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Gene Expression in Accumbens GABA Neurons from Inbred Rats with Different Drug-Taking Behavior

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Abstract

Inbred Lewis and Fisher 344 rat strains differ greatly in drug self-administration; Lewis rats operantly self-administer drugs of abuse including nicotine, whereas Fisher self-administer poorly. As shown herein, operant food self-administration is similar. Based on their pivotal role in drug reward, we hypothesized that differences in basal gene expression in GABAergic neurons projecting from nucleus accumbens (NAcc) to ventral pallidum (VP) play a role in vulnerability to drug taking behavior. The transcriptomes of NAcc shell-VP GABAergic neurons from these two strains were analyzed in adolescents, using a multidisciplinary approach that combined stereotaxic iontophoretic brain microinjections, laser-capture microdissection (LCM) and microarray measurement of transcripts. LCM enriched the gene transcripts detected in GABA neurons compared to the residual NAcc tissue: a ratio of neuron/residual > 1 and false discovery rate (FDR) <5% yielded 6,623 transcripts, whereas a ratio of >3 yielded 3,514. Strain-dependent differences in gene expression within GABA neurons were identified; 322 vs. 60 transcripts showed 1.5-fold vs. 2-fold differences in expression (FDR<5%). Classification by gene ontology showed these 322 transcripts were widely distributed, without categorical enrichment. This is most consistent with a global change in GABA neuron function. Literature-mining by Chilibot found 38 genes related to synaptic plasticity, signaling and gene transcription, all of which determine drug-abuse; 33 genes have no known association with addiction or nicotine. In Lewis rats, upregulation of *Mint-1*, *Cask*, *CamkIIδ*, *Ncam1*, *Vsnl1*, *Hpcal1* and *Car8* indicates these transcripts likely contribute to altered signaling and synaptic function in NAcc GABA projection neurons to VP.

Keywords

GABA; nicotine; nucleus accumbens; addiction; ventral pallidum; synapse; transcriptome; Lewis rats; Fisher 344 rats; laser capture microdissection

Introduction

In rats, the inbred Lewis and Fisher 344 strains differ greatly in drug self-administration behavior; while Lewis rats operantly self-administer several drugs of abuse, including nicotine, Fisher rats self-administer poorly (Brower *et al.*, 2002, Martin *et al.*, 1999, Suzuki *et al.*, 1988a, Suzuki *et al.*, 1988b). The nucleus accumbens shell (NAcc shell), located in ventral striatum, and its primary output target, the ventral pallidum (VP), are both critical to

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the integration and discrimination of information encoding the rewarding dimension of motivated behavior (Carlezon & Thomas, 2009, Heimer & Wilson, 1975, Shirayama & Chaki, 2006, Smith *et al.*, 2009). The vast majority of neurons in the NAcc are GABAergic medium spiny projection neurons (Meredith *et al.*, 1993). The normal function of VP is essential for aspects of reward learning and to motivational “wanting” and hedonic “liking” (Smith *et al.*, 2009). Based on their pivotal role in reward (Carlezon & Thomas, 2009, Meredith *et al.*, 1993), we hypothesized that differences in basal gene expression by these GABAergic neurons in the Lewis versus Fisher 344 strains may contribute to drug-taking vulnerability, which for cigarette smoking is usually manifest during adolescence (Chassin *et al.*, 1996, Dappen *et al.*, 1996, S.A.M.H.S.A., 2001).

Differences in the gene expression profiles or transcriptomes between the NAcc shell-VP GABAergic neurons from these two strains were analyzed in adolescents [postnatal day 41 (PN41)]. We used a multidisciplinary approach, combining stereotaxic iontophoretic brain microinjections, laser capture microdissection (LCM) and microarray detection of transcript expression levels. Neuroanatomical specificity was obtained by iontophoretic delivery of fluorogold (FG) into VP (Pieribone & Aston-Jones, 1988) followed by capture of all FG⁺ neurons in sections from the NAcc shell by laser capture microdissection (LCM). The efficacy of this approach was evaluated by measuring the ratio of gene expression for neurons compared to the remaining NAcc tissue after removal of the neurons. RNA extracted from both neurons and remaining NAcc was amplified and then analyzed by microarray. Differential gene expression by rat strain was validated for a subset of genes by real-time PCR.

Methods

Animals and surgeries

Lewis rats were bred in the laboratory, using breeders purchased from Harlan Laboratories (Indianapolis, IN). Adolescent male Lewis rats (PN35) were anesthetized with ketamine/xylazine (90 and 10 mg/kg, respectively, i.m.) and then placed in a stereotaxic frame. Fluorogold (2% in sterile saline, Fluorochrome, Englewood, CO) was deposited into ventral pallidum (AP: -0.22, ML: $\pm$ 2.2, DV: -8.0) bilaterally by iontophoretic injection, using a Midgard high voltage current source (Stoelting Co., Wood Dale, IL, USA). The current was set at +5 μ A (7s on, 7s off, for 5 min). Micropipettes were left in place for 15 min before the scalp incision was sutured. Five days later, rats were killed, brains removed, immediately frozen, and stored at -70°C. All procedures were conducted in accordance with the NIH Guidelines Concerning the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Tennessee.

Chronic nicotine self-administration (SA)

Rats were acclimated to a reversed 12:12 h light:dark cycle. Standard rat chow and water was provided *ad libitum*. Nicotine (pH = 7.2; calculated as free base) was prepared weekly in 200 U heparinized saline to deliver 50 μ l i.v. of 0.03 mg/kg b.wt. per injection. SA was performed according to our published protocol (Valentine *et al.*, 1997). Briefly, PN38 adolescent rats received jugular cannulae under xylazine-ketamine anesthesia and were individually housed in operant chambers (Coulbourn Instruments, Whitehall, PA) inside a sound-attenuating chamber. Operant chambers were equipped with 2 levers positioned 4 cm above the floor and a green cue light 1 cm above each lever was illuminated when nicotine was available. Lever presses were recorded and syringe pumps controlled by computers and interfaces, using L2T2 or Graphic State software (Coulbourn Instruments). Rats were given 3 d to recover from jugular surgery, during which time they received antibiotic daily and

hourly aliquots (50 μ l) of 200 U heparin. At PN41, rats received 0.03 mg/kg nicotine in heparinized saline iv with each lever press.

One lever was randomly designated as the active lever. Pressing this lever elicited a computer-driven i.v. injection of nicotine over 0.81 s on a fix ratio 1 (FR1) schedule. Each injection was followed by a 7 s period during which the green cue light was extinguished and lever presses were recorded, but nicotine was not injected. Pressing the inactive lever had no programmed consequence. Rats were given access to nicotine or saline SA 23 h/d and learned to self-administer nicotine without prior training, priming or food deprivation. The final hour of the lights-on cycle was reserved for housekeeping tasks. During this interval, environmental enclosure doors were opened and green cue lights turned off; levers were not retracted, and lever press activity was not recorded nor rewarded during this period. Self-administration d 1 (SA d 1) is the 1st day nicotine was made available and the rats were given 10 d unlimited free access to acquire nicotine SA.

Food self-administration

Beginning on PN41, a separate cohort of adolescent male rats were trained on an FR1 schedule to self-administer food pellets (45 mg) during daily 2 h sessions in the same operant chambers used for nicotine SA. Animals were food deprived overnight prior to the first session. Thereafter, they received 16 g food per d.

Laser capture microdissection

Brains were sectioned in a Leica cryostat at 10 μ m, and sections were mounted onto uncharged glass slides and maintained at -20°C until dehydration. Slides were dehydrated by sequential immersion in the following: 100% methanol (3 min), 95% EtOH (2 min), 100% EtOH (1 min, twice) and xylene (5 min, twice), and then air dried (15 min). The Arcturus XT (Life Technologies, Carlsbad, CA) was used to capture Fluorogold-labeled neurons. The infrared laser produced spots approximately 15 μ m in diameter, which allowed consistent capture of neurons onto CapSure LCM caps (Life Technologies). Approximately 750 neurons were captured from each brain.

RNA extraction

RNA trapped in the CapSure LCM caps was extracted using the PicoPure RNA isolation kit (Life Tech.); RNA was eluted with 13 μ l nuclease-free H_2O . RNA quality was analyzed using Bioanalyzer (Agilent Technologies, Foster City, CA). The mean value of RNA Integrity Number (RIN) was 7.9 for the 1.84 ± 2.4 ng of total RNA obtained from each rat.

RNA amplification and Affymetrix microarray

Total RNA were amplified by Nugen Ovation Pico WTA system, followed by cDNA synthesis using the WT Ovation Exon module. Ovation Biotin kits were then used to fragment and label the cDNA (Ovation reagents: NuGen Inc., San Carlos, CA). The labeled cDNA was then hybridized to the Rat Gene ST 1.0 array (Affymetrix, Santa Clara, CA), according to the manufacturer's protocols for Whole Transcriptome arrays. Microarrays were then processed on a Fluidics Station 450 and scanned by the Affymetrix GeneChip 3000 7G Scanner. Expression Console software (ver 1.1.2) was used to normalize data. All microarray samples were processed by the same technician consecutively, interleaving the two strains. No batch effect was observed. The Rat Gene ST 1.0 array probe sets are generated from the Brown Norway rat genome. Therefore, false positive differences between Lewis and Fisher rat samples may occur due to the assymetrical expression of single nucleotide polymorphisms (SNPs) by these 2 strains, differentially affecting cDNA hybridization to array probe sets.

Real-time PCR

Real-time quantitative PCR of 8 genes (and two reference genes *Ywhaz* and *Sdha*) was performed on the LightCycler 480 (Basel, Switzerland) using probes from the Universal Probe Library. The original unamplified RNA from each sample was used to prepare cDNA. The critical threshold (C_t) of genes of interest was normalized to the geometric mean of the two reference genes.

Data analysis

Microarray data were analyzed using the R-statistical package (Team, 2010). T-tests were used to compare the expression level of each gene from the two strains of rats and then all genes were analyzed for false discovery rate (FDR). Gene ontology analysis was conducted using DAVID Bioinformatics Resources (Huang *et al.* 2009). Nicotine and food self-administration data were analyzed using repeated measures ANOVA, with both lever and day treated as within subject variables. Strain was treated as a between subject variable.

Results

The capacity to acquire operant nicotine vs. food self-administration was evaluated in adolescent Lewis compared to Fisher rats. For nicotine SA, rats were housed in operant chambers and were not shaped, primed or food deprived. Three days after jugular vein surgery, nicotine was made available for 23 h per day. Figure 1 (data analyzed by repeated measures ANOVA) indicates that Lewis rats rapidly acquired nicotine self-administration, manifest by a significant increase in active lever presses in the absence of any change in the inactive lever [effect of lever on number of presses, $F(1,7)$: 18.2, $p<0.001$; effect of day on active lever presses, $F(9,63)$: 2.1, $p<0.05$; effect of day on inactive lever presses, $F(9,63)$: 0.8, $p>0.05$]. In contrast, both active and inactive lever presses by Fisher rats failed to increase over time [$F(9,81)$: 1.5, $p>0.05$]. The number of lever presses was different between strains [$F(1,16)$: 111.8, $p<0.001$] and the interaction between lever and strain was significant [$F(1,15)$: 17.5, $p<0.001$]. Thus, the number of active lever presses for nicotine was different between strains [$F(1,16)$: 66.6, $p<0.001$], as was the number of inactive lever presses [$F(1,16)$: 53.5, $p<0.001$]. The higher number of inactive lever presses in Lewis rats is likely the result of stronger locomotor activation induced by a larger amount of nicotine intake. Both strains showed a specific increase in active lever presses reinforced by food [Fisher, $F(1,7)$: 47.1, $p<0.001$; Lewis, $F(1,5)$: 35.1, $p<0.001$]. Neither active lever presses for food [$F(1,12)$: 0.7, $p=0.41$] nor inactive lever presses [$F(1,12)$: 0.7, $p=0.41$] differed by strain. The similar amount of inactive lever presses reflected similar level of arousal induced by food. Hence, both strains were capable of learning the operant task, but nicotine was only reinforcing in Lewis rats, under these conditions.

Neurons projecting from NAcc shell to VP were identified by retrograde uptake of fluorogold (FG) delivered by iontophoresis. Figure 2 illustrates coronal sections of rat brain, identifying both NAcc shell and VP. Within VP, the central area of FG fluorescence for each of six rats is marked by an asterisk. A representative deposit of FG is shown in the inset. Within NAcc shell, the center of the distribution of retrograde-labeled FG^+ neurons for each rat is shown by a green dot within the boxed area. The corresponding inset shows a representative fluorescence image of these FG^+ neurons, located in ventral medial NAcc shell (20X). In this region of NAcc shell, virtually all neurons are FG^+ . All individual FG^+ neurons were captured by LCM. Thus, this approach specifically captured a subpopulation of NAcc shell neurons that project from NAcc shell to VP, excluding neurons that project from NAcc shell to other brain regions.

Microarray analysis was used to detect the level of specific gene transcripts expressed by the transcriptome from laser-captured FG⁺ neurons compared to the tissue remaining in the area of NAcc shell after removal of these fluorescent neurons. A heat map of all genes was generated; figure 3A shows representative areas that demonstrate clustering of genes expressed at significantly greater or lesser levels in neurons vs. tissue remainder. At a ratio of neuron/remainder > 1 and false discovery rate (FDR)<5%, 6,623 gene transcripts were identified in both Lewis and Fisher rats; at ratios > 2.0 and > 3.0, 5,483 and 3,514 genes, respectively, were enriched in the neuron populations from both inbred rat strains (Fig. 3b). Amongst the enriched transcripts, Table 1 shows those related to the neurotransmitter, GABA (e.g., GABA A receptor alpha 1, glutamate decarboxylase 1 and 2), and/or associated with regulation of GABA neuron-related functions (e.g., dopamine receptors D1A and D2; GABA vesicular transporter). These data indicate that laser capture microdissection of individual FG⁺ neurons successfully enriched transcripts from GABA neurons projecting to VP. At three incremental levels of enrichment (i.e., neuron/remainder >1 to >3), the majority of these transcripts were common to both strains of rat. Nonetheless, LCM unavoidably introduces glial cells, in intimate contact with FG⁺ neurons, within each sample captured. For example, myelin basic protein and glial fibrillary acidic protein, standard markers for oligodendrocytes and astrocytes, respectively, were detected at neuron/remainder ratios of 2.7 and 1.5. This enrichment of glial markers indicates that targeting most of the neuronal soma for capture, by using 15 μ m diameter spot size, eliminates the ability to separate the neuronal vs. glial transcriptomes. This limitation of geometric capture by LCM of a high heterogeneous tissue was recently reported (Okaty *et al.*, 2011).

Genes that were differentially expressed in Lewis vs. Fisher NAcc shell GABA neurons projecting to VP were identified. One caveat about array analysis across strains concerns the potential for false positives due to asymmetrical expression of SNPs by the 2 strains, differentially affecting hybridization to the array probes. Table 2 shows the total number of gene transcripts as a function of the expression ratio in Lewis compared to Fisher rats (i.e., >1.5-fold and >2-fold); total transcript number declined with the application of increasingly stringent criteria to achieve statistical significance. A total of 322 genes were differentially expressed at a Lewis vs. Fisher ratio of >1.5-fold ($p<0.05$ and $FDR<5\%$; see supplemental file). Of these, 174 gene transcripts could be mapped to biological processes, as shown in Figure 4. Transcripts mapped across a wide range of biological processes and molecular functions, showing no specific enrichment by either biological or molecular ontology.

The differential gene expression detected by microarray analysis was validated by PCR using the same RNA samples. Eight gene transcripts were selected for validation. By microarray, the expression ratio of these transcripts in Lewis vs. Fisher rats varied by 1.5–2-fold (see Figure 5). Additionally, 6 of the 8 transcripts were expressed at higher levels in Lewis rats, whereas 2 (i.e., A disintegrin and metalloproteinase with thrombospondin motifs 3 and Neuromedin B) were greater in Fisher rats. Significant strain-dependent differences were detected by PCR analysis of all eight transcripts.

Gene interactions between the 322 differentially expressed transcripts were sought using Chilibot, an established data-mining software, to analyze relationships documented in all the abstracts compiled in PubMed (Chen & Sharp, 2004). We identified genes likely to determine drug abuse behavior based on their known relationships to cell signaling, synaptic plasticity, transcription, addiction and nicotine. Table 3 shows 22 genes that mapped to synaptic plasticity, 24 to signaling, 18 to transcription, 10 to addiction and 4 to nicotine. Twenty-one of 22 genes mapping to synaptic plasticity were also related to cell signaling. However, the strength of these gene relationships to signaling vs. synaptic plasticity varied in that gene $\times$ signaling interactions were based on a median of 14.5 unique publications, whereas gene $\times$ synaptic plasticity interaction were derived from a median of 3.5

publications. Only 4 of 18 genes mapped to transcription regulation were common to signaling and synaptic plasticity. Strong interactions between gene $\times$ transcription regulation were evident based on a median of 15.5 publications. Lastly, 7 of 10 genes mapped to addiction and all 4 genes mapped to nicotine were common to signaling and synaptic plasticity. Overall, 33 of the 41 genes in Table 3 have not previously been associated with addiction or nicotine. CASK (calcium/calmodulin-dependent serine protein kinase) exemplifies a gene strongly associated with all 3 biological processes, but not with addiction or nicotine.

Discussion

These studies demonstrate significant rat strain-dependent differences in the transcriptomes from neuroanatomically-defined sets of enriched GABA neurons that regulate the reinforcing dimension of motivated behavior. Differences in gene expression may impact the motivational function of these GABA neurons, since Lewis vs. Fisher rats differ greatly in their propensity to operantly self-administer nicotine and other drugs (Brower *et al.*, 2002, Martin *et al.*, 1999, Suzuki *et al.*, 1988a, Suzuki *et al.*, 1988b). In contrast, acquisition of operant food self-administration was similar between these strains. Therefore, differential gene expression is potentially related to drug reinforcement, rather than operant learning *per se*.

We combined neuroanatomical identification of specific neurons labeled with fluorogold and laser-capture of individual neurons. Fluorogold was transported from VP by GABA neurons in ventral medial NAcc shell. Despite RNA yield of only 1.8 ng/rat, requiring amplification before microarray, this protocol enriched >6,600 genes, including GABA-related transcripts. Strain-dependent effects on gene expression were identified; 322 vs. 60 transcripts showed 1.5-fold vs. 2-fold expression differences (FDR 5%). PCR validated the expression of selected gene transcripts. The LCM method unavoidably captures glial cells in close apposition to FG⁺ neurons, thus affecting the specificity of the transcriptome attributed to GABA neurons.

Classification by gene ontology showed these 322 strain-dependent transcripts lacked categorical enrichment. This wide-ranging alteration in the transcriptome from enriched NAcc GABA neurons is most consistent with a global change in neuronal function manifest throughout the cellular machinery. Since Chilobot literature-mining (Chen & Sharp, 2004) did not find networks amongst these genes, all 322 were analyzed for relationships to cellular processes pivotal to drug abuse (i.e., synaptic plasticity, signaling and regulation of gene transcription). Chilobot found genes with documented relationships to these 3 processes (Table 3). Almost all genes related to plasticity were common to signaling, whereas only 4 of 18 transcription genes were common to signaling/synaptic plasticity. Thirty-three of 38 genes related to plasticity, signaling and/or gene transcription have no association with addiction or nicotine. Based on novelty in the addiction literature, magnitude of the difference in strain-dependent expression, and prior knowledge of protein function, the following genes are highlighted.

Mint-1 (i.e., APBA1) has a relatively large number of associations with signaling, synaptic plasticity and transcription, but only 1 with addiction. Mint, an adaptor protein containing isoform-specific N-terminal sequences and common PTB and PDZ domains, is found in soma, axons and dendrites of excitatory and inhibitory neurons throughout the brain (Duclos & Koenig, 1995, Okamoto & Sudhof, 1997, Tanahashi & Tabira, 1999). Mints bind calcium channels, potentially affecting presynaptic calcium influx ((Maximov & Bezprozvanny, 2002, Maximov *et al.*, 1999). Only the N-terminus of Mint-1 binds Cask (Borg *et al.*, 1998, Butz *et al.*, 1998), itself a synaptic membrane-associated guanylate kinase that interacts with

membrane receptors affecting GABA release (Ho *et al.*, 2003). Cask is associated with all 3 cellular processes, but not addiction/nicotine. In hippocampal interneurons, expressing high levels of Mint-1 vs. Mint-2, Mint-1 deficiency is associated with impaired regulation of GABA release, (Ho *et al.*, 2003). Therefore, in Lewis vs. Fisher rats, the release of GABA by NAcc neurons regulating VP motivational functions might be affected by increased Mint-1-Cask heterodimers, reflecting increased basal levels of both transcripts (Lewis/Fisher ratios of *Mint-1* and *Cask*: 1.7 and 1.6, respectively).

Cask associates with synaptic vesicles through interactions with neurexins, Mints and synaptotagmin (Biederer & Sudhof, 2000, Hata *et al.*, 1993). Neurexins are presynaptic transmembrane cell-adhesion proteins (Nam & Chen, 2005) involved in initiating postsynaptic specializations at glutamate and GABA synapses (Graf *et al.*, 2004). Cask binds and phosphorylates the cytoplasmic tail of neurexin-1, which may be essential for regulation of the presynaptic cytoskeleton (Mukherjee *et al.*, 2008). Cask kinase activity is suppressed by neuronal activity and increased by silencing synapses. Two reports suggest that inhibitory synapses may undergo silencing (Bekkers, 2005, Kilman *et al.*, 2002). In Lewis rats, the enhanced basal expression of *Cask* may promote phosphorylation of neurexin concomitant to the silencing of GABA synapses involving NAcc GABA projections.

In contrast to Mint-1 and Cask, Ca²⁺/calmodulin-dependent protein kinase II δ (CamkII δ) is related to all 3 cellular processes and addiction/nicotine. Its expression ratio in Lewis vs. Fisher NAcc GABA neurons was 1.76 (p=0.03, 5% FDR). CamkII δ is present throughout rat brain (Takeuchi *et al.*, 1999). Nuclear CamkII δ is implicated in gene transcription, exemplified by mPer1 (Nomura *et al.*, 2003). Dopamine D₂ receptors (D₂R) activate CamkII δ to increase brain-derived neurotrophic factor (BDNF) (Takeuchi *et al.*, 2002), which is required for expression of activity-dependent inhibitory synapses (Hong *et al.*, 2008, Yamada *et al.*, 2002). These observations and the strain-dependent differences in basal *CamkII δ* expression indicate that neuronal plasticity, involving BDNF and inhibitory synapses encoding aspects of behavioral reward, may be facilitated in Lewis rats.

Table 3 shows that neural cell adhesion molecule 1 (Ncam1) has the strongest associations with signaling and synaptic plasticity and is also associated with addiction/nicotine. Its expression ratio in Lewis vs. Fisher NAcc projection neurons was 1.54 (p=0.023, 5% FDR). Ncams mediate extracellular interactions with matrix and cells. Membrane clustering of Ncams initiate intracellular signaling cascades implicated in synaptic plasticity (Luthi *et al.*, 1994, Maness & Schachner, 2007). By direct interaction with D₂R, Ncam1 promotes internalization and degradation, regulating D₂R signaling (Xiao *et al.*, 2009). Increased Ncam1 expression appears to reduce surface D₂R levels, which inhibit NAcc GABA neuron activity. Across 21 strains of recombinant inbred BXD mice, D₂R and *Ncam* expression were associated with models of ethanol preference (Hitzemann *et al.*, 2003). In Lewis rats, increased *Ncam1* expression in NAcc GABA projection neurons is likely to enhance synaptic plasticity and alter surface expression of D₂R, reducing dopamine-dependent inhibition.

Visinin-like 1 (Vsnl1; aka Vilip-1) and hippocalcin-like 1 (Hpcal1) are members of the neuronal Ca²⁺ sensor subfamily of visinin-like (Vsnl) proteins, which belong to the superfamily of EF-hand Ca²⁺ binding proteins (Braunewell & Gundelfinger, 1999). Vsnl interacts with multiple intracellular signaling cascades and affect exocytosis, modulation of adenylyl cyclase activity and regulation of both ligand- and voltage-gated ion channels (Burgoyne, 2007). Vsnl proteins are cytoplasmic at resting Ca²⁺ concentrations, and translocate to plasma or Golgi membrane with increased intracellular Ca²⁺ (Spilker *et al.*, 2002). Vsnl1 has low to moderate associations with signaling and synaptic plasticity and minimal relationships to addiction/nicotine. Similarly, Hpcal1 has relatively few

relationships with signaling and synaptic plasticity, and none with addiction/nicotine. The expression ratios for *Vsnl1* and *Hpcal1* in Lewis vs. Fisher were relatively high: 2.03 ($p=0.023$, FDR 5%) and 1.75 ($p=0.043$, FDR 5%), respectively. The *Vsnl1* ratio was confirmed by RT-PCR.

Vsnl1 interacts directly with the $\alpha 4$ subunit of the most abundant brain nicotinic cholinergic receptor (nAChR), $\alpha 4\beta 2$, increasing the surface expression of functional receptors, depending on Ca^{2+} concentration (Zhao *et al.*, 2009b). In contrast, $\alpha 7$ -containing nAChRs elicit a calcium-dependent membrane localization of *Vsnl1* (Zhao *et al.*, 2009a). The inward Ca^{2+} current induced by nicotinic stimulation of $\alpha 7$ nAChRs (Berg *et al.*, 2006) may drive the activation and membrane localization of *Vsnl1* that is associated with upregulation of $\alpha 4\beta 2$ nAChRs. The brain region-specific upregulation of $\alpha 4\beta 2$ nAChRs has been reported in rats that chronically self-administer nicotine (Moretti *et al.*, Parker *et al.*, 2004). Upregulation of nAChRs would likely increase neuronal sensitivity and responsiveness to nicotine (Nguyen *et al.*, 2004). However, in NAcc GABA projection neurons, the effect of chronic nicotine exposure on the level of nAChRs is not, to our knowledge, known. Nevertheless, the 2-fold increased expression of *Vsnl1* in Lewis vs. Fisher GABA projection neurons may contribute to the motivational effects of nicotine in Lewis rats and to their ability to acquire nicotine self-administration.

Carbonic anhydrase 8 (*Car8*) displayed the greatest difference in expression between Lewis and Fisher rats (3.36-fold; $p=0.024$, FDR 5%). This was confirmed by RT-PCR. *Car8*, an acatalytic carbonic anhydrase, binds inositol trisphosphate [IP(3)] receptors, inhibiting binding to inositol 1,4,5-trisphosphate (Hirota *et al.*, 2003). Thus, *Car8* modulates IP(3) receptor-dependent mobilization of Ca^{2+} from intracellular stores, which is critical to propagation of cytosolic Ca^{2+} signals (Taylor & Tovey, 2010). *Car8* mutant mice demonstrate the essential role of *Car8* in synaptogenesis and maintenance of synaptic function (Hirasawa *et al.*, 2007). The difference in basal expression by Lewis vs. Fisher rats suggests that *Car8* modulation of intracellular Ca^{2+} mobilization and signaling varies substantially between these inbred strains.

In summary, these studies demonstrate the feasibility of obtaining neuronal phenotype-enriched samples to ascertain strain-specific differences in gene transcript expression. Based on gene ontologies of 322 transcripts that differed between these 2 strains, there is a wide-ranging alteration in the transcriptome of GABA neurons projecting from NAcc shell to VP. This is most consistent with a global change in function. In Lewis rats, significant upregulation of *Mint-1*, *Cask*, *CamkII δ* , *Ncam1*, *Vsnl1*, *Hpcal1* and *Car8*, which participate in cellular signaling and synaptic plasticity, indicates that these gene transcripts may contribute to altered function of NAcc GABA neurons, potentially predisposing to nicotine self-administration in Lewis rats. However, data from a manifold of inbred strains and F1 crosses will be required to specify the potential role of individual genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Nicotine or food self-administration (SA) in Lewis vs. Fisher 344 adolescent male rats at postnatal day 41. Data shown in the left panels was from rats housed in operant chambers with nicotine available for 10d, beginning at PN41 (FR1 schedule), using our model of 23h drug access (Brower *et al.*, 2002). Lewis rats rapidly acquired nicotine SA [effect of lever on number of presses, $F(1,7)$: 18.2, $p<0.001$; effect of day on active lever presses, $F(9,63)$: 2.1, $p<0.05$; effect of day on inactive lever presses, $F(9,63)$: 0.8, $p>0.05$]. In contrast, Fisher rats failed to acquire nicotine SA. Right panels show results from both Lewis and Fisher rats that acquired operant food self-administration. Active lever presses for food did not differ by strain [$F(1,12)$: 0.7, $p=0.41$].

Figure 2.

Identification of GABA neurons in nucleus accumbens shell (NAcc shell) that project to ventral pallidum (VP) at postnatal day 41. Neurons projecting from NAcc shell to VP were identified by retrograde uptake of fluorogold (FG) delivered by iontophoresis bilaterally into VP (asterisks). Within VP, the central regions of FG fluorescence from six rats are marked by asterisks in a schematic coronal section. A representative deposit of FG is shown in the photomicrograph. Within NAcc shell, the center of the distribution of retrograde-labeled FG^+ neurons is shown by green dots within the boxed area. The corresponding inset shows a representative fluorescence image of these FG^+ neurons, located in ventral medial NAcc shell (20X). Individual FG^+ neurons were captured by laser-capture microdissection.

Figure 3.

Microarray detection of transcripts enriched in NAcc shell GABA neurons, obtained by laser-capture microdissection, compared to the remainder of tissue. Unsupervised cluster analysis of all genes was used to generate the data shown in Fig. 3a; representative areas of the heat map show the clustering of genes expressed at significantly greater or lesser levels in neurons vs. tissue remainder. These differences in neuron/remainder ratios were consistent throughout all samples. Venn diagrams (Fig. 3b) show the number of gene transcripts that were significantly (false discovery rate < 0.05) enriched in GABA neurons at various ratios of neuron/remainder (i.e., 1, 2, 3). At each ratio of neuron/remainder, most of the enriched neurons were common to both Lewis and Fisher GABA neurons. Neuronal transcripts with very low mean fluorescence values (<3 on microarray) were excluded from the analysis.

Figure 4. Distribution of 322 genes showing differential expression in Lewis vs. Fisher NAcc shell GABA projection neurons to VP. 174/322 of these genes could be mapped by biological process and molecular function. Gene transcripts were distributed across all biological processes, showing no enrichment in any particular process. Widespread distribution and lack of enrichment were also shown for mapping by molecular ontology.

Figure 5.

Validation of differential gene expression in Lewis vs. Fisher rats by real-time PCR. Eight genes that varied in expression by 1.5–2-fold (by microarray) between rat strains were selected for measurement by PCR. Significant strain-dependent differences were found for all genes. The eight genes were: A disintegrin and metalloproteinase with thrombospondin motifs 3 (Adamts3), carbonic anhydrase 8 (Car8), GABAA receptor γ -1 subunit (Gabrg1), neuromedin B (Nmb), neuronatin (Nnat), protein phosphatase 1 regulatory (inhibitor) subunit 14a (Ppp1r14a), cohesin subunit Rad21 (Rad21), Visinin-like protein 1 (Vsnl1). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 1

Enrichment of GABA and GABA Neuron-Related Genes *

Symbol	Gene Name	Neuron/Remainder (mean of both strains)	Neuron Fluorescence Intensity (mean of both strains)
Akap9	A kinase (PRKA) anchor protein (yotiao) 9	5.18	7.28
Dbi	diazepam binding inhibitor (GABA receptor modulator acyl-Coenzyme A binding protein)	8.25	8.43
Drd1a	dopamine receptor D1A	9.83	9.20
Drd2	dopamine receptor D2	2.74	6.81
Gabarap	GABA(A) receptor-associated protein	10.55	9.41
Gabarap11	GABA(A) receptor-associated protein like 1	9.18	8.73
Gabarap12	GABA(A) receptor-associated protein like 2	6.01	8.03
Gabarap12	GABA(A) receptor-associated protein like 2	5.32	7.78
Gabra1	gamma-aminobutyric acid (GABA) A receptor alpha 1	3.42	7.48
Gabra2	gamma-aminobutyric acid (GABA) A receptor alpha 2	3.90	6.69
Gabra4	gamma-aminobutyric acid (GABA) A receptor alpha 4	10.74	8.73
Gabra5	gamma-aminobutyric acid (GABA) A receptor alpha 5	1.72	5.97
Gabrb1	gamma-aminobutyric acid (GABA) A receptor beta 1	2.92	6.24
Gabrb2	gamma-aminobutyric acid (GABA) A receptor beta 2	6.04	7.45
Gabrb3	gamma-aminobutyric acid (GABA) A receptor beta 3	6.90	8.87
Gabrg1	gamma-aminobutyric acid (GABA) A receptor gamma 1	3.32	6.68
Gabrg2	gamma-aminobutyric acid (GABA) A receptor gamma 2	5.37	7.92
Gabrg3	gamma-aminobutyric acid (GABA) A receptor gamma 3	4.11	7.24
Gabbr1	gamma-aminobutyric acid (GABA) B receptor 1	3.93	7.42
Gabbr2	gamma-aminobutyric acid (GABA) B receptor 2	1.64	5.63
Gad1	glutamate decarboxylase 1	2.23	6.69
Gad2	glutamate decarboxylase 2	6.44	9.25
Pdyn	prodynorphin	3.73	6.84
Penk1	proenkephalin 1	4.01	6.50
Slc32a1	solute carrier family 32 (GABA vesicular transporter) member 1	6.41	8.38
Slc6a1	solute carrier family 6 (neurotransmitter transporter GABA) member 1	3.21	6.86

Symbol	Gene Name	Neuron/Remainder (mean of both strains)	Neuron Fluorescence Intensity (mean of both strains)
Slc6a11	solute carrier family 6 (neurotransmitter transporter GABA) member 11	2.90	6.59
Snap25	synaptosomal-associated protein 25	14.77	11.79
Stxbp1	syntaxin binding protein 1	9.05	9.15

* Neuron > Remainder for all genes, FDR 5%

Table 2

Differential Gene Expression in Lewis vs. Fisher Rats

Criteria	Number of genes
Lewis vs Fisher > 1.5 fold	1210
t-test $p < 0.05$	608
FDR 10%	573
FDR 5%	330
Lewis vs Fisher > 2 fold	267
t-test $p < 0.05$	90
FDR 10%	71
FDR 5%	60

Table 3

Genes Related to Cell Signaling, Synaptic Plasticity and Transcription Regulation

Gene Symb	Signaling	Synaptic plasticity	Transcription regulation	Addiction	Nicotine	Gene name
ADRBK1	345	14		5		adrenergic, beta, receptor kinase 1
AHI1	6	2				Abelson helper integration site 1
APBA1	15	30	15	1		amyloid beta (A4) precursor protein-binding, family A, member 1
CAMK2D	8	2	136	41	15	calcium/calmodulin-dependent protein kinase II delta
CAR8**	3	2				carbonic anhydrase 8
CASK	30	51	16			calcium/calmodulin-dependent serine protein kinase
DAAMI	25	2				dishevelled associated activator of morphogenesis 1
DSTN	37	29				destrin
EMD			72			emerin
EPHA5	8	34		2		EphA5
ETFA	1	3			1	electron-transfer-flavoprotein, alpha polypeptide
GRIA4				6		glutamate receptor, ionotropic, AMPA 4
GRB10	78					growth factor receptor-bound protein 10
GABRG1**		1				gamma-aminobutyric acid (GABA) A receptor, gamma 1
HBP1			48			HMG-box transcription factor 1
HMBOX1			4			homeobox containing 1
HPCAL1	6	2				hippocalcin-like 1
ILF3			35			interleukin enhancer binding factor 3
MTSS1			14			metastasis suppressor 1
NCAM1	277	675		11	5	neural cell adhesion molecule 1
NFATC3			76			nuclear factor of activated T-cells, calcineurin-dependent 3
NMI			15			N-myc (and STAT) interactor
NNAT**	6	1				neuronatin
NXPH1	5	3				neurexophilin 1
POLE3			5			polymerase (DNA directed), epsilon 3 (p17 subunit)
PPP1R14A**	26					protein phosphatase 1, regulatory (inhibitor) subunit 14A

Gene Symb	Signaling	Synaptic plasticity	Transcription regulation	Addiction	Nicotine	Gene name
PRDX1		44				peroxiredoxin 1
PSMD7				1		Psmd7 proteasome 26S subunit, non-ATPase, 7
PTAFR	68	10				platelet-activating factor receptor
RAD21			44			RAD21 homolog (S. pombe)
RICTOR	90	2		1		RPTOR independent companion of MTOR, complex 2
RUFY2	6	4	2			RUN and FYVE domain containing 2
SSH2	2	2				slingshot homolog 2
SNAPC2			9			small nuclear RNA activating complex, polypeptide 2
SPARC	99	21				secreted protein, acidic, cysteine-rich (osteonectin)
STXBP3				1		syntaxin binding protein 3
TERF1			111			telomeric repeat binding factor (NIMA-interacting) 1
VLDL-R	17	4				very low density lipoprotein receptor
VSNL1**	14	10		1	2	visinin-like 1
ZCCHC12	2		4			zinc finger, CCHC domain containing 12
ZFP410			6			zinc finger protein 410
Number of genes	24	22	18	10	4	
Median PubMed hits	14.5	3.5	15.5	1.5	3.5	

** denotes transcripts also measured by RT-PCR