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Mesencephalic Dopamine Neuron Number and Tyrosine Hydroxylase Content: Genetic Control and Candidate Genes

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Abstract

The mesotelencephalic dopamine system shows substantial genetic variation which fundamentally affects normal and pathological behaviors related to motor function, motivation, and learning. Our earlier radioenzyme assay studies demonstrated significantly higher activity of tyrosine hydroxylase (TH), the first and rate limiting enzyme in the biosynthesis of catecholamine neurotransmitters, in the substantia nigra – ventral tegmental area of BALB/cJ mice in comparison with that of C57BL/6ByJ mice. Here, using quantitative immunoblotting and immunocytochemistry, we tested the hypothesis that mesencephalic TH protein content and number of nigral TH-positive neurons show strain-dependent differences in C57BL/6ByJ and BALB/cJ parallel to those observed in the TH activity studies. Immunoblotting experiments detected significantly higher mesencephalic TH protein content in BALB/cJ in comparison to C57BL/6ByJ ($p<0.05$). Immunocytochemical studies demonstrated that the number of TH-positive cells in substantia nigra was 31.3% higher in BALB/cJ than that in C57BL/6ByJ ($p<0.01$), while the average dopamine neuron volume was not significantly different. In a search for candidate genes that modulate TH content and the size of mesencephalic dopamine neuron populations we also studied near-isogenic mouse sublines derived from the C57BL/6ByJ and BALB/cJ progenitor strains. A whole-genome scan with 768 single nucleotide polymorphism markers indicated that two sublines, C4A6/N and C4A6/B, were genetically very similar (98.3%). We found significantly higher mesencephalic tyrosine hydroxylase (TH) protein content in C4A6/B in comparison to C4A6/N ($p=0.01$), and a tendency for higher number of dopamine neurons in the substantia nigra in C4A6/B in comparison to C4A6/N, which, however, did not reach statistical significance. To identify the genetic source of the TH content difference we analyzed the SNP genotype data of the whole-genome scan, and detected two small differential chromosome segments on chr. 13 and chr. 14. Microarray gene expression studies and bioinformatic analysis of the two differential regions implicated two cis-regulated genes (*Spock1* and *Cxcl14*, chr. 13), and two growth factor genes [*Bmp6* (chr. 13), and *Fgf14* (chr. 14)]. Taken together,

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the results suggest that (1) nigral dopamine neuron number and TH protein content may be genetically associated but further studies are needed to establish unequivocally this linkage, and (2) *Spock1*, *Cxcl14*, *Bmp6*, and *Fgf14* are novel candidates for modulating the expression and maintenance of TH content in mesencephalic dopamine neurons *in vivo*.

Keywords

midbrain; dopamine system; gene mapping; recombinant QTL introgression

INTRODUCTION

Ground breaking observations on strain-dependent variation in brain regional levels of neurotransmitters and their elusive genetic correlation with complex behaviors have been puzzling for several decades (Maas, 1963, Sudak and Maas, 1964). Difficulties in explaining the genetic regulation of these pathways can be appreciated by considering that combinations of even a few polymorphic genes with individually small effects, and sensitive interactions with the environment, may lead to strikingly different, complex changes in the brain, and inbred mouse strains traditionally used in comparative studies may be polymorphic at thousands of genes, therefore chance association of complex traits in simple strain comparison studies is highly likely.

One step towards explaining this “neurotransmitter - behavior” genetic association puzzle was made by focusing on the cellular level as a substrate for the neurotransmitter to behavior paradigm. The foundations of an intriguing model which linked strain differences in midbrain tyrosine hydroxylase (TH) activity, to variation in midbrain dopamine neuron number, dopamine function and psychopharmacological response to dopamine agonists have been laid more than 25 years ago (Ross et al., 1976, Baker et al., 1980, Reis et al., 1981). These studies suggested that the strain difference in the activity of TH is determined entirely by a difference in the amount of enzyme protein, which in turn is a consequence of strain dependent difference in the number of TH-containing neurons located in the midbrain (Ross et al., 1976). The ultimate explanation, identification of the underlying genetic factor(s) which control variation in dopamine neuron number, however, has not been offered in the past decades, in spite of recent progress in complex trait genetics.

In our earlier studies we demonstrated that mesencephalic TH activity is significantly higher in the inbred mouse strain BALB/cJ than in C57BL/6ByJ, and the inheritance of the trait is polygenic, not Mendelian (Vadasz et al., 1982, Vadasz, 1990, Vadasz et al., 1994, Vadasz et al., 1998).

Here, we report that (1) mesencephalic TH protein content and the number of nigral dopamine neurons are positively associated in and BALB/cJ and C57BL/6ByJ, (2) there are significant differences in mesencephalic TH protein levels, but not in number of dopamine neurons, between advanced near-isogenic animal models which were derived from C57BL/6ByJ and BALB/cJ. Bioinformatic analysis of the genomic differences between the near-isogenic sublines identified four candidate quantitative trait genes, which may be responsible for the TH protein content differences in the midbrain dopaminergic region.

EXPERIMENTAL PROCEDURES

Animals

Initially, C57BL/6ByJ and BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) in 1978 and 1983, respectively. All breeding experiments were carried out first

at the New York State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, New York, and later at The Nathan Kline Institute for Psychiatric Research, formerly Rockland Research Institute, New York State - Office of Mental Health. Progenitors of B6By.C4A6B/Vad (C4A6/B) and B6By.C4A6N/Vad (C4A6/N) quasi-congenic RQI sublines were the C57BL/6ByJ background strain and the BALB/cJ donor strain. Recombinant QTL Introgression (RQI) strains were created to reduce genetic complexity of the dopamine-system differences between C57BL/6ByJ and BALB/cJ (Vadasz 1994, 1998). In 2002 using microsatellite DNA markers for a whole genome scan we detected residual heterozygosity in one of the RQI strains, C4A6. Because this strain showed the highest mesencephalic tyrosine hydroxylase activity among the tested RQI strains (same as that of the donor strain BALB/cJ), we wished to fix and preserve the unidentified loci responsible for the high expression of the phenotype in homozygous condition. Genome-wide genotyping of RQI strains with 247 microsatellite markers indicated 5.26% residual heterozygosity in the inbred C4A6 strain. Based on the genotype information for 10 chromosomes carrying heterozygous loci, for fine mapping the donor alleles responsible for the higher TH/MES we set up 16 mating pairs representing different combinations of homozygous and heterozygous loci. These founding pairs gave rise to 16 new genetically highly similar recombinant sublines, named as B6.C4A6/A through B6.C4A6/H. The sublines have been maintained by brother × sister (b × s) mating for more than 20 generations. The C4A6/B (b × s generations: F_{62+} as of 2006) and C4A6/N (b × s generations: F_{65+} as of 2006) inbred sublines and all other mice were bred and maintained in our colony. Experimental animals were housed in same-sex groups (3-5 per cage) under standard laboratory conditions (12 hr light / 12 hr dark cycle) with food and water available *ad libitum*. All reported data refer to male mice. The care and use of animals met the standards and recommendations of the IACUC of the NKI in accordance with US Department of Agriculture and US Public Health Service guidelines.

Dissection of ventral mesencephalon for Western blotting

Brains were quickly removed and placed on a saline-soaked filter paper on an ice-cold Peltier-cooled plate. First, using the ventral approach and holding scissor blades at an angle of about 30 degree, the area caudal to the optic chiasm including the middle part of the hypothalamus and the posterior or mammillary hypothalamic region was removed with four scissor cuts following the natural boundaries of the infundibular recess. Second, the cerebellum was removed to facilitate a precise cut in order to separate the rostral dopaminergic (A8, A9, A10 cell groups) and caudal noradrenergic (locus ceruleus) systems. Third, the posterior colliculus was gently pushed rostrally, and a perpendicular cut was applied with a razor blade to separate the pons from the midbrain. Using a thin, highly polished stainless steel spatula, and moving first rostrally over the colliculi, then ventrally and laterally, the mesencephalon was separated from the cortex (dorsally) and hippocampus (laterally), and the thalamus was cut away (rostrally). The isolated block of tissue was placed on its rostral surface, and a perpendicular cut was applied at the level of the aqueductus cerebri, thus removing the dorsal areas including the colliculi. This ventral mesencephalic tissue block included all the dopaminergic cell body areas: substantia nigra, the retrorubral area, and the ventral tegmental area (22). Approximate stereotaxic coordinates of the isolated tissue block are the following: Bregma −2.7 and −5.0, dorsoventral distance from the horizontal plane passing through the interaural line: 3.5 and −0.5 (23). Although most of the posterior or mammillary region was removed using this approach, we have to keep in mind that the dissected tissue block contains some lateral and posterior hypothalamic areas as well as various nuclei as presented in mouse brain atlases (23).

Quantitative Western blotting of mesencephalic tyrosine hydroxylase

Following dissection of ventral mesencephalon, the tissue samples were immediately frozen and stored at −80 °C. Tissue samples were rapidly homogenised (Kontes) in hypotonic protease inhibitor sample preparation buffer (SIGMA, St.Louis, Mo) containing 0.05 M Tris pH 7.4,

2.0 mM AEBSF, 1.0 mM EDTA, 130 μM Bestatin, 1.74 μM E-64, 1.0 μM Leupeptin, 0.3 μM Aprotinin. The homogenates were then centrifuged at 14,000 rpm for 30 min and the supernatants were divided into two aliquots A and B which were transferred into fresh tubes. These aliquots A and B were analysed separately in order to check the reproducibility of the method.

We quantitatively detected differences in the TH content by comparing to a standard curve generated using a striatal reference preparation with known TH content (0.34 ng TH / μg total protein, see details below). A reference standard curve was included with each Western blot.

Protein concentrations in each aliquot were determined using the Bradford method. (Bio-Rad, Hercules, CA). From each protein sample an aliquot containing 80 μg of total protein was transferred into a fresh Eppendorf tube and 5X Laemmli buffer solution (200 mM Tris Cl pH 6.8, 10 % SDS, 40 % glycerol, 0.2 % bromophenol blue, 10% β-mercaptoethanol) was added to each and prior to polyacrylamide gel electrophoresis (SDS-PAGE) they were boiled at 95 ° C for 3 min and were immediately put on ice. This 80 μg protein containing sample was separated on a polyacrylamide gel (3.5% SDS-polyacrylamide stacking gel, 10% SDS-polyacrylamide resolving gel). Then they were electrophoretically transferred to a PVDF membrane (Millipore, MA) at 395 V for 6 hours. Following transfer the PVDF membranes were blocked for 1 hour to prevent non-specific binding of antibody with 3% non-fat dried milk powder solution in TBST (TBS, 3% non-fat blotting grade milk powder/Bio-Rad, Hercules, CA/, 0.1% Tween) and incubated overnight (15 hours) at 4°C with mouse anti-TH IgG1 monoclonal antibody (STI, San Clemente, CA) at a dilution of 1:2000 in 3% non-fat dried milk powder solution (TBS, 3% non-fat blotting grade milk powder/Bio-Rad, Hercules, CA/, 0.1% Tween). Following incubation with primary antibody, blots were washed twice with TBST (TBS containing 0.1% Tween20) for 15 min followed by a third 15 min wash with blocking buffer of 3% non-fat dried milk powder solution in TBST. Following wash with blocking buffer blots were incubated for 3.5 hours at room temperature with alkaline phosphatase conjugated goat anti-mouse IgG1 antibody (Bio-Rad, Hercules, CA) in a solution of diluted (1:3000) 3% non-fat dried milk powder solution in TBST. The blots were washed once again in TBST for 15 minutes followed by a 15 minute wash in alkaline phosphatase colour development buffer (0.1M Tris, 0.1 M NaCl, 0.05 M MgCl2, pH 9.5). Immunoreactivity was visualized using BCIP (5-bromo-4chloro-3 indolyl-phosphate)/NBT(nitroblue tetrazolium) (Bio-Rad, Hercules, CA). The air dried PVDF membranes were scanned with a Hewlett Packard DeskScan II flatbed scanner. The images obtained with the scanner were imported into Adobe Photoshop and were saved as TIFF files for further analysis with the software NIH Image. TH immunoreactivity was quantified by densitometric analysis using NIH Image 1.67. Quantitation was achieved through calibration using a set of external standards prepared from a corpus striatum extract of known TH content. Therefore beside the mesencephalon samples each blot was prepared containing a dilution series (with 5μg, 10μg, 15μg, and 20μg total protein content corresponding to 1.7ng, 3.4ng, 5.1ng, and 6.8ng TH respectively) of this corpus striatum extract. The O.D. measurements from these known values were loaded into an spreadsheet file and O.D. values were plotted versus TH content creating a standard curve. The slope of this standard curve was used to calculate the amount of TH in the ventral mesencephalon samples.

For statistical analysis we used SPSS ver. 13. TH protein content data were subjected to Box plot tests. Two outliers were detected in the BALB/cJ group, which were excluded from further analysis.

Reference standard preparation for Western blotting

We quantitatively determined the TH content of a reference standard by comparing to a standard curve generated using pure TH protein. The TH content of the ‘corpus striatum

standard', used as reference, was determined by Western Blot analysis, comparing it to a control TH protein solution (obtained from STI, San Clemente, CA). The corpus striata of thirty mice were collected, combined and homogenized in a hypotonic protease inhibitor buffer solution (50 mM Tris pH 7.4, 5 mM EDTA, 2 mM EGTA, 500 μM Benzamidine, 5 mM Dtt, 500 μM AEBSF, 0.33 mg/l Pepstatin A). The homogenate was centrifuged for 15 min at 15,000 rpm at 4 °C. The supernatant was collected and its total protein content was determined with the Bradford protein analysis method. Then the supernatant was divided into working aliquots which were kept in an ultra low freezer (-80 °C). The corpus striatum extract and the TH control protein were subjected to a 10% SDS-PAGE then they were transferred to a PVDF membrane (Millipore Corp., Bedford, CA). Each blot contained a dilution series from the corpus striatum standard with total protein contents of 5μg, 7μg, 10μg, 15μg, and 20μg, and a dilution series from the pure control TH protein solution with TH content of 1 ng, 2 ng, 5ng, 7ng, 10 ng, 12 ng, 15 ng, and 20 ng. The optical density values from the control TH showed linearity in the range of 1ng - 7 ng TH. The slope of the standard curve was used to determine the TH concentration in the corpus striatum extract (3.4 ng TH/10 μg total protein). The aliquots from this extract were used as reference for the quantification of TH in mesencephalon protein samples.

Histology

Mice were anesthetized deeply with Ketamine-Xylazine (100 mg/kg -10 mg/kg) and perfused through the left ventricle with 0.1 M phosphate buffered saline (PBS, pH 7.2-7.4) for 2 minutes followed by perfusion with a fix solution (4% paraformaldehyde, 4% sucrose in PBS) for 7 minutes. The heads were left in fixative for 24 hours before removal of the brains to PBS at 5° C. Embedding, sectioning, and staining was performed by NeuroScience Associates, Knoxville, TN. Brains were treated with 20% glycerol and 2% dimethyl sulfoxide to prevent freeze-artifacts and multiply embedded (25 mouse brains per block) in a gelatin matrix using MultiBrain™ Technology (NeuroScience Associates, Knoxville, TN). After curing, the block was rapidly frozen by immersion in isopentane chilled to −70°C with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The MultiBrain™ block is sectioned coronally at the designated thickness, i.e. 35μ for the comparison of C57BL/6By and BALB/cJ, and 50μ for the comparison of C4A6B and C4A6N. All sections cut (none were discarded) were collected sequentially into a 4×6 array of containers which were filled with antigen preserve solution (50% PBS pH 7.0, 50% Ethylene glycol, 1% Polyvinyl Pyrrolidone) for sections to be stained immunohistochemically. Every 6th or 4th section was stained immunohistochemically for TH in the C57BL/6By and BALB/cJ or C4A6B and C4A6N comparisons, respectively.

For TH immunochemistry, free-floating sections were incubated in a 1:1500 dilution of TH primary antibody (Pelfreez, Milwuakee, WI), a goat anti-rabbit secondary antibody, and an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector, Burlingame, CA). The peroxidase was visualized with diaminobenzidine reaction, and the sections were mounted on gelatinized (subbed) glass slides.

Exhaustive cell counting

The following strains were represented in each multi-brain section: C57BL/6ByJ (n=7), BALB/cJ (n=6), C5A3 (n=6), and I5B25A (n=6). Only results of the analysis of C57BL/6ByJ and BALB/cJ brains are reported here. Because these sections were too thin for stereological analysis (final thickness ~7um), the number of TH cells was estimated by counting all of the labeled cell in the substantia nigra, and dividing by the section sampling fraction (i.e. 1/6). Final cell counts were corrected using the method of Abercrombie (Abercrombie, 1946). Cells were counted by plotting their location onto high-resolution (100×) video montages of the sections, using customized software developed with the program Object-Image (http://

simon.bio.uva.nl/object-image). The pixel coordinates of the montage were linked to the X-Y coordinates of a motorize microscope stage, and software was designed to allow the user to systematically step through the region of interest at high magnification (×100 to ×400) and plot all cells in each field of view, and record the X-Y coordinates of every cell. Final counts were derived from the digital plots. Cell size was estimated on every 10th cell plotted in the left hemisphere substantia nigra. Cell size was measured using the nucleator method, evaluating ×3 radii per cell centered at the nucleolus, or in most cases at the approximate nuclear center because the nuceolus was not easily visualized.

Unbiased stereology

A second set of 12 mouse brains (C4A6/N, n=6, and C4A6/B n= 6) was sectioned at 50um thickness in a single block using MultiBrain™ Technology, and every 4th section stained for TH. The number of TH cells in the left substantia nigra was estimated by the optical fractionator method (West et al., 1991, Dorph-Petersen et al., 2001) using “Stereo Investigator” software (Microbrightfield, Inc). The tissue was sampled with a 100× objective using disector X-Y-Z dimensions of 50×50×6 microns, using 2 micron guard zones above and below the counting box. The sampling grid was adjusted so that 209 +/- 43 neurons (mean +/- S.D.) were sampled per substantia nigra, and the estimated coefficient of error (m =1 of Gundersen et al., 1996) ranged from 0.07 to 0.09. Estimates of section thickness per brain was 11.6 +/- 0.77 μm (mean +/- S.D., n = 12). Total cell number was estimated by number weighted section thickness. Cell size was measured using the nucleator method as described above for exhaustive cell counting. Coefficients of error for cell volume ranged from 0.003 to 0.006.

Gene expression microarray data analysis

All gene expression data are publicly accessible and were based on Affymetrix Mouse Gene Expression Array 430 2.0. We used the Vadasz/Saito Whole Brain B6vsC 430 2.0 May_2007 RMA Database (Gene Expression Omnibus series entry GSE7155 (http://www.ncbi.nlm.nih.gov/geo/) for establishing gene expression strain differences between C57BL/6ByJ and BALB/cJ. For testing cis-regulation of genes we used the INIA Whole Brain BXD mRNA M430 (Jun06) RMA Database (http://www.genenetwork.org).

RESULTS

The histogram of TH protein levels of the ventral mesencephalon in progenitor and near-isogenic mice are shown in Fig. 1. One-way ANOVA indicated significant strain differences in TH protein amount ($F_{1,3}$=7.84, p=0.001), which was followed up by preplanned comparisons. Independent Samples T tests for C57BL/6ByJ (n=5) versus BALB/cJ (n=7), and C4A6/N (n=6) versus C4A6/B (n=5) detected statistically significant differences p=0.03 (t_{10}=2.55) and p=0.01 ($t_{5.78}$=3.76), respectively.

To confirm the prediction that higher TH/MES can be attributed to higher mesencephalic dopamine neuron number, we used multibrain matrix technology for simultaneous sectioning and immunocytochemically staining 25 mouse brains for TH. The population size of nigral dopamine neurons were estimated in two immunocytochemical studies using the same delineation of the substantia nigra region (Fig. 2).

In the first immunocytochemical study, using exhaustive cell counts, we estimated the number of TH cells in the SN in the progenitor strains C57BL/6ByJ and BALB/cJ (Fig. 3A). Cell counts were adjusted using the Abercrombie correction factor to compensate for overcounting of split cells that are sampled in multiple sections. These measurements, from the summed left and right SN, showed significantly more cells in the BALB/cJ (9530 +/- 195; mean±SE, n=6) than in C57BL/6ByJ (7257+/- 516; mean±SE, n=7) with a peak difference at around -3.6mm from

Bregma in both the left and right hemisphere (p=0.003). (Hemispheric comparisons, not shown, showed nearly identical cell numbers in the left and right hemispheres). Cell size was estimated using the nucleator method on every 10^{th} labeled neuron in the SN, and was not significantly different between BALB/cJ (1346±53; mean±SE, n=6) and C57BL/6ByJ (1495± 104; mean ±SE, n=7).

These data, and results of earlier radioenzyme assays (Vadasz et al., 1994, Vadasz et al., 1996b, Vadasz et al., 1998), provided robust evidence of genetically controlled higher number of nigral dopamine neurons and higher mesencephalic TH activity in BALB/cJ in comparison to the other commonly used standard mouse strain, C57BL/6By.

In the second study, using unbiased stereology, the average volume and the population size of dopamine neurons was estimated in near-isogenic strains (Fig. 3B). The estimate for the number of TH positive neurons in the left substantia nigra was higher in C4A6/B (3978.33 ±337.58; mean±SE, n=7) than in C4A6/N (3539.43±242.61; mean±SE, n=7), however, the difference was not significant (p>0.05). Likewise, estimate of the average dopamine cell volume for C4A6/B (1735.29±86.78 μm^3; mean±SE, n=7) was also higher than that in C4A6/N (1543.33±35.32 μm^3; mean±SE, n=7) without significant statistical difference (p=0.075). Power analysis for the near-isogenic lines indicated that at power (1-β)=0.8 with the observed effect sizes d=0.57 for the cell count, and d=1.1 for average cell volume, the necessary sample sizes to reach the error probability α=0.05 are n=40, and n=12, respectively.

For mapping the potential sources of detected differences in neuron number and TH content, we used a high-resolution genotyping panel of 768 single nucleotide polymorphism DNA markers (SNPs) for genome-wide screening of C4A6/B, C4A6/N, and the progenitors to identify the genomic sources of the dopamine system-related differences. Comparison of the four genomes indicated 1.17% differential donor genome in subline C4A6/B on chrs. 13 and 14 (Fig. 3C and Table 2). The chr. 13 segment was flanked by background-type SNP markers rs3710348 (31.28Mb) and rs13481826 (57.57Mb) defining a maximum interval-length of 26.29Mb (NCBI Build 36); the chr. 14 segment was flanked by rs30259301 (117.35Mb) and the telomere (approx. at 124Mb) defining a maximum interval-length of 6.65Mb. These results suggested that DNA polymorphisms located on the identified chromosome intervals are the ultimate cause of differences in mesencephalic TH content.

Bioinformatic analysis indicated that the chr. 13 donor segment between 31.28Mb and 57.57Mb harbors 269 known or predicted genes, or gene models (http://www.informatics.jax.org/searches/marker_report.cgi). Database query for genes polymorphic between C57BL/6J and BALB/cByJ or its sublines narrowed the list to 79 genes featuring SNPs in various categories, such as coding synonymous, coding nonsynonymous, intron, and mRNA-UTR. A similar analysis of the chr. 14 donor interval between 117.35Mb and the telomere detected 39 genes of which 8 were polymorphic.

It has been suggested that most inbred laboratory mouse strains (Beck et al., 2000), including the progenitor strains, shared a common ancestor (Wade et al., 2002), and some regions of their genome will be largely Identical By Descent (IBD). Such IBD regions, which are not considered strong positional candidates (Mehrabian et al., 2005), can be identified by low frequency of SNPs between two inbred strains. Candidate genes are more likely to fall in non-IBD regions, therefore positional cloning should focus on sub regions where the progenitors differ. We analyzed the SNP distribution histogram between C57BL/6J and BALB/cByJ on chr. 13 between 31.28 Mb and 57.57 Mb (http://www.jax.org/phenome/snp.html), and identified IBD regions where SNP frequency<20/0.1Mb at the following regions (Mbp): 41.1-42.5, 47.8-50.1, 50.9-52.6. Testing of the 79 polymorphic genes on chr. 13 indicated that one of them (Nedd9, 41.32Mbp) was in a low SNP frequency region, while genes in the other

identified IBD regions were not polymorphic. On the chr. 14 differential chromosome segment we identified the following regions with low SNP frequency (Mbp): 118.3-119.05, 121.4-122.15, 122.8-123.4. Of the 8 polymorphic genes two (Hs6st3 and Pcca) fell on these IBD regions, thus reducing the list of candidates to 6 genes.

A set of candidate genes may also be narrowed by requiring the gene to be expressed, to be correlated with the phenotype of interest, and to have cis-regulated expression (Brem et al., 2002, Klose et al., 2002, Schadt et al., 2003, Monks et al., 2004, Morley et al., 2004, Bystrykh et al., 2005, Chesler et al., 2005). To filter the remaining 78 (chr. 13) and 6 (chr. 14) genes for cis-regulation, first we analyzed whole brain gene expression data from the progenitor strains (Vadasz/Saito Whole Brain B6vsC 430 2.0 May_2007 RMA Database, Gene Expression Omnibus series entry GSE7155). Comparison of transcription abundance of the candidate genes indicated significant strain differences between C57BL/6ByJ and BALB/cJ for Serpinb9c, Ssr1, Gmpr2, Unc5a, Cxcl14, Spock1, and Ibrdc2 (T-test, $p<0.05$). Gene expression signal values of these genes were subjected to interval QTL mapping using the BXD INIA Brain mRNA M430 (Jun06) RMA Database (http://www.genenetwork.org). The LOD (logarithm of the odds to the base 10) scores for expression of the 7 genes were tested for genome-wide significance (1000 permutation (Churchill and Doerge, 1994)). The criterion of cis-regulation was the identification of a single peak of the transcript abundance QTL with genome-wide significance ($p<0.05$) and a peak position identical or near to the genomic location of the gene. Only two genes had genome-wide statistically significant LOD scores: Spock1 (sparc/osteonectin, cwcv and kazal-like, chr. 13: 57,430,817bp 1419672_at, LOD=9.1, and Cxcl14 (chemokine (C-X-C motif) ligand, chr. 13: 56,298,265bp, 1418457_at, LOD=6.33). Peaks of the transcript abundance QTLs for these genes fell in bins which included their genomic locations indicating the possibility of cis-regulation of these genes. Brain mRNA expression signal values of candidate genes Spock1, and Cxcl14 in the C57BL/6ByJ vs. BALB/cJ comparison were 652.7 vs. 477.4 ($p<0.01$), and 398.9 vs. 336.7 ($p<0.05$), respectively.

Although application of the IBD strategy and the requirement of cis-regulation may be useful in narrowing the set of candidate genes, none of them should be regarded as sufficient and strictly required criteria. There is no evidence that the mouse genome has a rigidly identifiable mosaic structure, and it was suggested that methods for QTL mapping may fail if they assume a simple block-like structure (Yalcin et al., 2004). Also, if we require the gene to be expressed, to be correlated with the phenotype of interest, and to have cis-regulated expression, all genes which show temporally regulated differential gene expression and in the experimentally targeted developmental stage are not significantly expressed will be lost for further consideration as candidate genes. One may assume that neuron number, for example, does not change as dynamically in response to environmental stimuli as neurotransmitter or protein content in regions of interest in the brain. Therefore, we also used alternative approaches. Considering that mesencephalic TH content depends on number of TH positive cells in the region of interest, average cell volume, and within-cell TH protein concentration, we searched for Gene Ontogeny (GO) terms, which approximate our phenotype. We chose the GO definition “the function that stimulates a cell to grow or proliferate” (GO:0008083). Analysis of the overlap between the set of genes on the chr. 13 and chr. 14 segments, and the set of 146 genes covered by GO:0008083 identified three genes (*Bmp6*, *Ogn*, and *Il9*) from the chr. 13 donor interval and one gene (*Fgf14*) from chr. 14 donor interval (http://www.informatics.jax.org/searches/GO.cgi?id=GO:0008083). Of the identified genes on chr. 13 *Bmp6* (rs29589226, rs29764424) and *Il9* (rs29251134), on chr. 14 *Fgf14* (rs3709982, rs3692681, rs13482415), were polymorphic between C57BL/6J and BALB/cByJ.

DISCUSSION

The original goal of our project was to identify genetic factors, which significantly affect the natural variation in mesencephalic dopamine neuron number. In this endeavor we adopted the notion that mesencephalic TH activity (TH/MES) and TH abundance are index traits for number of dopamine neurons in the midbrain A8, A9, A10 dopaminergic cell groups (Ross et al., 1976, Baker et al., 1980). Our general strategy focused on reduction of irrelevant genetic and environmental complexity. While the concept of congenic strains, and introgression of a monogenic qualitative trait into a pure line was well known, the idea that complex, quantitative traits with continuous distribution could be transferred onto a homogeneous genetic background was contrary to contemporary genetic belief (e.g., (Green, 1981)). We hypothesized that a set of genes underpinning a specific quantitative trait with continuous distribution, such as complex neural or behavioral phenotypes, can be introgressed into a homogeneous genetic background by the combination of artificial selection and concomitant backcrosses (Vadasz, 1990, Vadasz, 1994, Vadasz et al., 1996a, Vadasz et al., 1998).

To this end we used the BALB/cJ donor strain with 38% higher mesencephalic TH activity (TH/MES) than that of the C57BL/6ByJ background mouse strain (Vadasz et al., 1982), and in earlier studies, we applied artificial selection for high and low TH/MES in replicated mouse lines with concomitant backcrosses to the C57BL/6ByJ background mouse strain (Vadasz, 1990, Vadasz et al., 1994, Vadasz et al., 1998). This procedure allowed us to retain the selectively favored QTLs for TH/MES. After 5 backcross-intercross cycles, on the average about 3% of the genome remained BALB/cJ donor-type in the RQI strains (b_5i_7 series), i.e., the background heterogeneity was about 3%. Then we initiated brother × sister (b × s) mating in 100+ closed lines to develop inbred recombinant quantitative trait locus introgression (RQI) strains containing short chromosome segments of the donor mouse strain BALB/cJ (with high TH/MES) on the background of the C57BL/6ByJ strain characterized by average TH/MES in comparison to other strains (Vadasz et al., 1982, Vadasz et al., 1998). For testing our QTL introgression hypothesis, we screened an initial set of RQI strains and their progenitor strains for TH/MES. As expected, BALB/cJ showed significantly higher TH/MES than that of B6By (Vadasz et al., 1998), and we identified one introgression strain, C4A6, which rescued the BALB/cJ-type high TH activity phenotype on the C57BL/6ByJ background. These results provided proof of the QTL introgression principle (Vadasz et al., 1998).

In theory, when introgression is completed, a set of recombinant QTL introgression (RQI) strains on the same genetic background can provide a more powerful tool for the genetic dissection of the targeted complex traits than other existing tools such as recombinant inbred (RI) or recombinant “congenic” (RC) strains because RI and RC strains receive *random* genomic contribution from the progenitor strains, and background heterogeneity is *high* (50%) or *relatively high* (12.5%), respectively.

In the present study we showed that the progenitor strain difference in mesencephalic TH protein content (abundance) was comparable to the difference in mesencephalic TH enzyme activity between C57BL/6ByJ and BALB/cJ in earlier reports (Vadasz et al., 1998). We also found a robust strain difference in the number of nigral dopamine neurons. Thus, for the progenitor strains a positive association between TH content and dopamine cell number could be demonstrated.

The genetically highly (98.7%) similar near-isogenic sublines, C4A6/B and C4A6/N, significantly differed in mesencephalic TH content, and the difference could be attributed to two small differential chromosome segments. Similarly to the progenitor strains the sublines showed a difference in number of TH positive neurons in the substantia nigra. However, the difference did not reach statistical significance because of small sample size. Thus formally

this indicates a genetic dissociation of mesencephalic TH content and number of nigral TH positive neurons. However, we would like to point out that further studies can show some degree of common genetic control, or genetic correlation, because power calculations suggested that with increased sample sizes the detected differences in average volume and population size of nigral dopamine neurons may become statistically significant.

A complicating factor in the interpretation of the cell counting results is the use of exhaustive cell count method for progenitors (Fig. 3. panel A) and a different method, unbiased stereology, for the same analysis in the near-isogenic strains (Fig. 3. panel B). The two methods may differ in sensitivity and potential artefacts. In general, unbiased stereology is considered as a more advanced methodology, however, at the time of the first study on the progenitors it was not available in our laboratory. Although the difference in cell number between C57BL/6J and BALB/cJ was obtained by exhaustive counting, we are confident that the difference is valid because of the large effect size, and the very similar histological processing which was ensured by the multibrain matrix technology. Designing the second study on the near-isogenic lines, we had to consider the consequences of using phenotypic introgression to develop the near-isogenic lines, i.e., one usually expects a smaller difference between the near-isogenic lines than that between the progenitor lines because of elimination of most of the background interaction effects, and potential loss of some of the involved polygenes. Therefore, we wanted to make sure that the detected difference will not be “biased”, and we will not obtain by chance false positive results. Because at the time of the second study unbiased stereology was already available in our laboratory we switched methodology. We could not apply the same approach for the progenitors because the sections from the first study were too thin for unbiased stereology. As to the source of genetic differences in mesencephalic TH content, bioinformatic analysis highlighted four candidate genes whose relevance will be discussed.

Spock1/testican, which contains osteonectin-like domains, a Kazal-like sequence, and a cys-trp-cys-val (CWCV) domain, is related to protein families that are involved in adhesion, migration, and cell proliferation. It was suggested that it is a multidomain testicular proteoglycan resembling modulators of cell social behavior (Alliel et al., 1993). The cis-regulated, polymorphic Spock1/testican core protein is 95% identical to its human counterpart. In situ hybridization investigations revealed that mouse testican mRNA is mainly present in a subpopulation of pyramidal neurons localized in the CA3 area of the hippocampus. Testican includes several putative functional domains related to extracellular or pericellular proteins associated with binding and/or regulatory functions. On the basis of its structural organization and its occurrence in postsynaptic areas, this proteoglycan might contribute to various neuronal mechanisms in the central nervous system (Bonnet et al., 1996). The suggested functions do not exclude a role in modulation of dopamine neuron population size.

Little is known regarding the physiological function of Cxcl14 (chemokine (C-X-C motif) ligand 14, BRAK). Several reports implicate CXCL14 in regulation of tumor development, it is a potent inhibitor of angiogenesis and a chemotactic factor for immature dendritic cells (Shellenberger et al., 2004). It was also reported that CXCL14 is a potent chemoattractant and activator of dendritic cells and might be involved in dendritic cell homing in vivo (Shurin et al., 2005). In fish CXCL14 chemokine is expressed early in ontogeny predominantly in the brain suggesting that these chemokines must play key roles in the patterning and maintenance of the developing vertebrate central nervous system (Huising et al., 2004). Our results, i.e., detection of Cxcl14 expression in the mouse brain with cis-regulation, are consistent with a developmental role in modulating the mesotelencephalic dopamine system. Published data on midbrain dopamine number and expression of the cis-regulated candidate genes (Spock1 and Cxcl14) are not available across several strains for correlation analysis. However, it has been reported that the number of tyrosine hydroxylase (TH) immunoreactive cell bodies was significantly higher in the midbrain of DBA/2J mice than in C57BL/6J mice (D’Este et al.,

2007). Moreover, mining of the BXD INIA Brain mRNA database (see Results) shows that brain expression of Spock1, and Cxcl14 in C57BL/6J is lower, and higher, than those in DBA/2J, respectively. This information indicates that expression of Cxcl14, but not Spock1, shows the same pattern of association with dopamine neuron number in both strain comparisons (C57BL/6ByJ vs. BALB/cJ and C57BL/6J vs. DBA/2J), thus suggesting that Cxcl14 may be a more likely candidate than Spock1.

Comparison of the functions of the remaining three candidate genes revealed the following. *Bmp6* (bone morphogenetic protein 6) was expressed in the developing midbrain floor (Jordan 1997), it was as effective as fibroblast growth factor-2 (FGF-2) and GDNF in promoting survival of rat mesencephalic dopaminergic neurons cultured from the embryonic midbrain floor at embryonic day (E) 14 (Jordan et al., 1997). BMP6 increased the uptake of [^{3}H] dopamine without affecting the uptake of [^{3}H]5-hydroxytryptamine and [^{3}H]GABA, underscoring the specificity of the trophic effect (Espejo et al., 1999); it significantly increased the number of TH-positive neurons and induced nuclear translocation of the phosphorylated BMP-restricted Smad in a substantial number of TH- and microtubule-associated protein 2 (MAP2ab)-positive cells, and it was capable of inducing, in a dose and time dependent manner, moderate levels of TH in cultured neurons from the mouse embryonic striatum (Brederlau et al., 2002). BMP6 was shown to be a neurotrophic factor for calbindin-positive striatal neurons (Gratacos et al., 2001, Gratacos et al., 2002). BMP-6 treatment induced dopaminergic neuronal differentiation of the IMR-32 cells as judged by expression of GAP-43, tyrosine hydroxylase and microtubule-associated protein 2ab (Sumantran et al., 2003). We found no evidence of effects of Spock1, Cxcl14, Fgf14 on TH.

Interleukin-9 (IL9) is a relatively new cytokine that supports the growth of helper T-cell clones, mast cells, and megakaryoblastic leukemia cells. *Il9* encodes a protein, which is involved in the immune response (Lu et al., 2006), its role in the central nervous system, if any, is not well known.

Fgf14 (fibroblast growth factor 14, is a member of a distinct subclass of fibroblast growth factors) which is expressed in the developing and adult nervous systems, suggesting a role in nervous system development and function (Smallwood et al., 1996). Genetic ablation of the *Fgf14* gene in mice or a missense mutation in *Fgf14* in humans caused ataxia and cognitive deficits. Neuropharmacological studies showed that *Fgf14*-deficient mice had reduced responses to dopamine agonists. The paroxysmal hyperkinetic movement disorder phenocopies a form of dystonia, a disease often associated with dysfunction of the putamen (Wang et al., 2000). These phenotypes suggest that the neuronally expressed *Fgf14* gene is essential for regulating normal neuronal activity, and may have dopamine-system related functions (Wang et al., 2000, Wang et al., 2002, Dalski et al., 2005, Lou et al., 2005).

Comparison of in situ hybridization data from the Allen Brain Atlas (http://www.brain-map.org) indicate that growth factor genes *Bmp6* and *Fgf14* show rather limited expression density and level in the adult C57BL/6J mouse brain, which is consistent with our hypothesis of their influence on the development of midbrain dopamine system, i.e., their expression may show significant temporal variation. Ubiquitous high level expression of *Spock1* suggests a housekeeping function, while *Cxcl14* shows regionally variable expression levels with the highest expression in the lateral septal complex (Fig. 5). Both *Spock1* and *Cxcl14* are expressed in the midbrain (scaled expression levels are 100 and 17.34, respectively, in a range of 1-100), which strengthens their candidate gene status.

Taken together, the functions, the genomic positions, and the genetic attributes of these genes in the quasi-congenic sublines suggest that *Spock1, Cxcl14, Bmp6* and *Fgf14* are likely candidates for playing a role in the developmental control of nigral dopamine neuron growth,

proliferation, and survival, and affecting mesencephalic TH activity and abundance. Nomination of these four genes for modulation of natural variation in mouse mesencephalic TH protein content is consistent with our earlier studies on the transfer of dopamine system - specific QTLs onto C57BL/6ByJ background, in which the best model for estimating the number of effective genetic factors suggested the involvement of at least two unlinked loci (Vadasz et al., 1994). Further studies are needed to confirm the candidacy of these genes, and to test our hypothesis that with increased statistical power the observed differences in number of TH positive cells and in cell volume will become significant, and thus some degree of common genetic control for the cellular properties and TH content can be established. Our working hypothesis is that these genes have pleiotropic effects, and (although their effect size may be small) play a significant role in the development of the midbrain dopaminergic system affecting not only the steady-state level of TH, but also the average volume and number of nigral and ventral tegmental dopamine neuron populations. Identification of new genes for dopamine neuron growth, proliferation, and survival can open new avenues of research for understanding the development of natural and pathological variation in brain structure, function, and behavior.

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List of Abbreviations

A8-A10

catecholaminergic cell groups according to Dahlstrom and Fuxe (1964)

B6

C57BL/6ByJ

Bmp6

bone morphogenetic factor 6

C
BALB/cJ

CB
cerebellum

CTX
Cerebral cortex

Cxcl14
chemokine (C-X-C motif) ligand 14

Fgf14
fibroblast growth factor-14

fr
fasciculus retroflexus

HIP
Hippocampal region

HPF
Hippocampal formation

HY
Hypothalamus

IBD
Identical By Descent

IP
interpeduncular nucleus

LSX
Lateral septal complex

MB
mammillary bodies

MB
Midbrain

MES
mesencephalon

ml
medial lemniscus

MY
Medulla

OLF
Olfactory areas

P
Pons

PAG
periaqueductal gray

PAL
Pallidum

pc
posterior commisure

Pn
pontine nuclei

QTG
quantitative trait genes

QTL
quantitative trait locus

RC
recombinant “congenic”

RHP
Retrohippocampal region

RI
recombinant inbred

RQI
recombinant QTL introgression

sAMY
Striatum-like amygdalar nuclei

SN
substantia nigra

SNc
substantia nigra, pars compacta

SNP
single nucleotide polymorphism

SNr
substantia nigra, pars reticulata

SPOCK1
sparc/osteonectin, proteoglycan (testican) 1

STR
Striatum

STRv
Striatum ventral region

TH
Thalamus

TH
tyrosine hydroxylase

TH/MES
mesencephalic tyrosine hydroxylase enzyme activity

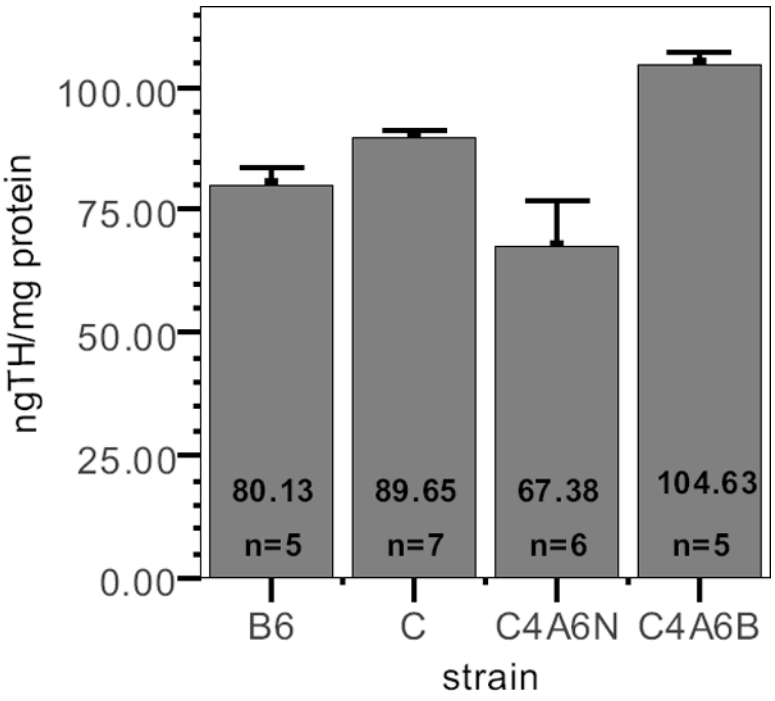

Fig. 1.

Ventral mesencephalic TH content in B6 (C57BL/6ByJ), and C (BALB/cJ) progenitor strains, and in near-isogenic lines C4A6/B and C4A6/N. Bars show means, error bars show +/- 1 SE. For genomic similarity of strains see Table 1.

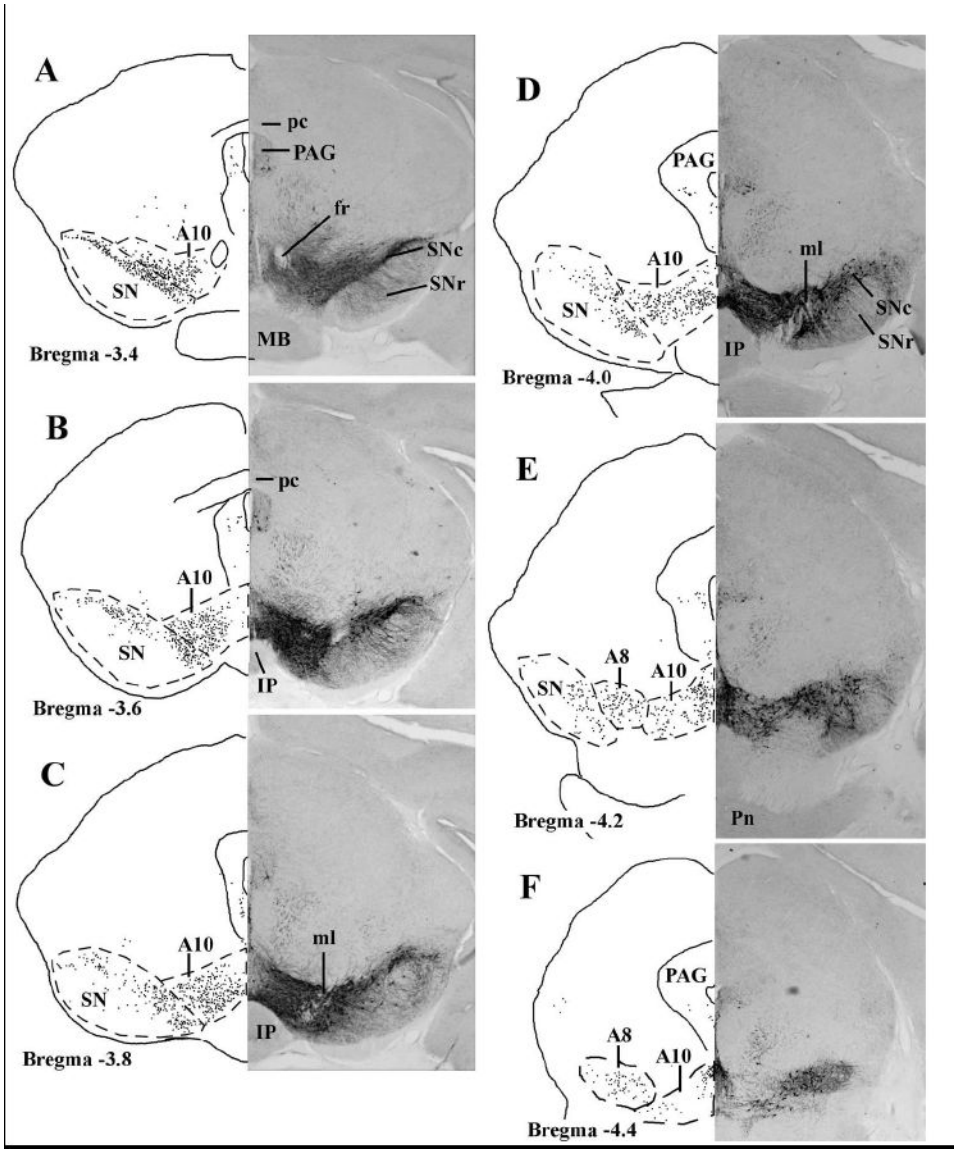

Fig. 2.

Delineation of substantia nigra. A series of sections labeled with an antibody to tyrosine hydroxylase demonstrates our method of delineating the substantia nigra. Shown is a case from the progenitor strain C57BL/6ByJ that was evaluated by plotting all cells in each section ("exhaustive counting" method). The section spacing in figures A-F is 210 um, and the section thickness is 35 um. Bregma locations are approximate, and were determined by from local landmarks (the medial lemniscus and the interpeduncular nucleus) (Franklin and Paxinos, 1997). Each dot represents a single dopamine cell. In all preparations, counts of TH cells in the substantia nigra included both the pars compacta and pars reticulata. For quantification of TH cells, we identified the border of SN as described previously (Zaborszky and Vadasz, 2001) with minor modifications. The rostral-caudal length of the substantia nigra (SN) in our mice was approximately 1.4 mm. Using sections spaced every 200 or 210 um, SNc extended 3 to 4 sections rostral to the medial lemniscus, where it was defined as the compact band of cells ventral to the less compact and less organized cells of A10. In the 2-3 sections at the level of the medial lemniscus, we included all TH cells lateral to the medial lemniscus, including clusters of cells that extended into the SN pars reticulata. This definition likely includes a small

part of rostral A8, but these are very few cells and not readily distinguished from the SN at this level. In 2-3 sections caudal to the medial lemniscus, the SN diminishes as the adjacent dorsally located A8 enlarges. Here we defined SN as the cells found at the same relatively lateral position in immediately rostral sections, and A8 as the cells found at the same relatively medial position in more caudal sections. Borders of SN in all sections were drawn by an investigator (J.S.) who was blind to the animal' s identity. Abbreviations: A8-A10 catecholaminergic cell groups are according to Dahlstrom and Fuxe (1964); fr - fasciculus retroflexus; IP - interpeduncular nucleus; MB - mammillary bodies; ml - medial lemniscus; PAG - periaqueductal gray; pc - posterior commisure; Pn - pontine nuclei; SN - substantia nigra; SNc - substantia nigra, pars compacta; SNr - subhstantia nigra, pars reticulata.

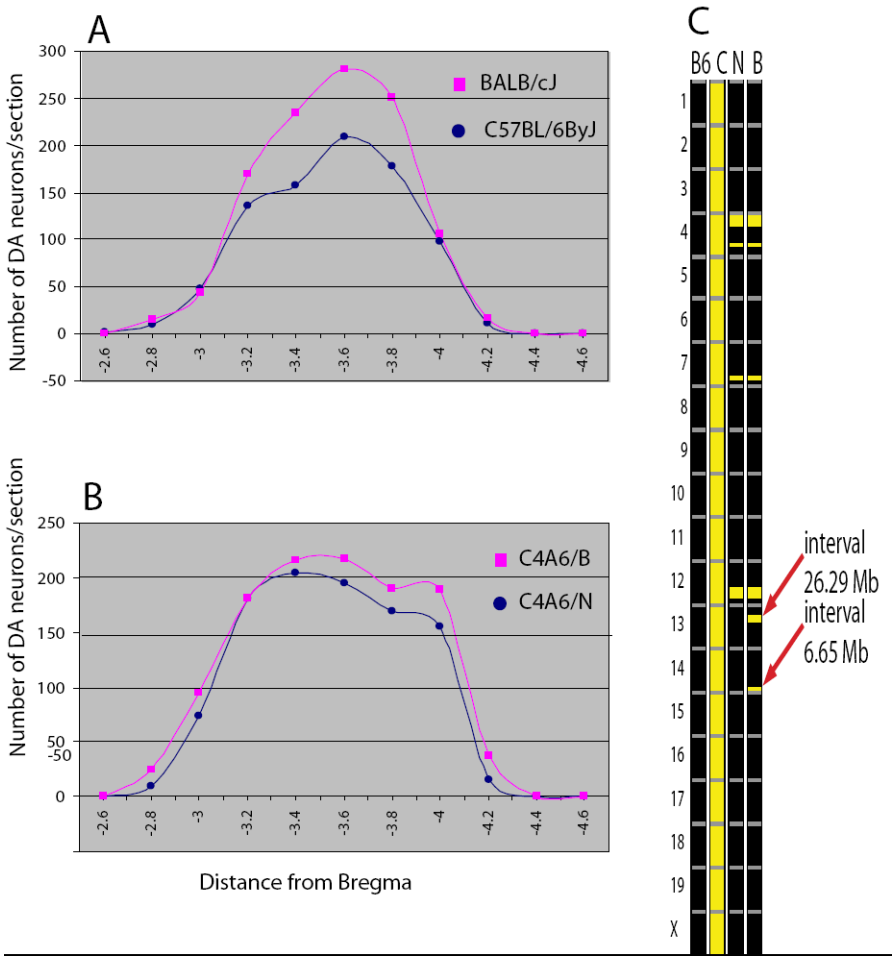

Fig. 3.

Rostro-caudal distribution of dopamine neurons in the substantia nigra.

A. Progenitor strains (BALB/cJ and C57BL/6ByJ) showed significant difference in total number of nigral dopamine neurons (31.33% $p<0.01$). **B.** Near-isogenic strain C4A6B showed 12.4% more nigral dopamine neurons than C4A6N, however, the difference was not statistically significant. **C.** Whole genome scanning with a panel of 768 mouse SNPs. C57BL/6ByJ-type polymorphic alleles are shown in black, BALB/cJ-type alleles are shown in yellow. Comparison of the SNP genotypes indicated that the differential BALB/cJ-type SNPs, which were present in C4A6B and absent in C4A6N, were located on chr. 13 (a block of 5 SNPs), and on chr. 14 (one telomeric SNP).

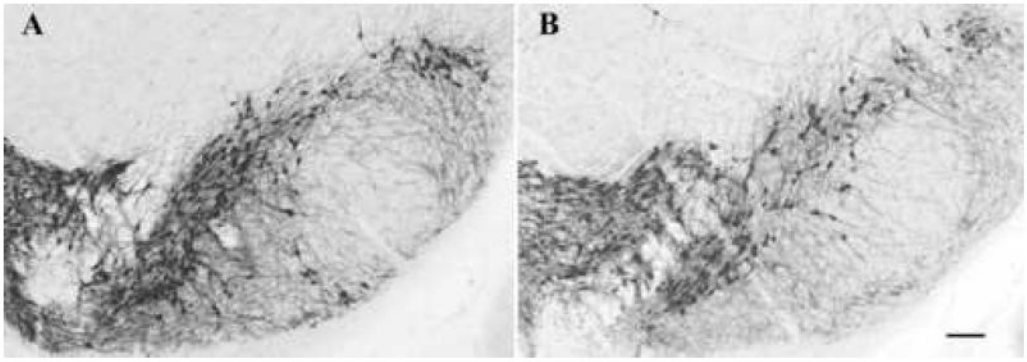

Fig. 4.

Shown is an illustration of the dopamine cell number difference in the progenitor mouse strains. The cell number was significantly higher in the BALB/cJ strain (shown in A, id#C29/39) compared to the C57BL/6ByJ (shown in B, id#B32/39). Both sections are at approximately -3.8 mm from Bregma. Scale bar = 0.1mm.

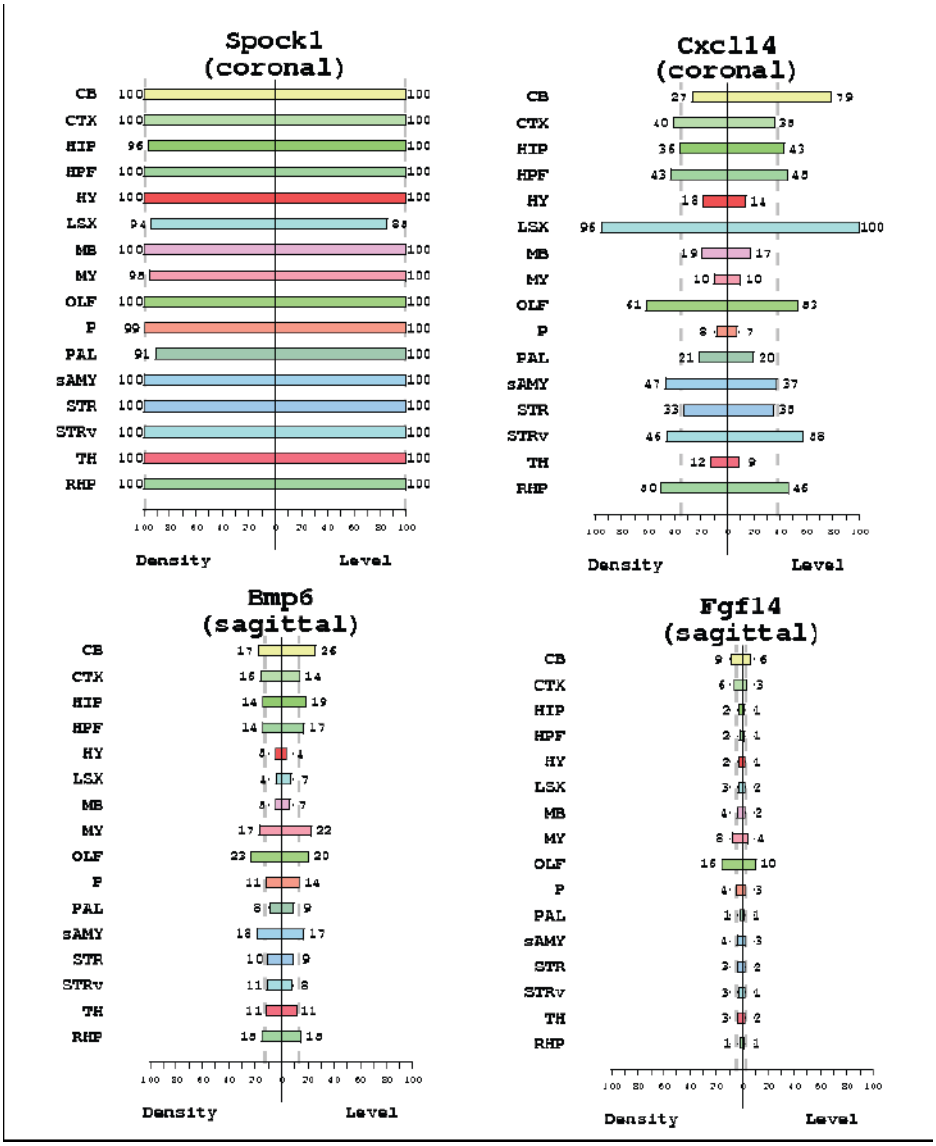

Fig. 5.
Expression summary graphs of candidate genes from the Allen Brain Atlas.

Table 1

Genomic similarity to C57BL/6ByJ

Comparison	number SNPs*	similarity (%)
C57BL/6ByJ vs. BALB/cJ	461	39.97
C57BL/6ByJ vs. C4A6B	26	96.61
C57BL/6ByJ vs. C4A6N	21	97.27
C4A6B vs. C4A6N	9	98.83

* Total number of SNP markers in the mapping panel was n=768