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The search for the neurobiological basis of vulnerability to drug abuse: using microarrays to investigate the role of stress and individual differences

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Abstract

Basic neurobiological studies have led to great progress in our understanding of the mechanisms of action of drugs of abuse. Much has been learned about the brain response from the moment a psychoactive drug enters the organism onwards, including the psychological, neurobiological and peripheral effects of repeated drug administration, withdrawal and re-exposure. However, to relate this knowledge to the human experience requires further research on *the antecedents of drug-taking behavior and the factors that predispose particular individuals to drug seeking and drug abuse*. Thus, it is important to address several issues at the fundamental level: (1) Why are some individuals more vulnerable to drugs of abuse more than others? Is there a broader dimension or dimensions of emotional reactivity that contribute to this difference in vulnerability? (2) What is the effect of psychosocial stress on drug-seeking and drug-taking behavior, and are the effects distinct across individuals? (3) Since both drug-taking behavior and stress have sustained and pervasive effects on the brain, can we use microarrays to discern the "neural signature" or "neural phenotype" associated with these processes, and can we distinguish this signature across individuals with differing propensities to taking drugs?

In the present paper, we summarize some of our early attempts at addressing these questions. We rely on animal studies aimed at characterizing the emotional and stress reactivity of rats with different propensities to self-administer drugs (high responders and low responders); we briefly describe the effect of a psychosocial stressor on these animals; we then detail a study using microarray technology aimed at investigating the "neural phenotype" associated with social defeat stress in the high vs. low responder animals. This "discovery" approach is used as a starting place for identifying novel mechanisms that might alter the vulnerability of different individuals to drug-seeking behavior. The power and limits of this approach, and its future directions, are discussed within this general framework.

Keywords: Drug abuse; Vulnerability; DNA chip; Hippocampus; High responders; Low responders; Social defeat; Novelty

1. Introduction

Drug-taking behavior can be characterized as a complex genetic disorder that results from the interplay of genetic predisposition and environmental factors, with the drug itself representing one of these non-genetic factors. An analogy can be drawn between the drug and a pathogen (virus, bacterium) that triggers other diseases. Indeed, the interaction host–pathogen determines who will become ill and who will not, and modifies the outcome of the disease process. Similarly, in substance abuse, it is important to uncover the fundamental mechanisms of action of the drug itself on a "typical" organism. But is it also important to understand the properties of the "host" and to decipher the interplay between the characteristics of individuals and the impact of a particular drug upon them. This is all the more important as the initial drug-taking behavior

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appears to be "voluntary" on the part of some individuals. It is therefore of paramount importance to understand the antecedents of the initial drug-seeking behavior, as well as the unique impact of the drug on those vulnerable individuals.

An additional complexity to be considered is that while some individuals have a drug of choice, many others become addicted to a number of drugs, sometimes belonging to many different pharmacological classes (psychostimulants, opioids, benzodiazepines among others). These two patterns suggest rather different views of drug vulnerability: one is a specific neurochemical dysfunction that has behavioral consequences and that leads an individual to seek a drug of choice to repair that imbalance (Goldstein, 2001). An example would be a "hypo-endorphenia" leading to opiate addiction. The other view would suggest a broader propensity to use drugs either because of the reward and novelty that they provide, or as a means of coping with emotional and environmental stress, or both. This latter view would lead to the search for personality variables (thrill-seeking, neurosis and anxiety), and their genetic and neural correlates, as the "endophenotype" mediating the likelihood to seek drugs. This latter view is supported by a number of studies showing that thrill-seeking can predispose to drug-taking behavior in some individuals (Cloninger et al., 1995, 1998) and that there is a great deal of comorbidity between substance abuse and mood disorders (Franken and Hendriks, 2001; Haver and Dahlgren, 1995; Kell, 1995; Milby et al., 1996). Whether the thrill-seeking and the anxiety-coping factors represent distinct paths to drug abuse, or whether they converge at least in some individuals, remains to be determined.

Based on the above perspective, we considered various strategies for investigating the neurobiology of individual differences in emotionality and the impact of these differences on responsiveness to stress and drug-seeking behavior. We exploited a model first described in the pioneering work of Piazza et al. (1989) as a framework for understanding the neurobiological antecedents of differences in drug self-administration during the acquisition phase. Since social stress is a primary activator of the stress response in humans and since subjects suffering from mood disorders are particularly prone to it (de Wit et al., 2003; Young et al., 2000), we chose to study the differential impact of a social stress on animals with different propensities to self-administer drugs.

The behavioral paradigms to be detailed below were correlated with changes in the expression of specific candidate genes that have been associated with stress, substance abuse or both. However, in addition, we have begun to use microarray technology as a means of discovery of novel mechanisms that might illuminate the interface between stress and substance abuse and reveal why distinct classes of individuals differ in both.

2. Individual differences in novelty seeking and drug-taking behavior: a basic dimension of emotional reactivity and its neural correlates

Piazza and co-workers were the first to demonstrate a relationship between behavior in a novel environment and propensity to self-administer drugs. When outbred rats are exposed to the mild stress of a novel environment, some rats defined as high responders (HR) exhibit high rates of exploratory locomotion, while others defined as low responders (LR) exhibit low rates of locomotor activity (Piazza et al., 1989). Importantly, these two groups of rats differ in their drug-seeking behavior, as HR rats learn to self-administer psychostimulants faster than LR rats (Piazza et al., 1989, 2000; Marinelli and White, 2000; Kabbaj et al., 2001).

2.1. The HR–LR phenotype reflects a basic difference in emotional reactivity to the environment

Over the last several years, our laboratory undertook the examination of the neural and behavioral correlates of the HR–LR dimension. We demonstrated that the locomotor response in a novel environment predicts anxiety-related behavior of these animals in a number of tests. When compared to LR rats, HR rats exhibit low basal levels of anxiety in the light–dark box and elevated plus maze (Dellu et al., 1996b; Kabbaj et al., 2000). In addition, they show significant differences in climbing and floating behavior in the initial phases of the forced swim test, with LR rats showing less climbing and more floating (Calvo et al., in preparation). Finally, HR and LR mothers exhibit significantly different maternal behavior towards their offspring in the second week of life. Indeed, when compared to LR mothers, HR mothers spend less time with their pups and perform less ano-genital licking (Vazquez et al., in preparation). Repeated testing shows that this trait is fairly stable, and ongoing selective breeding of this suggests that this trait is likely to be a heritable phenotype (Stead et al., in preparation). Together, this body of work shows the HR–LR distinction in novelty seeking behavior based on the locomotion test to have significant power to predict differences in a range of other behaviors involving interaction with the environment, including the acquisition of drugs of abuse.

2.2. Basal and exploration-induced differences in gene expression in HR vs. LR animals

The behavioral differences described above are accompanied by basal differences in gene expression of

key molecules in the emotional circuitry of the HR vs. LR rats. A critical component of the differences in anxiety- and drug self-administration related behaviors between HR and LR rats is revealed by our finding that hippocampal expression of glucocorticoid receptors (GR) was significantly higher in the LR rats. This differential levels of hippocampal GR in HR and LR rats may be responsible for their differential corticosterone secretion in response to an acute stressor (Dellu et al., 1996a; Kabbaj et al., 2000). This increase in GR expression is responsible also, at least in part, for the increased anxiety-like behavior exhibited by LR animals. Thus, the administration into the hippocampus of a GR antagonist, RU38486, dose dependently decreases the anxiety behavior of the LR rats and renders them as exploratory as their HR counterparts (Kabbaj et al., 2000). In light of the role of GR in drug addiction, the basal GR difference between HR and LR rats may be also relevant to their differential drug-taking behavior. Indeed, in mice, selective inactivation of the GR gene in the brain flattened the dose–response function for cocaine intravenous self-administration and sensitization (Deroche-Gamonet et al., 2003). Furthermore, in rats, administration of a GR antagonist dose-dependently reduced the motivation to self-administer cocaine. By contrast, overexpression of GR in the mouse forebrain leads to enhanced sensitization to cocaine (Wei et al., in press).

HR and LR rats also differ in terms of expression of the hippocampal serotonergic receptor, 5HT1a (Calvo et al., in preparation). This finding is relevant because these receptors are dysregulated in depressive humans (Arango et al., 1990; Schechter et al., 1990), and are directly or indirectly, targets of various psychotropic drugs (Schechter et al., 1990). Numerous other genes show basal altered expression between the two groups, including CRH in the amygdala (higher in LR, consistent with higher anxiety) and some of the adrenergic receptor subtypes in the bed nucleus of the stria terminalis. Additionally, we have used the properties of the immediate early gene *c-fos* to characterize the neuronal activation of HR and LR rats in response to an anxiogenic stressor. When compared to LR, HR rats showed low expression of *c-fos* mRNA in the CA1 area of the hippocampus, but high *c-fos* mRNA levels in the olfactory area, the orbital cortex, the cingulate cortex, the dorsal striatum and the paraventricular nucleus of the hypothalamus (Kabbaj and Akil, 2001). Given that *c-fos* is a transacting factor, these findings suggested that the short- and long-term consequences of the exposure to the anxiogenic stressor may also be quantitatively and anatomically different in these two groups of animals. Thus, these *c-fos* results demonstrated how experience may further exaggerate individual differences in gene expression and may cause further divergence in emotional reactivity between the two groups.

2.3. The effect of social stress on the HR–LR phenotype: two paths to substance abuse?

Interestingly, the behavioral profiles of HR and LR rats tend to be equalized after psychosocial stress. For example, one week of social isolation tend to inhibit HR's novelty seeking behavior, rendering them more like LR rats (Kabbaj et al., 2001). We have interpreted this and other observations on the effect of stress on the HR–LR phenotype to suggest that differences in novelty-seeking behavior are best observed when animals are not stressed or threatened or need to deploy fundamental coping strategies that are critical for survival. Rather, individual differences in novelty seeking behavior can allow a given species to exhibit a range of risk-taking behavior that allows it to adapt to a broader range of environmental conditions, some involving greater threat and others requiring greater exploration.

Among the most interesting interactions between the HR–LR dimension and stress is the effect of social stress on the acquisition of drugs. We have shown that chronic social defeat equalizes individual differences in cocaine self-administration such that a week after the termination of a chronic social defeat stressor, HR and LR are no longer distinguishable with both exhibiting high levels of drug-taking behavior. This happens because social defeat slightly delays self-administration in HR rats without increasing their maximal response, and produces a dramatic enhancement in self-administration in LR rats (Kabbaj et al., 2001). These results suggest that some animals (HR) might seek drugs because of their high propensity for novelty-seeking, while others (LR) might seek drugs as a reaction to social stress. While the direct relevance to human drug-seeking behavior remains to be established, the identification of these "two paths to substance abuse" in this paradigm can serve as a useful animal model for investigating at the neural and molecular level the mechanisms that lead to vulnerability to addictive behavior. The search for genes whose expression is differentially altered by social defeat in HR vs. LR rats (described below) is a first step in identifying mechanisms that distinguish these two paths to drug addiction.

3. A microarray studies of neural reactivity to social stress in HR vs. LR rats

In this study, our aim was to identify novel hippocampal genes that may contribute to individual differences in emotional responses in HR vs. LR rats either basally or following social defeat. The hippocampus was chosen because HR and LR differ basally in hippocampal expression of GR (Kabbaj et al., 2000), hippocampal expression of serotoninergic receptors

5HT1a (Calvo et al., in preparation) and interestingly exhibit differential *c-fos* expression in the hippocampus in response to an anxiogenic stressor (Kabbaj and Akil, 2001). Given that *c-fos* is a transacting factor, we hypothesized that other genes in the hippocampus in HR and LR rats would be differentially regulated in response to stressors, like social defeat. Our *c-fos* study also revealed differential activation in HR and LR rats in the olfactory area, the orbital cortex, cingulate cortex, striatum and paraventricular nucleus of the hypothalamus (Kabbaj and Akil, 2001). These brain areas represent candidate areas that will be investigated in future microarray studies.

Potentially relevant genes were ascertained using Affymetrix microarray technology which allows the simultaneous examination of approximately 8600 transcripts. Each subject was studied independently (i.e. without pooling). To minimize false positives, the experiment was fully replicated and the experiments analyzed together to identify those genes that showed reliable differences in gene expression across the two experiments. We have also used a stringent statistical criterion that limits the likelihood of false positives, the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995). This test is more lenient than the standard Bonferroni correction, which is not appropriate for microarray studies because of the large number of observations (probes on the array). An FDR correction set at a p-value threshold of 0.05 will adjust the stringency so that only 5% of the "significant" findings are likely to be false positives. Using this approach, we have identified a set of genes that may be partly responsible for determining individual differences in emotional responses, and the differential effect of psychosocial stress on HR vs. LR animals

4. Material and methods

4.1. Subjects

Seventy-two male Sprague–Dawley rats from Charles River (Wilmington, MA), weighing 250–300 g were used. Those animals were housed 3/cage ($43 \times 21.5 \times 25.5$ cm plexiglass cages). In addition, 24 vasectomized male Long–Evans rats weighing 500–550 g were housed with female Long–Evans rats. These males were used as resident attackers, and were chosen from an original group of 30 rats for their consistent aggressive behavior. From the 72 Sprague–Dawley rats, 10 rats (in the intermediate group IR) were used to test aggressive behavior of Long–Evans rats. This test consisted of a single 5 min encounter. All animals were housed on a 12:12 light–dark cycle (light on at 7 p.m.), and food and water were available ad libitum.

All experiments were conducted in accordance with the guidelines of the animal ethics committee at the University of Michigan following the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

4.2. Experimental design

This study was completed using two cohorts of 36 animals that were run at a three month interval. Each cohort contained the same four experimental groups: (1) HR non-defeated ($n = 6$), (2) LR non-defeated ($n = 6$), (3) HR defeated ($n = 6$) and (4) LR defeated ($n = 6$).

Sprague–Dawley rats were acclimated to the animal colony for 1 week and then screened for their locomotor response in a novel environment, by placing them in a $43 \times 21.5 \times 24.5$ cm (high) clear acrylic activity monitor. Locomotor activity was monitored by means of two banks of photocells connected to a microprocessor. This 60 min test was conducted during the light portion of the light–dark cycle (7–10 p.m.). After assessing that the locomotor activity has a normal distribution, the rats were identified as HR (the 12 rats that scored locomotor counts in the highest third of the sample) or LR (the 12 rats that scored locomotor counts in the lowest third of the sample). Some of the other 12 rats who scored in the intermediate counts (Intermediate responders or IR) were either used to test for Long–Evans aggressive behavior or were not used in the experiment.

Seven days after the locomotion test, equal numbers of HR and LR animals were assigned to one of two groups: socially defeated or controls. Rats were sacrificed by decapitation 2 h 30 min following the fourth session of either social defeat or exposure to novel cages. Their brains were immediately removed, and hippocampi rapidly dissected and frozen. Tissues were stored at $-80\ ^{\circ}$C until total RNA extraction.

4.3. Social defeat

The social stress paradigm consisted of four encounters with an aggressive Long–Evans male rat. Each Long–Evans male rat was vasectomized and housed with a normal cycling female rat to enhance territorial behavior and aggressiveness. Prior to each agonistic interaction, the resident's mate was transferred to another cage. A Sprague–Dawley male rat was then placed as an intruder into the resident male's cage and the rats were allowed 5 min to interact. After this initial 5 min, the rats had another extra 10 min to interact and if the fighting became too intense, the intruder rat was transferred to a protective metal cage and placed back into the resident's home cage, which allowed for intense visual, auditory and olfactory inter-

actions (Miczek, 1991). This cage was of sufficient size to allow animals to move freely ($10 \times 10 \times 15$ cm). Intruder males exhibited the following behaviors during an agonistic interaction: immobility (four paws on ground, orienting towards resident), escape (fleeing the resident), crouching (four paws on ground, not orienting towards resident), defensive upright (standing erect with forepaws extended), walking, rearing and grooming. The agonistic interactions occurred under red light conditions, during the dark phase of the light–dark cycle (9–11 a.m.). The control animals were simply exposed to novel cages for 15 min on four consecutive days during the dark phase of light–dark cycle (9–11 a.m.).

4.4. Gene Chip studies

In total, 48 Affymetrix RG-U34A Gene Chips were hybridized with cRNA derived from individual hippocampi from Sprague–Dawley rats so that each Gene Chip represented the hippocampus of one rat.

Total RNA was extracted separately from individual hippocampi using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) per manufacturers' instructions followed by a cleanup step using RNeasy RNA purification columns (Qiagen, Valencia, CA, USA). Using 10 μg total RNA as determined by absorbance at 260 nm, first and second strand synthesis was performed per instructions in the Affymetrix Expression Analysis Technical Manual, ver. 3 (Affymetrix, Santa Clara, CA, USA). cRNA was synthesized using the Bioarray High Yield RNA Transcript Labeling Kit (Enzo, New York, NY, USA) and prepared for hybridization with RG-U34A Gene Chips per Affymetrix instructions. Gene Chips were hybridized for 18 h at 42 °C, washed and stained on an Affymetrix fluidics station using the standard EukGEws2v3 protocol and scanned with a Gene Array Scanner.

4.5. Data analysis of array results

CEL files from the Affymetrix Gene Chip arrays were normalized by RMA (Irizarry et al.,) and imported into Partek Pro 5.1 for statistical analysis. A multivariate ANOVA, using categorical factors for experiment cohort (1 and 2), phenotype (HR and LR) and treatment (defeat and control) was used to generate p-values for phenotype, treatment and interaction differences. An FDR correction was applied to post hoc pairwise group comparisons to determine threshold *p*-values at the 5% false positive level. In addition, only transcripts that received a "present" call using MAS 5.0 algorithms on at least 50% of the chip were considered. Transcripts meeting the above criteria were then entered into a gene ontology (GO) analysis using the "function mapper" at http://brainarray.mhri.med.umich.edu/brainarray/. Briefly, this tool calculates *p*-values for the representation of GO terms using a hypergeometric distribution after inputting a gene list and the array type (RGU34A).

4.6. Real-time PCR

For real-time polymerase chain reaction (RT-PCR) studies 2 μg of total RNA from hippocampus of each rat was pooled within a group to generate four pools: HR non-defeated, HR defeated, LR non-defeated and LR defeated. RNA was DNase treated then cleaned up with RNeasy columns. cDNA was synthesized using random hexamers with the Novagen cDNA synthesis kit and quantified by a pico-green assay. Equal amounts of cDNA were input into real-time PCR reactions which were monitored using the BioRad iCycler machine using SyberGreen incorporation as a tracking dye. The primer sequences for Ca+/calmodulin kinase II (Cam-KII) were: ccgatccacaccactatcct (5′ primer) and gccgtcgatgtactgtgtga (3′ primer). Real-time PCR C_t values were normalized to four control genes (GAPDH, mt-NDI, snRNPB and Actg). Primer sequences for control genes are as follows: GAPDH, tcaaggctgagaatgggaag (5′ primer), tactcagcaccagcatcacc (3′ primer); mt-NDI, aaatcgagctcccttcgact (5′ primer), tgaagaatagggcgaatggt (3′ primer); snRNPB, cccctcctccaggaattaga (5′ primer), agggaaagagaagggctgag (3′ primer); Actg, cgagccttaggacccagttt (5′ primer), tacaggtgtcgatgcaaacg (3′ primer). Fold-changes for the appropriate groups being compared (HR defeat, HR non-defeat, LR defeat, LR non-defeat) were calculated separately by normalization to each of the four control genes and a one-way *t*-test was performed to determine if the fold-change significantly differed from 1.0.

5. Results

All Sprague–Dawley rats were defeated at least three times. A rat was considered not defeated if it was not attacked and did not show any behavioral reactivity (supine, escape, and upright) to the aggressor during the 15 min of encounter. In a previous experiment, no differences in the behavioral response to attack were apparent between the HR and LR groups (Kabbaj et al., 2001).

Tables 1 and 2 list genes found significantly differentially expressed between groups as described below. Since each gene can be associated with multiple GO terms, the table is organized into general functional categories and two GO terms that were significantly enriched are indicated in separate columns. A significant enrichment of a GO term within a gene list suggests that a specific functional class of molecules might contribute to the differences being studied. However, in

Table 1
Overview of the genes that were differentially expressed between the HR and LR phenotypes. The direction of the difference is given as HR > LR for genes with higher expression in HR relative to LR and LR > HR for genes with higher expression in LR relative to HR

Functional class	GenBank ID	Difference	Gene symbol	Enriched GO terms	
				Binding	Development/ neurogenesis
Extracellular signaling	D49653	LR > HR	Lep	Yes	
	AF055065	LR > HR	Ptpns1	Yes	
	S69161	LR > HR	Trhr		
	AB006914	HR > LR	Trip10		
	AF022952	LR > HR	Vegfb	Yes	
Receptor activity	AB016160	LR > HR	Gabbr1		
	X15468	LR > HR	Gabrb3		
	M36419	LR > HR	Gria2		
	M55291	HR > LR	Ntrk2	Yes	Yes
	U57500	LR > HR	Ptpra		
	U87305	LR > HR	Unc5h1		Yes
Synaptic function	M64780	HR > LR	Agrn		
	AA924772	HR > LR	Mt3	Yes	
	AF091834	LR > HR	Nsf		
	L10362	LR > HR	Sv2b		
	X52772	LR > HR	Syt1		
Intracellular signaling	L12382	LR > HR	Arf3	Yes	
	M16112	HR > LR	Camk2b	Yes	
	AI233421	LR > HR	Dgkg	Yes	
	M17526	HR > LR	Gnao		
	S61973	LR > HR	Grina		
	U38812	LR > HR	Itpr1		
	D14591	LR > HR	Map2k1	Yes	
	M64300	LR > HR	Mapk1	Yes	
	S79213	LR > HR	Ppp1r2		
	D38261	LR > HR	Ppp2r2c		
	D14568	LR > HR	Ppp3r1	Yes	
	M15523	LR > HR	Prkce	Yes	
	AA799981	HR > LR	Prkch	Yes	
	D01046	LR > HR	Rab11b	Yes	
	AF025506	HR > LR	Rabac1		
	AF041107	LR > HR	Tulip1		
Transcription	AI170685	LR > HR	Dnaja2		
	Y09507	LR > HR	Hif1a	Yes	
	AB012235	LR > HR	Nfix	Yes	
	U14398	LR > HR	Pdx1		
	U56862	LR > HR	Znf260		
Nucleic acid binding	AJ010386	LR > HR	Cugbp2	Yes	
	D84418	HR > LR	Hmgb2	Yes	
	U20181	LR > HR	Ireb2	Yes	
	AI233173	HR > LR	Nme1	Yes	
Cell cycle	D16309	LR > HR	Ccnd3		
	U36444	LR > HR	Pctk1		
Cell–cell interaction	D38492	LR > HR	Cntn1		
	X77934	LR > HR	LOC64312	Yes	
	M96376	HR > LR	Nrxn2		
	X99338	LR > HR	Sdfr1		
Chaperone activity	L18889	LR > HR	Canx		
	AI228738	HR > LR	Fkbp1a	Yes	
	AI176658	LR > HR	Hspb1		
	AF023621	LR > HR	Sort1		
Biosynthesis/metabolism	L07736	HR > LR	Cpt1a		
	X13527	LR > HR	Fasn	Yes	
	X02231	LR > HR	Gapd		
	L01793	LR > HR	Gyg		
	Y17295	LR > HR	Prdx6		
	AF036761	LR > HR	Scd2		
	U63923	LR > HR	Txnrd1	Yes	

Table 1 (*continued*)

Functional class	GenBank ID	Difference	Gene symbol	Enriched GO terms	
				Binding	Development/ neurogenesis
Protein degradation	M76426	LR > HR	Dpp6		
	M62388	LR > HR	LOC81816		
	AB000491	HR > LR	LOC81827		
	D50694	HR > LR	Psmc2	Yes	
	D50695	HR > LR	Psmc4	Yes	
Ribosome structure	D25224	HR > LR	Lamr1		
	X78327	HR > LR	Rpl13		
	AA891713	HR > LR	Rpl13a		
	X51536	HR > LR	Rps3		
Small molecule transport	M74494	LR > HR	Atp1a1	Yes	
	D38101	LR > HR	Cacna1d	Yes	
	M86621	HR > LR	Cacna2d1		
	AF031642	LR > HR	Slc14a2		
	S75687	LR > HR	Slc1a3		
Structural component	AA891812	HR > LR	Add1	Yes	
	AA892506	HR > LR	Coro1a		
	AA875659	HR > LR	Inexa		
	M75148	HR > LR	Klc1		
	AI227608	HR > LR	Mapt		
	D28110	LR > HR	Mobp		Yes
	U05784	HR > LR	MPL3	Yes	
	AF004811	HR > LR	Msn		

Table 2
Overview of the genes that were altered by social defeat in HR and LR rats. The direction of change is given as the effect of defeat relative to controls. The changes in italics were significant in post-hoc analyses for HR vs. LR or defeated vs. non-defeated, independent of their significance in the interaction test

General function	GenBank ID	Gene symbol	Direction of change induced by defeat		Basal difference
			In HR	In LR	HR vs. LR
Extracellular signaling	D49653	Lep		Up	*LR > HR*
	AF055065	Ptpns1	*Up*		*LR > HR*
	AF022952	Vegfb		*Down*	*LR > HR*
Receptor activity	M36419	Gria2		*Up*	*LR > HR*
Synaptic function	AF089839	Nsf		Down	*LR > HR*
	L10362	Sv2b	*Up*		*LR > HR*
Intracellular signaling	L12382	Arf3	Up	Down	*LR > HR*
	M16112	Camk2b		*Up*	*HR > LR*
	M17526	Gnao	Down	Up	*HR > LR*
	U38812	Itpr1	Up		*LR > HR*
	M64300	Mapk1	*Up*		*LR > HR*
	AA799981	Prkch	Down		*HR > LR*
	D01046	Rab11b	Up	Down	*LR > HR*
	AF041107	Tulip1		*Down*	*LR > HR*
Nucleic acid binding	AI233173	Nme1	Down	Up	*HR > LR*
Cell–cell interaction	D38492	Cntn1		*Down*	*LR > HR*
Chaperone activity	AA893328	Canx		*Down*	*LR > HR*
	AI228738	Fkbp1a		*Up*	*HR > LR*
Biosynthesis/metabolism	Y17295	Prdx6	Up	Up	*LR > HR*
	AF036761	Scd2	Up	Down	*LR > HR*
Protein degradation	M62388	LOC81816		*Down*	*LR > HR*
Ribosome structure	AA800054	Rpl19	*Down*	*Up*	HR > LR
Small molecule transport	AA946532	Abcd3	*Down*		HR > LR
	S75687	Slc1a3	Up		*LR > HR*
Structural component	D14441	Basp1	*Down*		HR > LR
	D28110	Mobp	*Up*		*LR > HR*

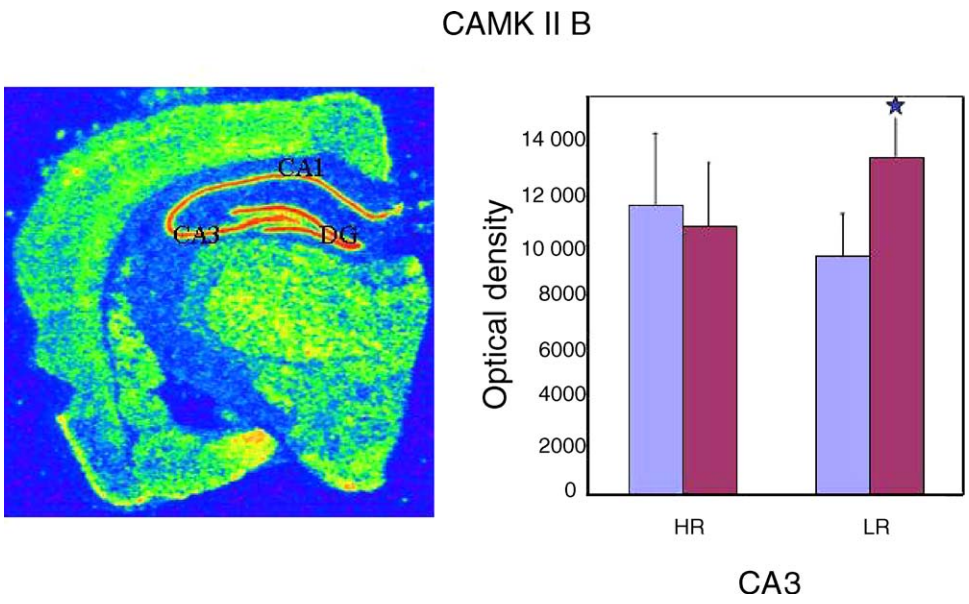

Fig. 1. Color enhanced photomicrograph from X-ray film exposed for 2 days after in situ hybridization with antisense cRNA probe against rat CAM-KIIb mRNA. The bar graph shows the results of the in situ hybridization study. Social defeat significantly increased the mRNA expression of CAM-KIIb in LR rats but not in HR rats in the CA3 area of the HC. Social defeat also increased CAM-KIIb in the CA1 area of the HC in LR rats (not shown). Social defeat had no effect on CAM-KIIb expression in the DG area of the HC (not shown). Results are expressed as mean ± SEM. * $p < 0.05$. The sample size was $n = 6$ rats per experimental group.

many cases, specific molecules might contribute to biological differences without the involvement of a larger functional class, in which case the enrichment of GO terms might not be observed. Also, the assignment of GO terms to genes is still in progress as our understanding of gene function progresses. Thus, the use of this approach in data analysis should be thought of as supplemental to provide biological context to mathematically significant results but should also be viewed as incomplete, especially in rat where gene annotation is far behind that of mouse and human.

Table 1 lists genes that were significantly differentially expressed between HR and LR animals after FDR multiple testing correction with a threshold of $p < 0.05$. The GO term "binding" was enriched with a p-value of 0.009 and the GO terms "development" and "neurogenesis" were enriched with p-values of 0.005 and 0.002, respectively. The latter two terms are grouped in the table since "neurogenesis" is a subterm of "development" and the genes in this table that are associated with "development" are also associated with "neurogenesis". Also shown are the direction of the differential expression between HR and LR animals and the gene symbol and GenBank accession numbers.

Table 2 lists genes that had a significant interaction term between phenotype (HR and LR) and treatment (defeat and no defeat) after an FDR multiple testing correction with a threshold of $p < 0.05$. The table shows the direction of differential expression between HR and LR and the direction of change induced by social defeat in HR and LR separately. The changes shown in italics were also significantly different between HR and LR or by social defeat, independent of their significance in the interaction test. No GO terms were significantly enriched in this gene list.

5.1. Confirmation studies

While the results described in Tables 1 and 2 were derived from a conservative analysis, involving two independent cohorts of animals, it would be optimal to confirm each of the genes using other measures of gene expression, such as RT-PCR and in situ hybridization (ISH). An optimal outcome is to discover a novel candidate gene that is detected by Gene Chip analysis, confirm the differential expression by RT-PCR and further support and enhance these findings using ISH. An example of a gene that has been analyzed in this fashion is CAM-KIIb, which showed an induction by social defeat, but only in the LR animals. Fig. 1 shows the effects of chronic social defeat on the expression of CAM-KIIb in the HC in HR and LR rats.

6. Discussion

In this study, we analyzed individual differences in basal and stress induced hippocampal gene expression using Affymetrix Gene Chips. We discovered sets of genes that may be partly responsible for determining individual differences in emotional reactivity, either under control conditions or following a psychosocial

stressor. The list subsumes a number of gene ensembles, including a number of genes implicated in extracellular cell signaling (e.g. leptin, vascular endothelial factor b), neurotransmitter receptors related genes (e.g. GABA, glutamate receptors, neurotrophic receptors), genes implicated in synaptic function (e.g. agrin, synaptic vesicle glycoproteins), intracellular signaling related genes (e.g. calmodulin kinases, Map kinases). The list also includes some transcription factors (e.g. hypoxia inducible factor, nuclear factor X), cell cycle regulator genes (e.g. PCTAIRE-1 protein kinase) as well as other genes implicated in biosynthesis/metabolism, ribosomal and structural functions.

It is important to emphasize that Affymetrix technology is not highly sensitive and is unlikely to reveal subtle regulation of low-abundance genes, which represent a large percentage of the hippocampal transcriptome (Evans et al., 2002). This lack of sensitivity will leave a significant number of regulated transcripts undetected and these would represent false negatives. Two examples of such false negatives are GR and 5HT1a. These two genes were represented with different set of probes that did not perform adequately across all chips (mismatch probes show greater signal than the perfect match probes) and thus contributed to the false negative results.

Furthermore, because of the large number of observations in microarray experiments, an unavoidable number of false positives will also be present in any single data set. We have attempted to minimize false positives by independent replication. Additional methods including RT-PCR followed by in situ hybridization for selected genes can provide further validation in neuroanatomically more precise manner. Below, we discuss some of the highlights of the observations we have made in contrasting the different groups.

6.1. HR and LR rats exhibit differential basal expression of several gene classes

As we have shown, HR and LR rats exhibit differential expression of several genes across multiple functional classes. Interestingly, the term ''neurogenesis'' was significantly enriched within these genes suggesting a potential basal difference in neuronal turnover within the HC between HR and LR rats. Furthermore a few genes involved in cell cycle progression (Ccnd3 and Pctk1) were also in this list. With the exception of the BDNF receptor, Ntrk2, all of the genes in this class were increased in LR relative to HR. These findings suggest that compared to LR rats, HR rats may have a slower rate of cell proliferation, differentiation and transformation. This hypothesis is reinforced by the findings of Lemaire et al. (1999) who showed that cell proliferation in LR rats was twice that observed in HR rats especially within the granule cell layer of the dentate gyrus.

The identified ''neurogenesis'' genes that are differentially regulated in the hippocampus of HR and LR rats may well play a role in drug addiction. Indeed, there are many similarities between the molecular and cellular substrates for addiction and memory (for review see Nestler, 2002). BDNF for example, promotes LTP in the hippocampus as well as learning and memory in several behavioral measures (Patterson et al., 1996, 2001). Similarly, BDNF promotes the locomotor activating and rewarding actions of drugs of abuse by acting on the mesolimbic dopaminergic circuitry (Horger et al., 1999). At first blush, it may not be clear how molecular changes in neurotrophic factors within the hippocampus alter the activity of the mesolimbic dopaminergic circuitry. Note, however, that glutamatergic afferents from the hippocampus to the nucleus accumbens exert a potent excitatory effect on VTA DA neurons, influencing both DA neuron population activity and the regulation of the firing properties of these neurons (Floresco et al., 2001). Thus, it is reasonable to hypothesize that HR and LR differences in neurotrophic factors in the hippocampus play a role in regulating glutamatergic inputs to the accumbens and may be implicated in the differential dopamine release observed in that area in the same animals (Rouge-Pont et al., 1993).

Since the hippocampus is highly implicated in contextual learning and memory (Sharp, 1999), the differential basal levels of the ''neurogenesis'' genes in HR and LR could be also implicated in how these animals differentially respond and adapt to contextual cues associated with drugs of abuse (Lu et al., 2004; Pierce and Bari, 2001).

6.2. Social defeat leads to differential alterations of gene expression in HR vs. LR rats

We have shown that several changes in genes responsive to psychosocial stress of social defeat can depend on the background in which the stress is imposed. In fact most of the genes listed in Table 2 show opposite regulation of gene expression, with regard to direction of change, in HR and LR animals. Furthermore, in general, genes that are up-regulated by defeat in LR animals are basally lower relative to HR animals and genes that are down-regulated by defeat in LR animals are basally higher relative to HR animals. The same pattern is true for genes regulated by defeat in HR animals. This is consistent with previous reports demonstrating that social stressors act to ''equalize'' the HR and LR phenotype (Kabbaj et al., 2000, 2001).

CAM-KIIb as an example of a novel candidate gene differentially affected by social defeat in HR and LR rats.

CAM-KIIb was one the genes differentially regulated by social defeat in HR and LR rats. This was seen in the arrays and confirmed by the RT-PCR analysis. In situ hybridization further confirmed that the expression of this gene was increased in LR, but not HR, after social defeat (Fig. 1). CAMKinase II is one of the kinases that are highly expressed in the brain especially in the hippocampus where it represents 2% of total proteins (Fig. 1 demonstrates its abundance). Numerous studies have reported that Hippocampal CAM-KII plays a major role in the induction of long-term potentiation (LTP). Indeed, this molecule is considered a leading candidate in the search for the molecular basis of memory (for reviews see Lisman et al., 2002 Fukunaga and Miyamoto, 2000). These findings have led us to hypothesize that learning and memory functions may be differentially modulated by social stress in HR vs. LR rats. We are currently examining this hypothesis.

7. Future directions

The studies above show that investigating differences in emotionality can be a fruitful avenue of research aimed at understanding differences in vulnerability to substance abuse. In addition, these basal differences in emotionality can interact with social stress, resulting in multiples paths that lead to drug-taking behavior, and might therefore be responsive to different types of intervention.

Using a combination of hypothesis driven and discovery strategies, we have begun to uncover neural and molecular correlates of these individual differences in emotional reactivity. The candidate approach suggests a key role of stress-related genes, as well as a number of monamine-related genes. The genomic approach points to a broad number of differences in signaling and neurogenesis between HR and LR animals. Remarkably, a subclass of genes shows both basal differences between the groups, as well as a differential reactivity to social stress across the groups. This suggests that they may be important in the expression and maintenance of differences in emotionality between HR and LR. However, they need to be tested in behavioral, pharmacological and genetic paradigms to ascertain whether or not they play a role in the differential vulnerability to substance abuse.

We have shown above an example with CAM-KII, and how it led us to studies of the differential impact of social stress on learning and memory in different animals. Another example might be leptin, which was found to be more highly expressed in HR vs. LR animals, and to be further induced by social defeat in LR animals only. Does leptin play a role in either the initial differences in emotionality or the differential responses of the two groups to social stress? Though leptin had been first discovered in the context of food intake and obesity, more recent work has shown the system to be broadly expressed in the brain and suggested its importance in learning and memory, neural plasticity, and drug addiction. For instance, leptin has been proposed to be a ‘‘cognitive enhancer’’ because it selectively increases NMDA receptor function (for review, see Harvey, 2003). Leptin also attenuated acute food deprivation-induced heroin seeking behavior (Shalev et al., 2001). Thus, its higher basal levels and greater responsiveness to social stress is in line with the view that LR rats may indeed show greater neural plasticity than HR rats and may weather the effects of social stress quite differently? Would leptin affect the impact of stress on either drug seeking or drug relapse? Would these effects be distinct in different groups of animals? Leptin represents another example of a novel candidate that arises from the genomic discovery approach which may be implicated in individual differences in drug addiction and emotional responsiveness.

The above view puts modulation of specific genes (CAM-KBII, leptin) in a new context of relevance to substance abuse. This coupled with the fact that the novelty seeking phenotype in rodents holds some parallels to human thrill-seeking behavior leads opens new avenues for investigating the interplay between the thrill-seeking trait, the impact of social stress and the modulation of neural plasticity in humans. Each of these elements may alter not only the vulnerability to seeking drugs, but the neural response to the drug once taken—a great example of pathogen–host interaction.

Since many of the observed differences in gene expression between HR and LR may be secondary to the differential impact of experience on them, it would be of great interest to identify early differences in gene expression, be they genetic or epigenetic that may be the precursors to the distinct profiles observed in the adult. We have therefore undertaken a selective breeding study of HR vs. LR rats, and a developmental study using microarray analysis across the life trajectory. These studies should allow us to ascertain early differences in gene expression that may be the initial predisposing factors to differences in emotionality, and that can lead to differences in response to experience, which in turn can lead to other differences in gene expression, amplifying the uniqueness of the neural phenotypes The combination of such genomic developmental analysis with genetic linkage or QTL analysis (Danzinger et al., in press), may prove particularly powerful in identifying novel candidate genes that lead to differences in emotionality, stress reactivity and consequent vulnerability to substance abuse

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References

Arango, V., Ernsberger, P., Marzuk, P.M., Chen, J.S., Tierney, H., Stanley, M., Reis, D.J., Mann, J.J., 1990. Autoradiographic demonstration of increased serotonin 5-HT2 and beta-adrenergic receptor binding sites in the brain of suicide victims. Arch. Gen. Psychiatry 47, 1038–1047.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57, 289–300.

Cloninger, C.R., Sigvardsson, S., Przybeck, T.R., Svrakic, D.M., 1995. Personality antecedents of alcoholism in a national area probability sample. Eur. Arch. Psychiatry Clin. Neurosci. 245, 239–244.

Cloninger, C.R., Sigvardsson, S., Bohman, M., 1988. Childhood personality predicts alcohol abuse in young adults. Alcohol Clin. Exp. Res. 12, 494–505.

Danzinger, R.S., You, M., Akil, H. Discovering the genetics of complex disorders through integration of genomic mapping and transcriptional profiling. Nature Rev., in press.

de Wit, H., Soderpalm, A.H., Nikolayev, L., Young, E., 2003. Effects of acute social stress on alcohol consumption in healthy subjects. Alcohol Clin. Exp. Res. 27, 1270–1277.

Dellu, F., Mayo, W., Vallee, M., Maccari, S., Piazza, P.V., Le Moal, M., Simon, H., 1996a. Behavioral reactivity to novelty during youth as a predictive factor of stress-induced corticosterone secretion in the elderly—a life-span study in rats. Psychoneuroendocrinology 21, 441–453.

Dellu, F., Piazza, P.V., Mayo, W., Le Moal, M., Simon, H., 1996b. Novelty-seeking in rats—biobehavioral characteristics and possible relationship with the sensation-seeking trait in man. Neuropsychobiology 34, 136–145.

Deroche-Gamonet, V., Sillaber, I., Aouizerate, B., Izawa, R., Jaber, M., Ghozland, S., Kellendonk, C., Le Moal, M., Spanagel, R., Schutz, G., Tronche, F., Piazza, P.V., 2003. The glucocorticoid receptor as a potential target to reduce cocaine abuse. J. Neurosci. 23, 4785–4790.

Evans, S.J., Datson, N.A., Kabbaj, M., Thompson, R.C., Vreugdenhil, E., De Kloet, E.R., Watson, S.J., Akil, H., 2002. Evaluation of Affymetrix Gene Chip sensitivity in rat hippocampal tissue using SAGE analysis. Serial analysis of gene expression. Eur. J. Neurosci. 16, 409–413.

Floresco, S.B., Todd, C.L., Grace, A.A., 2001. Glutamatergic afferents from the hippocampus to the nucleus accumbens regulate activity of ventral tegmental area dopamine neurons. J. Neurosci. 21, 4915–4922.

Franken, I.H., Hendriks, V.M., 2001. Screening and diagnosis of anxiety and mood disorders in substance abuse patients. Am. J. Addict. 10, 30–39.

Fukunaga, K., Miyamoto, E., 2000. A working model of CaM kinase II activity in hippocampal long-term potentiation and memory. Neurosci. Res. 38, 3–17.

Goldstein, A., 2001. Addiction: From Biology to Drug Policy, second ed. . Oxford University Press.

Harvey, J., 2003. Leptin: a multifaceted hormone in the central nervous system. Mol. Neurobiol. 28, 245–258.

Haver, B., Dahlgren, L., 1995. Early treatment of women with alcohol addiction (EWA): a comprehensive evaluation and outcome study. I. Patterns of psychiatric comorbidity at intake. Addiction 90, 101–109.

Horger, B.A., Iyasere, C.A., Berhow, M.T., Messer, C.J., Nestler, E.J., Taylor, J.R., 1999. Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. J. Neurosci. 19, 4110–4122.

Irizarry, R., Hobbs, B., Collin, F., Beazer-Barclay, Y., Antonellis, K., Scherf, U., Speed, T., 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4 (2), 249–264.

Kabbaj, M., Akil, H., 2001. Individual differences in novelty-seeking behavior in rats: a c-fos study. Neuroscience 106, 535–545.

Kabbaj, M., Devine, D.P., Savage, V.R., Akil, H., 2000. Neurobiological correlates of individual differences in novelty-seeking behavior in the rat: differential expression of stress-related molecules. J. Neurosci. 20, 6983–6988.

Kabbaj, M., Norton, C.S., Kollack-Walker, S., Watson, S.J., Robinson, T.E., Akil, H., 2001. Social defeat alters the acquisition of cocaine self-administration in rats: role of individual differences in cocaine-taking behavior. Psychopharmacology (Berlin) 158, 382–387.

Kell, M.J., 1995. Opiate dependence, comorbidity and seasonality of birth. J. Addict. Dis. 14, 19–34.

Lemaire, V., Aurousseau, C., Le Moal, M., Abrous, D.N., 1999. Behavioural trait of reactivity to novelty is related to hippocampal neurogenesis. Eur. J. Neurosci. 11, 4006–4014.

Lisman, J., Schulman, H., Cline, H., 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. 3, 175–190.

Lu, L., Dempsey, J., Liu, S.Y., Bossert, J.M., Shaham, Y., 2004. A single infusion of brain-derived neurotrophic factor into the ventral tegmental area induces long-lasting potentiation of cocaine seeking after withdrawal. J. Neurosci. 24, 1604–1611.

Marinelli, M., White, F.J., 2000. Enhanced vulnerability to cocaine self-administration is associated with elevated impulse activity of midbrain dopamine neurons. J. Neurosci. 20, 8876–8885.

Miczek, K.A., 1991. Tolerance to the analgesic, but not discriminative stimulus effects of morphine after brief social defeat in rats. Psychopharmacology (Berlin) 104, 181–186.

Milby, J.B., Sims, M.K., Khuder, S., Schumacher, J.E., Huggins, N., McLellan, A.T., Woody, G., Haas, N., 1996. Psychiatric comorbidity: prevalence in methadone maintenance treatment. Am. J. Drug Alcohol Abuse 22, 95–107.

Nestler, E.J., 2002. Common molecular and cellular substrates of addiction and memory. Neurobiol. Learn. Mem. 78, 637–647.

Patterson, S.L., Abel, T., Deuel, T.A., Martin, K.C., Rose, J.C., Kandel, E.R., 1996. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16, 1137–1145.

Patterson, S.L., Pittenger, C., Morozov, A., Martin, K.C., Scanlin, H., Drake, C., Kandel, E.R., 2001. Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron 32, 123–140.

Piazza, P.V., Deminiere, J.M., Le Moal, M., Simon, H., 1989. Factors that predict individual vulnerability to amphetamine self-administration. Science 245, 1511–1513.

Piazza, P.V., Deroche-Gamonent, V., Rouge-Pont, F., Le Moal, M., 2000. Vertical shifts in self-administration dose–response functions predict a drug-vulnerable phenotype predisposed to addiction. J. Neurosci. 20, 4226–4232.

Pierce, R.C., Bari, A.A., 2001. The role of neurotrophic factors in psychostimulant-induced behavioral and neuronal plasticity. Rev. Neurosci. 12, 95–110.

Rouge-Pont, F., Piazza, P.V., Kharouby, M., Le Moal, M., Simon, H., 1993. Higher and longer stress-induced increase in dopamine concentrations in the nucleus accumbens of animals

predisposed to amphetamine self- administration. A microdialysis study. Brain Res. 602, 169–174.

Schechter, L.E., Bolanos, F.J., Gozlan, H., Lanfumey, L., Haj-Dahmane, S., Laporte, A.M., Fattaccini, C.M., Hamon, M., 1990. Alterations of central serotoninergic and dopaminergic neurotransmission in rats chronically treated with ipsapirone: biochemical and electrophysiological studies. J. Pharmacol. Exp. Ther. 255, 1335–1347.

Shalev, U., Yap, J., Shaham, Y., 2001. Leptin attenuates acute food deprivation-induced relapse to heroin seeking. J. Neurosci. 21, RC129.

Sharp, P.E., 1999. Comparison of the timing of hippocampal and subicular spatial signals: implications for path integration. Hippocampus 9, 158–172.

Wei, Q., Lu, X.-Y., Liu, L., Schafer, G., Shieh, K.-R., Burke, S., Robinson, T., Watson, S., Seasholtz, A., Akil, H. Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. Proc. Natl. Acad. Sci. USA, in press.

Young, E.A., Lopez, J.F., Murphy-Weinberg, V., Watson, S.J., Akil, H., 2000. Hormonal evidence for altered responsiveness to social stress in major depression. Neuropsychopharmacology 23, 411–418.