine activity between bHRs and bLRs have yet to be defined. The current study took advantage of recent advances in mass spectrometry-based small molecule measurements which enabled the development of a 17-analyte neurochemical panel (19) and a SIL assay for improved extracellular quantitation (20). We applied these methods under basal conditions (SIL) or in response to stimuli known to evoke dopamine release (23), including exposure to a novel environment and cocaine administration.

Experiment 1: Baseline neurochemical differences between awake bHRs and bLRs

To determine whether bHRs and bLRs differ in their NAc neurochemical profiles at rest, microdialysis was performed in both phenotypes and samples were analyzed for 17 neurochemicals, including dopamine, norepinephrine, serotonin, histamine, glutamate, GABA, acetylcholine, adenosine, DOPAC, 3-MT, HVA, 5-HIAA, normetanephrine, taurine, serine, aspartate and glycine (Table 1). Results indicate that only dopamine in dialysate samples was significantly different between phenotypes ($T_{2.45}$, dF_{15} , $p=0.027$; $q=0.096$), with elevated levels in bHRs (1.42 nM, $n=10$) compared to bLRs (0.87 nM, $n=7$; see Table 1).

Since previous studies have identified a relationship between locomotor activity and accumbal dopamine and NE (27), we exploited the same bHR and bLR rats used above to estimate absolute extracellular levels of these transmitters in the NAc. To do so, we used a recently developed SIL technique which accounts for variability in probe recovery (Fig 1) (20). This required the addition of isotopically labeled dopamine ($^{13}C_6$ -dopamine; 5 nM) and NE (d_6 -NE; 5 nM) in the perfusate of the dialysates collected under baseline conditions. The average extraction fraction (E_d) taken at baseline for dopamine was 0.34 ± 0.03 for bHRs and 0.35 ± 0.02 for bLRs. Applying these E_d values generated absolute baseline dopamine concentrations of 5.09 ± 0.38 nM ($n=10$) and 2.76 ± 0.48 nM ($n=7$) for bHRs and bLRs, respectively (Fig 1). The average E_d taken at baseline for NE was 0.24 ± 0.02 for bHRs and 0.36 ± 0.03 for bLRs. Applying these E_d values generated absolute NE concentrations of 2.20 ± 0.29 nM ($n=10$) and 1.01 ± 0.20 nM ($n=7$) for bHRs and bLRs, respectively (Fig 1). Similar to the data without SIL correction, dopamine was significantly higher in bHRs compared to bLRs ($T_{3.86}$, dF_{15} , $p=0.002$). The analysis also showed that bHRs had significantly higher basal NE levels in NAc compared to bLRs ($T_{3.05}$, dF_{15} , $p=0.008$). These results are consistent with previous work in outbred HRs (e.g. 28–29). In particular, outbred HRs have been shown to have higher basal firing rates in the VTA (14), which is congruent with bHRs showing increased spontaneous dopamine events in the NAc (1). E_d for catecholamines has been shown to be relatively insensitive to release inhibition, metabolism and synthesis, but strongly affected by uptake inhibition (30). Thus, under

baseline conditions, there appears to be disrupted catecholamine uptake in bHRs, which may contribute to the behavioral phenotype of these animals.

Experiment 2: Behavioral and neurochemical effects of cocaine in bHR and bLR rats

In order to assess behavioral differences in a dopamine-stimulated state, we treated bHR (n=6) and bLR rats (n=8) with a single injection of cocaine (15 mg/kg i.p.) (Fig 2). To determine phenotypic differences in cocaine-mediated behavioral responses relative to baseline activity, we performed a two-way repeated measure (RM) ANOVA analysis across all time points (–60 to 60 min; Fig. 2). There was a significant effect of time ($F_{23,288}=8.83$, $p<0.0001$), phenotype ($F_{1,288}=121.90$, $p<0.0001$), and a time $\times$ phenotype interaction ($F_{23,288}=2.71$, $p<0.0001$). Bonferroni post-hoc comparisons showed that bHRs did not differ from bLRs at baseline (i.e. –60 - 0 min), but they did show a greater locomotor response immediately after cocaine treatment (time 5 – 55 min; Fig. 2).

While monitoring cocaine-induced hyperlocomotion, we simultaneously applied microdialysis to sample broad neurochemistry in the NAc. Three bLRs from this study had occluded probes, resulting in neurochemistry samples from n=5 bLRs and n=6 bHRs. All concentrations were baseline normalized by setting the mean of all 12 basal fractions to 100%. Cocaine effects on neurochemistry were then analyzed using a two-way RM ANOVA across all time points (–60 to 60 min) using phenotype and time as independent variables; followed by Bonferroni post hoc comparisons. All statistical results from the two-way RM ANOVA from this study are reported in Table 2, and these statistics correspond to the traditional microdialysis traces which are shown in Fig S1 for visualization purposes. The percentage change in cocaine-induced neurochemical efflux relative to baseline is illustrated in the heatmap in Fig 3. Cocaine elicited a greater maximal increase in NAc dopamine in bHRs (385%) compared to bLRs (143%; $p<0.0001$) (Fig 3). This same trend was also found for the dopamine metabolite 3MT with a maximal 152% increase in bHRs and an 83% in bLRs ($p=0.0007$) (Fig 3). 3MT is a direct metabolite of dopamine via extracellular catechol-O-methyltransferase (COMT) activity and is often used as a marker of synaptic dopamine. Since cocaine reliably increases striatal synaptic dopamine, increases in 3MT are expected to follow the same trend (31). NE also showed greater increases in bHRs (572%) as compared to bLRs (446%; $p=0.015$) in response to cocaine (Fig 3, Fig S1). Although cocaine primarily targets the dopamine transporter (DAT), it is relatively non selective over other monoamine transporters and has also been shown to increase striatal NE through NE transporter inhibition (NET) (32). Therefore, since bHRs showed a significantly greater NE and dopamine response to cocaine (Fig 3, Table 1, Fig S1), it is plausible that bHRs have a downregulation or disruption of striatal NET, causing a lower DAT/NET BMax as indicated by the E_d . In this case, cocaine would more fully occupy NET binding sites thereby preventing NE clearance and leading to more accumulation. This hypothesis remains speculative since the current study did not directly assess NET or DAT activity, nor other substrates of catecholamine uptake such as the organic cation transporter (OCT3) (33). However, it is interesting to note that NET blockers, like atomoxetine, are used clinically to treat impulse control disorders, including ADHD (34). Importantly, these treatments enhance cortical monoamine transmission, while having little or no effect on accumbal monoamine release, suggesting a differential modulatory role for NE in these 2 brain areas (35). Thus,

while NE tone in cortical regions may modulate impulsive behaviors, NE activity in the NAc may act as a modulator to promote dopamine responsiveness.

An intriguing and unexpected finding from the current study is the phenotype-specific effects of cocaine treatment on glycine (Fig 3, Table 2, Fig S1). Comparing bHR and bLR percent changes across all time points (–60–60 min), a RM ANOVA followed by a Bonferroni posthoc test showed that bLRs had a maximal increase of 190% while bHRs had a maximal increase of 64% ($p=0.012$; Fig 3, Table 2, Fig S1). It has previously been shown that glycine microinjections in the NAc cause dose-dependent effects on dopamine-mediated behaviors, with low doses decreasing and high (non-physiological) doses increasing dopamine-mediated locomotor activity (36). Therefore, it is possible that bLRs, but not bHRs, possess a compensatory mechanism whereby endogenous glycine is released to counter the effects of excessive dopamine stimulation. This notion that striatal glycine can play a role in bHR/bLR phenotypes is novel and warrants further investigation.

Experiment 3: Noradrenergic modulation of novelty-induced behavior and neurochemical changes

Since basal (Fig 1) and cocaine-evoked NE (Fig 3) were elevated in bHRs compared to bLRs, our goal for Experiment 3 was to demonstrate the proof of principle that NE actions through $\alpha 1$ ARs in the NAc contribute to the primary phenotype of bHRs – i.e. their exaggerated behavioral response to novelty. To do this, a selective $\alpha 1$ AR antagonist (terazosin) was perfused bilaterally (through microdialysis probe) into the NAc of a subset of bHRs. We then compared the behavioral and neurochemical responses of this group to untreated bHRs and bLRs after placing animals in a novel environment (Fig 4).

Baseline and post novel environment activity scores were compared across all time points (i.e. –5 to 30 min) among the 3 groups using a RM ANOVA with time and group included as independent variables followed by a Bonferroni post hoc test. This analysis showed a significant effect of time ($F_{7,147}=11.76$, $p<0.0001$), group ($F_{2,21}=10.36$, $p=0.0007$), and a time $\times$ group interaction ($F_{14,147}=3.49$, $p<0.0001$). Post hoc comparisons showed that bHRs had a significantly higher locomotor response at the first time point (5 min after being introduced to novel environment) compared to bLRs or bHRs + terazosin (Fig 4A). The differences in locomotor activity between the two phenotypes did not persist as long as previously reported (e.g. Ref. 1), likely due to the prolonged habituation (24 hrs) in the microdialysis testing environment prior to exposure to the novel testing cage.

Another goal of this study was to examine the neurochemical underpinnings of the interaction between $\alpha 1$ AR blockade and novelty-induced hyperactivity in bHRs. To do this, we monitored dopamine (Fig 4B) and NE (Fig 4C) in the NAc in the 3 treatment groups during exposure to the novel testing apparatus. A RM ANOVA using time and group as independent variables and a Bonferroni post hoc test was used to assess differences among the groups across all time points, i.e. from –5 min to 30 min. The analysis showed that, after exposure to novelty, bHRs had elevated dopamine levels relative to bLRs or bHRs perfused with terazosin (Fig 4B). Specifically, there was a significant effect of group ($F_{2,16}=6.69$, $p=0.0077$), an effect of time ($F_{7,112}=2.49$, $p=0.02$), and a time $\times$ group interaction ($F_{14,112}=1.79$, $p=0.05$). Bonferroni post hoc comparisons of dopamine levels showed that

bHRs differed from bLRs at the 5- and 15-min time points, while they differed from the bHR + terazosin group at 5-25 min (Fig 4B). Similarly, for NE, differences were found between bHRs and the other 2 groups. There was a significant effect of group ($F_{2,15}=5.15$, $p=0.02$), an effect of time ($F_{7,105}=3.13$, $p=0.005$), and a time $\times$ group interaction ($F_{14,105}=1.86$, $p=0.04$; Fig 4C) for NE levels. Bonferonni post hoc analyses showed that bHRs only differed from the bHR + terazosin group at the 10-, 15-, and 25-min time points (Fig 4C). These results show that exposure to a novel environment induces dopamine and NE release in bHRs, and this trend is diminished through bilateral NAC $\alpha 1$ AR blockade.

Terazosin treatment also disrupted the correlative relationship between dopamine and NE in the NAc. Kendall τ analyses demonstrated a significant positive correlation between novelty-evoked accumbal dopamine and NE levels in bHRs ($\tau=0.43$, $p<0.0001$; $R^2=0.191$, $y=0.693 \times + 82.4$; Fig 5A) and bLRs ($\tau=0.48$, $p<0.0001$; $R^2=0.366$, $y=0.708 \times + 33.5$; Fig 5B). However, the significant correlation was lost in the bHR + terazosin group ($\tau = -0.04$, $p=0.7$; $R^2=0.001$, $y=0.034 \times + 77.9$; Fig 5C). Taken together, the $\alpha 1$ AR antagonist terazosin was capable of attenuating the release of dopamine as well as the exaggerated locomotor response to novelty in bHRs, rendering them indistinguishable from bLRs, the more anxiety-prone phenotype. Our current findings demonstrate the effectiveness of $\alpha 1$ AR blockade with terazosin in the NAc to modulate novelty-induced hyperlocomotion in accordance with a previous mouse study (37). Further, bilateral injections of terazosin in the NAc has previously been shown to attenuate cocaine-induced locomotion and cocaine-evoked dopamine overflow in rats (27), suggesting that the locomotor-enhancing effects of cocaine are dependent on dopamine release, which is dependent, at least in part, on NE signaling in the NAc. Furthermore, a recent study showed that systemically-administered terazosin reduced cocaine preference in cocaine-preferring rats (38). Since many of the behavioral effects of cocaine are mediated through mesolimbic dopamine signaling, it is reasonable to speculate that $\alpha 1$ AR blockade in NAc is partially responsible for these effects. While the localization of $\alpha 1$ ARs is of major importance for understanding how this interaction may come about, the current study was, unfortunately, not designed to specifically address this. Nonetheless, others (27) have reported $\alpha 1$ ARs at terminal sites in the NAc, possibly on mesolimbic dopamine neurons. Therefore, the most parsimonious explanation for our results is that $\alpha 1$ ARs on dopamine terminals regulates the amount of both tonic and phasic dopamine that can be released in the NAc. Our pharmacological data with terazosin suggest that NE positively regulates evoked dopamine levels since the antagonist blocked novelty-evoked dopamine release and disrupted the relationship between NE and dopamine. It is possible, therefore, that the elevated NE amplifies dopamine tone, which enhances bHR response to novelty and psychostimulants, as found in the current study. This is in line with previous microdialysis studies which have shown that NE signaling can modulate extracellular dopamine levels (39).

An interesting and novel finding in the current study was that, in addition to dopamine, NE levels also rose in response to a novel environment in bHRs, and this increase in both catecholamines was inhibited by $\alpha 1$ AR blockade. One simplistic explanation for this finding is based on the known metabolism of dopamine to NE via dopamine- β -hydroxylase activity. That is, reduced dopamine may cause reduced extracellular NE. Another possibility, however, is that dopamine and NE are co-released, which is consistent with previous studies

which have demonstrated this phenomenon in cortical neurons (40). In particular, DeVoto and colleagues showed that presynaptic α_2 ARs terminals in the cortex could unidirectionally modulate both NE and dopamine as co-transmitters. Another possibility is that dopamine release in response to novelty has downstream actions on striatal medium spiny neurons (MSNs) expressing dopamine D1 receptors, which is a main contributor to dopamine-mediated movement in the basal ganglia, i.e. the direct pathway (41). In this instance, activation of accumbal D1R-expressing MSNs could have triggered GABA release from local axon collaterals, thereby inhibiting the release of NE from adjacent NE projection neurons. That is, GABA release may act as an intermediate to modulate NE levels. Although these possibilities are all speculative, the fact that both NE and dopamine release both appear sensitive to α_1 AR receptor blockade in bHRs suggests a local functional relationship between these systems in the NAc in this phenotype. Follow up studies using specific pharmacological tools and receptor localization techniques will be required to examine this relationship in more detail.

Behaviorally, bHRs and bLRs differ in anxiety-like behavior and stress responsivity (see 3 for review), and NE and dopamine are known regulators of the stress response (42–44). In fact, NE in the NAc has also been shown to be elevated in response to stressful stimuli (45). Together with the current findings, it is therefore conceivable that NE in the NAc plays an important role in the behavioral phenotypes of bHRs and bLRs, but more studies would be required to prove this relationship. Specifically, the current study (Experiment 3) did not include a group in which bLRs were treated with terazosin, nor additional controls such as delivering NE enhancing compounds into NAc of bHRs to further confirm the relationship between NE and dopamine in either phenotype. In addition, future studies should investigate the role of β -adrenergic receptors to the bHR/bLR phenotype since these receptors were previously shown to be important in differentiating bHR/bLR behaviors (3) and have also been found in the striatum and shown to modulate dopamine release (46).

In summary, our findings provide further evidence for differences in the dopamine system in the NAc of bHRs and bLRs, and demonstrate, for the first time, a role for NE signaling via α_1 ARs in the NAc in the distinct behavioral phenotypes of these bred lines. Importantly, we demonstrate “true” baseline differences in neurochemical signaling in the NAc, with bHRs showing elevated dopamine and NE. Furthermore, administration of an α_1 AR antagonist, terazosin, rendered bHRs more bLR-like, attenuating the locomotor response to novelty and diminishing the relationship between dopamine and NE. Taken together, these findings highlight a role for α_1 ARs in the behavioral phenotype of bHR rats, with data to suggest that antagonizing these receptors depresses dopamine activation. Thus, these data further support a role for dopamine and NE in mediating individual differences in response to novelty. Future studies will investigate additional aspects of co-regulation of dopamine and NE and other adrenergic receptor systems of this unique genetic animal model as potential targets for the treatment of addiction and other impulse control disorders.

Materials and Methods

Reagents

Cocaine HCl was from Malinckrodt Pharmaceuticals (St. Louis, MO). Neurochemicals for stock solutions, terazosin HCl and all LC-MS reagents including water, acetonitrile, ammonium formate, benzoyl chloride, $^{13}\text{C}_6$ benzoyl chloride, formic acid and sulfuric acid were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise noted. Chemical isotopes including $^{13}\text{C}_6$ -dopamine and deuterated NE (d_6 -NE) were from C/D/N Isotopes (Porte Claire, Quebec, Canada).

Subjects

Male bHR (n=28) and bLR rats (n=22) (Sprague–Dawley) from generations 35 and 37 were obtained from the selective-breeding colony in the Akil lab (6). Rats weighed between 350 and 450 g for all experiments. Rats were pair-housed in a temperature and humidity controlled room with 12 hr light/dark cycles with food and water available *ad libitum*. Adequate measures were taken to prevent the rats from experiencing pain and discomfort. All rats were treated as approved by the University of Michigan Unit for Laboratory Animal Medicine (ULAM) and in accordance with the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Probe Implantation

Rats were anesthetized using an isoflurane vaporizer. For bilateral NAc studies (Experiment 1 and 3), 1 mm microdialysis probes were implanted according to the following coordinates AP: +1.7, ML: ± 1.5 , DV: -7.1 which targeted the area bordering the shell and core of the nucleus accumbens (47). In Experiment 2 (cocaine treatment), rats were unilaterally implanted in this same region. Probes were secured to the skull by acrylic dental cement and metallic screws. Upon completion of the experiments, animals were euthanized and brains were extracted and frozen at -80°C until further processing for histological analysis to verify probe placements (Fig S2).

In vivo Microdialysis

Following unilateral or bilateral NAc implantation, rats were allowed to recover for 24 hr in a Rattun plexiglass bowl (BASi, West Lafayette, IN) with free access to food and water. All experiments were conducted in awake, behaving rats. On the day of the experiments, probes were flushed with artificial cerebrospinal fluid (aCSF; composition in mM: CaCl_2 1.2; KCl 2.7, NaCl 148 and MgCl_2 0.85) at a flow rate of 2 $\mu\text{l}/\text{min}$ for 2 hr using a Chemyx Fusion 400 syringe pump (Chemyx, Stafford, TX). Perfusion flow rate was then reduced to 1 $\mu\text{l}/\text{min}$ for 1 hr prior to beginning fraction collection (i.e. 3 hr total habituation time). All samples were collected at 1 $\mu\text{l}/\text{min}$ at 5-min intervals (5 μl samples). For baseline concentration experiments (Experiment 1), raw dialysate concentrations ($\pm\text{SEM}$) are reported. For experiments that combined behavioral analyses and microdialysis sampling (Experiments 2 and 3), all concentrations were normalized to baseline values (i.e. the average of baseline is set to 100%) to correct for variability due to individual differences and probe recoveries. For

experiments that used bilateral probe implants (Experiments 1 and 3), analyte concentrations between the 2 hemispheres were averaged.

Neurochemical LC-MS analysis

Samples from all experiments were analyzed using benzylation and the LC-MS method previously described by the Kennedy lab (19, 48). Briefly, the 5 μ l dialysate samples were derivatized by adding 2.5 μ l of 100 mM sodium carbonate monohydrate, 2.5 μ l 2% benzoyl chloride in acetonitrile, and then 2.5 μ l of a stable ^{13}C benzoylated isotope internal standard mixture (made up in a 50:50 water:acetonitrile solution containing 1% sulfuric acid) was added to improve quantitation. A Thermo Fisher Accela UHPLC (Waltham, MA) system automatically injected 5 μ l of the sample onto a Waters (Milford, MA) HSS T3 reverse phase HPLC column (1 mm $\times$ 100 mm, 1.8 μ m). Mobile phase A consisted of 10 mM ammonium formate and 0.15% formic acid. Mobile phase B was pure acetonitrile. Analytes were detected by a Thermo Fisher TSQ Quantum Ultra triple quadrupole mass spectrometer operating in positive multiple reaction monitoring (MRM) mode.

For Experiment 1 and 2 a panel of 17 neurochemicals was measured including dopamine, NE, serotonin (5HT), histamine, glutamate, GABA, acetylcholine (ACh), adenosine, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3MT), homovanillic acid (HVA), 5-hydroxyindole acetic acid (5-HIAA), normetanephrine (NM), taurine, serine, aspartate and glycine. Also included in Experiment 1 is a stable isotope labeling (SIL) technique for assessment of extracellular levels of dopamine and NE (20) (see below). For Experiment 3 we focused on dopamine and NE levels during behavioral monitoring.

Stable Isotope Labeled Quantitation

Inclusion of 5 nM $^{13}\text{C}_6$ -dopamine and d_6 -NE in the aCSF perfusate (Experiment 1) allowed us to calculate an extraction fraction (E_d), i.e. the ratio of the isotope that exits the probe

compared to the amount retained in the dialysate sample. We used $E_d = 1 - \frac{{}^{13}\text{C}_6\text{dopamine}_{out}^{SIL}}{{}^{13}\text{C}_6\text{dopamine}_{in}^{SIL}}$

for dopamine and $E_d = 1 - \frac{d_6\text{NE}_{out}^{SIL}}{d_6\text{NE}_{in}^{SIL}}$ for NE. Absolute extracellular concentrations (i.e. C_{app})

in the striatum were determined by dividing dialysate concentrations of dopamine and NE by their respective E_d values.

Behavioral Testing

For Experiments 2 and 3, Logitech (Apples, Switzerland) webcams were placed 30 cm above the Rattun chambers as previously described (49). The camera was connected via a USB port to a PC running Matlab 2009 (Mathworks, Natick, MA). Cameras captured video which was then digitally rendered to compute detected movement events which correspond to general locomotor activity such as running around the cage and rearing. Smaller movements such as whisking are not detected as motion. The program collected data points every 60 seconds and we then binned the data into 5-min intervals to correspond with neurochemical measures.

Experimental details

Experiment 1: Baseline neurochemical differences between awake bHRs and bLRs

Microdialysis probes were implanted bilaterally into NAc of bHRs (n=10) and bLRs (n=7). ACSF perfusate contained isotopically labeled dopamine ($^{13}\text{C}_6$ -dopamine; 5 nM) and NE (d_6 -NE; 5 nM) for 1 hr prior to baseline sampling (i.e. 3 hr total equilibration period). Baseline samples were collected every 5 min for 30 min (6 fractions). The mean ($\pm$ SEM) of each neurochemical was taken across all 6 fractions *prior to SIL correction* and are reported in Table 1. After E_d was calculated, these values were applied to dopamine and NE dialysate concentrations to calculate C_{app} , i.e. absolute extracellular concentrations in both phenotypes (Fig 1). Differences between phenotypes were assessed using an unpaired t-test for all 17 analytes using the Bioconductor “qvalue” package (<http://www.bioconductor.org>) to correct for false discovery rate (Q). For the SIL experiment a simple unpaired t-test was performed in Prism (Graphpad, La Jolla, CA) for both dopamine and NE (Fig 1). Differences between phenotypes were considered significant when the p-value was <0.05 and the q-value <0.10 , or a less than 10% chance of false positives to occur.

Experiment 2: Behavioral and neurochemical effects of cocaine in bHR and bLR rats

bHRs (n=6) and bLRs (n=8) were implanted unilaterally in the NAc and then allowed to recover for 24 hr. After connecting microdialysis probes and a 3 hr habituation period, locomotor activity recording started and baseline microdialysis fractions were collected every 5 min for 1 hr. After these baseline collections, cocaine (15 mg/kg i.p.) was administered and behavioral recording and microdialysis sample collection continued simultaneously for an additional 1 hr.

To examine whether any behavioral differences could be detected between phenotypes (bHR vs. bLR) across the full time course of the experiment, a RM two-way ANOVA was performed with time as the repeated variable and phenotype as the independent variable using Prism 6 (GraphPad, La Jolla, CA) or Statview (SAS, Cary, NC) software. When significant main effects or interactions were detected, Bonferroni post hoc analyses were conducted to determine differences at specific time points (Fig 2).

Three bLRs had microdialysis probe occlusions during the study, therefore neurochemistry was collected from a total of n=5 bLRs, and n=6 bHRs. Neurochemical concentrations were normalized to baseline (i.e. 100%) and are expressed in heatmap format (Fig 3) and traditional trace format (Fig S1). A two-way RM ANOVA was used across all time points (–60 – 60 min) with phenotype (bHR vs. bLR) and time considered independent variables. All statistical results are reported in Table 2.

Experiment 3: Noradrenergic modulation of novelty-induced behavior and neurochemical changes

Three groups were included in this experiment: bLRs (n=7), bHRs (n=5) and bHRs + terazosin (n=7). Animals were habituated to their microdialysis chambers for 3 hrs before including terazosin (10 μM) in the microdialysis perfusate of a subgroup of bHRs (for 30 min prior to start of behavioral analysis). Terazosin perfusion continued until the end of the behavioral assessment. Baseline activity scores were collected for 10 min and binned into two 5-min intervals prior to novel environment exposure. To

introduce animals to a novel environment while undergoing microdialysis (i.e. at time 0) rats were gently lifted by the tail and a new Rattun bowl with different bedding was placed underneath them. We then compared motor activity across all time points (i.e. –10 to 30 min) among the 3 groups. Locomotor activity was binned into 5-min intervals and averaged to time lock behavior and neurochemical changes (Fig. 4). A RM ANOVA using group (bLR, bHR and bHR + terazosin) and time as independent variables was applied to compare locomotor responses between groups across all time points (i.e. –5 to 30 min) (Fig. 4A).

During behavioral monitoring animals were undergoing bilateral microdialysis to deliver terazosin and to track changes in accumbal dopamine and NE levels. Microdialysis samples were continually collected every 5 min for 10 min during baseline (2 fractions) and then for an additional 30 min following introduction to the novel environment (6 fractions). A RM ANOVA was applied to compare each neurochemical response between groups, after introduction to a novel environment, using group and time as independent variables and the percent of baseline levels as the dependent variable (Fig 4B–C). To further examine the relationship between dopamine and NE, correlational analyses was conducted, for which, each of the 6 time points was treated as a distinct data point for each animal (Fig 5). Data were loaded into Python data structures where implementations of Kendall's tau (τ) in the SciPy and NumPy libraries were used to inspect correlations. Given the use of arbitrary units and the relatively modest sample size, Kendall's τ was used as a more robust test coefficient that would indicate dependence if two variables were linearly correlated, but also if there was a monotonic relationship between the parameters. Furthermore, Kendall's τ is advantageous because it doesn't rely on any assumptions regarding the distribution of the data. For visualization, a linear regression is shown using the % baseline dopamine on the Y-axis and % baseline NE on the X-axis for bHRs (Fig 5A), bLRs (Fig 5B) and bHRs + terazosin (Fig 5C).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Absolute extracellular baseline concentrations of dopamine and norepinephrine in the nucleus accumbens were calculated using a stable isotope labeling (SIL) technique. This method was used to determine the extraction fraction (E_d) which is used to estimate microdialysis probe recovery. Concentrations (C_{app}) were calculated as the mean dialysate concentration (6 fractions, 30 min total) divided by E_d for dopamine or norepinephrine. Data are expressed as mean + SEM nM concentration and show that both dopamine and norepinephrine are significantly higher in bHRs compared to bLRs after SIL correction (** $p < 0.01$).

Fig. 2. Time course of behavioral activation in bHRs (blue squares) and bLRs (red circles) following cocaine (15 mg/kg i.p.) treatment. Locomotor data were collected every minute and then pooled into 5-min fractions and are expressed as mean locomotor activity counts $\pm$ SEM (shaded gray and red areas surrounding data points) (A). Black arrow indicates time point when cocaine was administered. Significant group differences at a given time point indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3.

Heat maps showing the percent baseline responses of measured neurochemicals in response to cocaine treatment relative to baseline in bHRs (A) and bLRs (B). Black arrow indicates time point when cocaine (15 mg/kg i.p.) was administered. Large positive changes (increases) are shown in red while negative changes (decreases) are shown in blue (range -100% to 600% as indicated in heatmap scale). Corresponding traditional microdialysis traces are shown in Fig S1 and statistical analyses are found in Table 2.

Fig. 4.

Behavioral and neurochemical responses to a novel environment in bHRs (blue square), bLRs (red circle) and bHRs bilaterally perfused with 10 μ M terazosin (green triangle) in the nucleus accumbens. Locomotor data were collected every minute and then pooled into 5-min fractions and are expressed as mean locomotor activity counts $\pm$ SEM (shaded gray, red and green areas surrounding data points) (A). bLRs and bHRs perfused with terazosin showed depressed novelty-induced locomotor activity when compared to bHRs not treated with terazosin (A). Dopamine (B) and NE (C) are reported as % baseline $\pm$ SEM (shaded gray, red and green areas surrounding data points). Levels were normalized to 100% using the 2 baseline fractions (10 min) prior to exposure to novel environment (black arrow). Exposure to a novel testing apparatus enhanced NAc dopamine release in bHRs, and to a lesser extent in bLRs and bHRs treated with terazosin (B). Exposure to novelty also affected NE release in bHRs, and this effect was diminished by terazosin treatment (C). Significant

group differences at a given time point indicated as: # $p < 0.05$, ### $p < 0.001$ for bHR vs bLR; and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for bHR vs bHR + terazosin.

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Fig. 5.

Linear regression correlation analysis between dopamine and NE signaling in the nucleus accumbens in (A) bHRs, (B) bLRs, and (C) bHRs treated with terazosin (10 μ M). Data are represented as %baseline concentration at each 5-min time point after exposure to a novel environment (i.e. Experiment #3). Correlation between dopamine and NE signaling is disrupted when adrenergic receptors are blocked by terazosin.

Table 1

Basal dialysate concentrations of 17 neurochemicals measured in awake behaving bHRs and bLRs in the nucleus accumbens. Concentrations were calculated as the mean of baseline fractions (6 fractions, 30 min total) and are expressed as mean $\pm$ SEM nM concentration. Only dopamine showed a significant (sig.) basal difference between phenotypes in this assay (* $p < 0.05$). Since assay was multiplexed, a false discovery rate (0.1) was applied to correct for the multiple comparisons (q).

Analyte in NAc	bHR (nM)	bLR (nM)	t ratio	p value	sig.	q value
Dopamine	1.42 $\pm$ 0.16	0.87 $\pm$ 0.14	2.45	0.027	*	0.096
Norepinephrine	0.47 $\pm$ 0.07	0.40 $\pm$ 0.07	0.64	0.531	–	0.156
Serotonin	0.06 $\pm$ 0.01	0.08 $\pm$ 0.01	2.05	0.074	–	0.096
DOPAC	339 $\pm$ 50	258 $\pm$ 63	1.01	0.325	–	0.132
HVA	254 $\pm$ 29	347 $\pm$ 116	0.91	0.373	–	0.132
3-MT	0.34 $\pm$ 0.06	0.31 $\pm$ 0.05	0.35	0.728	–	0.177
Normetanephrine	0.06 $\pm$ 0.01	0.10 $\pm$ 0.04	1.41	0.176	–	0.126
5-HIAA	985 $\pm$ 113	1589 $\pm$ 381	1.76	0.099	–	0.096
Histamine	2.25 $\pm$ 0.45	3.84 $\pm$ 0.77	1.88	0.079	–	0.096
ACh	3.41 $\pm$ 0.59	4.82 $\pm$ 1.10	1.21	0.242	–	0.126
Glutamate	101 $\pm$ 18	149 $\pm$ 35	1.32	0.206	–	0.126
GABA	15.81 $\pm$ 2.69	13.6 $\pm$ 2.5	0.59	0.564	–	0.156
Adenosine	72.4 $\pm$ 21.5	58.7 $\pm$ 17.3	0.41	0.687	–	0.177
Taurine	475 $\pm$ 71	759 $\pm$ 235	1.24	0.234	–	0.126
Glycine	992 $\pm$ 310	616 $\pm$ 154	0.95	0.356	–	0.132
Aspartate	128 $\pm$ 76	67.3 $\pm$ 21.9	0.70	0.502	–	0.156
Serine	7377 $\pm$ 2329	7639 $\pm$ 3298	0.06	0.948	–	0.216

Table 2

Comparison of cocaine (15 mg/kg i.p.) induced changes in striatal neurochemistry between bHRs and bLRs across all time points (–60 to 60 min) using a two-way RM ANOVA. Only dopamine, norepinephrine, 3MT and glycine showed time × cocaine treatment interactions. Specific time points where bHRs and bLRs differed were shown by a Bonferroni post hoc test and are shown in the right most column.

Neurochemical in NAc	F _{Time}	F _{Phenotype}	F _{Time × Phenotype}	P _{Time}	P _{Phenotype}	P _{Time × Phenotype}	sig.	Post hoc (min)
Dopamine	18.28	11.64	5.05	<0.0001	0.01	<0.0001	***	15-30, 45
Norepinephrine	19.93	2.46	1.82	<0.0001	0.15	0.015	**	25-30
Serotonin	19.89	0.01	0.64	<0.0001	0.96	0.90	–	–
DOPAC	2.83	0.69	0.51	<0.0001	0.43	0.96	–	–
HVA	0.94	0.22	0.59	0.55	0.65	0.93	–	–
3-MT	23.42	3.73	2.35	<0.0001	0.08	0.0007	**	15-20, 30
Normetanephrine	9.91	1.07	1.54	<0.0001	0.33	0.06	–	–
5-HIAA	1.50	0.10	0.44	0.07	0.75	0.99	–	–
Histamine	7.40	0.99	1.19	<0.0001	0.34	0.26	–	–
ACh	1.50	1.46	1.18	0.07	0.25	0.27	–	–
Glutamate	3.37	0.58	1.32	<0.0001	0.47	0.16	–	–
GABA	2.26	0.33	1.05	0.0013	0.58	0.40	–	–
Adenosine	1.33	1.59	0.77	0.15	0.24	0.77	–	–
Taurine	3.04	0.96	0.82	<0.0001	0.35	0.71	–	–
Glycine	2.25	2.09	1.86	0.0013	0.18	0.012	*	10-15
Aspartate	1.95	0.02	1.21	0.0072	0.88	0.24	–	–
Serine	1.46	2.07	1.58	0.085	0.18	0.051	–	–

Significance (sig.) indicated by

* p<0.05,

** p<0.01,

*** p<0.001.