Altered Gene Expression in Mice Selected for High Maternal Aggression

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Abstract

We previously applied selective breeding on outbred mice to increase maternal aggression (maternal defense). In this study, we compared gene expression within a continuous region of the CNS involved in maternal aggression (hypothalamus and preoptic regions) between lactating selected (S) and non-selected control (C) mice (n = 6 per group). Using microarrays representing over 40,000 genes or expressed sequence tags, two statistical algorithms were used to identify significant differences in gene expression: robust multi array and the probe logarithmic intensity error method. ~ 200 genes were identified as significant using an intersection from both techniques. A subset of genes were examined for confirmation by real-time PCR. Significant decreases were found in S mice for neurotensin and neuropeptide Y receptor Y2 (both confirmed by PCR). Significant increases were found in S mice for neuronal nitric oxide synthase (confirmed by PCR), the K+ channel subunit, Kcna1 (confirmed by PCR), corticotrophin releasing factor binding protein (just above significance using PCR; p = 0.051), and GABA A receptor subunit 1A (not confirmed by PCR, but similar direction). S mice also exhibited significantly higher levels of the neurotransmitter receptor, adenosine A1 receptor, and the transcription factors, c-Fos, and Egr-1. Interestingly, for 24 genes related to metabolism, all were significantly elevated in S mice, suggesting altered metabolism in these mice. Together, this study provides a list of candidate genes (some previously implicated in maternal aggression and some novel) that may play an important role in the production of this behavior.

Keywords (5-10)

maternal aggression; microarray; lactation; hypothalamus; selection; preoptic area; nNOS; NPY; CRF

Introduction

Maternal aggression (also termed maternal defense behavior) is conserved in mammals and birds. In rodents, maternal defense behavior involves attacks against intruders by lactating females that is hypothesized to protect the offspring from potential harm (Agrell *et al.* 1998;Gammie & Lonstein 2005;Parmigiani *et al.* 1999;Wolff 1985,1993). However, not all studies find maternal aggression to be a deterrent to infanticide, including in mice (Ebensperger

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1998) and common voles (Heise & Lippke 1997), although in the latter study heightened aggression was associated with decreases in rate of infanticide. For reviews of the ecological relevance of maternal aggression, see (Lonstein & Gammie 2002;Wolff & Peterson 1998). One previous approach for understanding the genetic basis of defense behavior involved using quantitative trait loci to identify chromosomal regions that corresponded with levels of maternal behavior, including aggression (Peripato *et al.* 2002). However, the actual genes contributing to the phenotype have yet to be isolated. Another, more common approach has been to study whether or how this behavior is altered in knockout mice. For example, maternal aggression is decreased in mice missing either neuronal nitric oxide synthase (Nos1) (Gammie & Nelson 1999), a subset of pheromone receptors (Del Punta *et al.* 2002), or the trp2 ion channel that transduces pheromonal inputs (Leypold *et al.* 2002). Conversely, maternal aggression is significantly increased in mice missing estrogen receptor β (Ogawa *et al.* 2005). Another approach for examining the genetics of behavior has been to conduct selection studies and then perform high density gene expression analysis of the CNS to uncover gene candidates (Bronikowski *et al.* 2004;Feldker *et al.* 2003a;Feldker *et al.* 2003b). To date, this approach has not been used for studies on maternal aggression.

We have recently applied selection for high maternal aggression on outbred mice of the hsd:ICR (CD-1) strain (Gammie *et al.* 2006). We found a realized heritability of this trait of 0.40 and by maintaining selected (S) and non-selected control (C) lines, we set up the possibility of examining gene expression differences between groups that could provide insights into the genetic basis of maternal aggression. This study involved examining differences in gene expression between S and C mice in continuous portion of the CNS (including preoptic and hypothalamic regions) that contains regions previously implicated in maternal aggression. For example, medial preoptic area and nucleus show altered brain activity in association with maternal aggression (Gammie & Nelson 1999,2001;Hasen & Gammie 2005). Paraventricular nucleus likewise exhibits altered neuronal activity with maternal aggression testing (Gammie & Nelson 2001;Hasen & Gammie 2005) and lesions of this region alter maternal aggression output (Consiglio & Lucion 1996;Giovenardi *et al.* 1998). Lateral hypothalamus is the sole brain region containing of hypocretin neurons and recent work found this peptide to modulate maternal aggression (D'Anna & Gammie 2006). The use of high-density oligonucleotide microarrays allowed for the simultaneous examination of ∼ 40,000 genes or expressed sequence tags. The aim of study was to identify genes that could contribute to maternal aggression output. We report here the gene expression profiles of S and C mice using high-density oligonucleotide microarrays, identify new genes of interest, compare results to known regulators of maternal aggression, and discuss the relevance of gene expression changes to the biology of maternal aggression.

Materials and methods

Experimental Subjects

Female (focal) mice came from an on-going selection study for high maternal aggression. The founding population of S and C mice were derived from outbred hsd:ICR mice (*Mus domesticus*) (Harlan, Madison, WI). The test mice in this study came from the second litter of S and C dams tested in Generation 5 of the selection study (Gammie *et al.* 2006). All animals were age matched (∼70 days old at time of dissection). For mating, each female was housed with a single breeder male (hsd:ICR strain; purchased separately from Harlan and not related to focal mice) for 2 weeks. When breeder males were removed, each female was housed singly and provided precut nesting material until dissections. Polypropylene cages were changed once weekly, but when pups were born (postpartum Day 0), cages were not changed for any animals for the remainder of the experiment. Pups were culled to 12 on postpartum Day 0. All animals were housed in the same room and cages of S and C females were alternated with one another

on the same shelves. All dissections occurred on postpartum Day 5. A 14:10 light/dark cycle with lights on at 0600 CST was used. Female mice were given ad lib access to breeder chow (Harlan) and tap water whereas breeder and intruder males were provided with regular rodent chow. Intruder male mice were of the hsd:ICR strain and were group housed 4 per cage. All animal work was conducted with accepted standards of humane care and studies were approved by the University of Wisconsin animal care and use committee.

Maternal aggression testing

On postpartum Day 5, each dam was exposed to an intruder male for 4 min in her home cage between 0900 and 1130 h. The pups were removed from the cage just prior to the behavioral test. Removal of the pups from a dam just before an aggressive test does not diminish the expression of maternal aggression in mice (Svare *et al.* 1981). The day of testing occurred within the window of peak maternal aggression that occurs from postpartum Day 4 though 10 in mice (Svare 1990). An intruder male mouse was placed in the dam's home cage and the test session was recorded on videotape and subsequently analyzed off-line to quantify maternal aggression. Maternal aggression scoring was conducted by individuals blind to experimental conditions and treatments. For quantification of maternal aggression total duration of attacks was examined. At the completion of each test, the dams were immediately killed and brain tissue collected as described below.

Tissue collection

On postpartum Day 5, brains were removed from 13 S and 14 C females immediately following a 4 min aggression test. Dissections occurred between 0900 and 1130h. Dissections of the 12 mice (6 S and 6 C) used for gene expression analysis all occurred within the same week. S mice used for array analysis all met criterion (at least 40 sec of total time aggression) and C mice were randomly chosen from a group that was within 2 standard deviations of the C group mean time aggressive (4 sec). Animals were killed by cervical dislocation and then decapitated. The whole brain was removed and immediately placed ventral side up on a covered Petri dish filled with ice. Major cuts to the whole brain were performed using a razor blade and smaller cuts were performed using a scalpel. All cuts were made under a dissecting microscope. Vertical cuts were made at Bregma −0.70mm and Bregma −2.06mm using external landmarks and were verified after cutting by identifying key landmarks on the cut surface of the brain. Focal tissue was separated by running the scalpel blade along the line of the optic nerve as it ascends into the brain and then cutting laterally toward the midline using the bottom of the lateral ventricle as a landmark (see Fig. 1).

The dissected region was frozen in a plastic tube on dry ice and stored at -80 C until processing. Total RNA was isolated using a GenElute Mammalian Total RNA Miniprep Kit (Cat#RTN-70, Sigma-Aldrich, St. Louis, MO). Following isolation, RNA concentration was determined using a BioMate 3 spectrophotometer (Thermo Spectronic, Lanham, MD) and stored at −70°C until being processed for either gene array analysis or real-time PCR.

High-Density Oligonucleotide Array Hybridization

Microarray analysis was performed with Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) using targets derived from total RNA isolated from mouse CNS as described above. RNA was prepared and labeled following the protocols and procedures described in the Affymetrix Expression Analysis Technical Manual (www.affymetrix.com). In brief, after total RNA was isolated, double stranded cDNA was synthesized from purified RNA and was used as a template to synthesize biotin-labeled cRNA by *in vitro* transcription using the GeneChip array IVT Labeling kit (Affymetrix). Amplified cRNA was fragmented and hybridized to the arrays according to the manufacturer's procedures (www.affymetrix.com). Target preparation, hybridization and data collection were performed

by the Gene Expression Center at the University of Wisconsin-Madison. To extract the fluorescent signal from each feature on the GeneChip array, all arrays were scanned at 570 nm using the Affymetrix GC3000 Scanner. Fluorescent signals corresponding to hybridization intensities were analyzed with the Affymetrix GCOS vs 1.2.1 software using the following settings: Algorithm defaults, Alpha1, 0.05; alpha2, 0.065; Tau, 0.015; Gamma 1L 0.0045; Gamma 1H 0.0045; Gamma 2L 0.006; Gamma2H 0.006; Perturbation 1.1. In all analyses the probe sets were scaled to a target signal of 1000 using the "Scale" function in the GCOS software.

Statistical Analysis

Two statistical approaches were used for analysis. First, robust multiarray analysis (RMA) (Han *et al.* 2004;Irizarry *et al.* 2003) was run on the data set using ArrayAssist (Iobian Informatics, La Jolla, CA). RMA models are additive on the log2 intensity scale with additive error and are fit iteratively for each probe set. Second, the Probe Logarithmic Intensity Error (PLIER) method (Affymetrix) employing perfect match signaling was used. PLIER accounts for the difference between probes by means of probe affinities which represent the strength of signal produced at a specific concentration for a given probe. Probe affinities are calculated using experimental data across multiple arrays. PLIER was designed to improve analysis of both high and low intensity signals.

Verification of microarray with real-time PCR

RNA was purified as described above and was taken from the same mice as used for array analysis. A StrataScript First Strand Synthesis system kit (Cat# 200420, Stratagene, Cedar Creek, TX) was used to reverse transcribe 1 μg of RNA to cDNA in an Eppendorf MasterCycler Personal PCR machine. The cDNA was then amplified using Invitrogen LUX primers (Invitrogen, Carlsbad, CA) in combination with hypoxanthine phosphorybosyltransferase 1 (HPRT1, Cat#105M-02, Invitrogen). All primers were designed using Invitrogen D-LUX Designer (Invitrogen) (locations shown in Table 1) and were targeted to regions of the gene identified as being significantly altered in expression by the Affymetrix gene array probes. The genes were chosen for PCR analysis with a focus on possible maternal aggression pathways. For example, Nos1 had previously been implicated in maternal aggression. Although neither Crhbp, neurotensin, Gabr1a, nor Npy2r had specifically been implicated in aggression, they are parts of pathways regulating stress reactivity that could modulate aggression. The K+ channel, Kcna1, was examined because more than one probe set identified this gene as significant (see Supplemental Table 1) and other K+ channel subunits were upregulated in S mice (see Table 2), so altered K+ channel activity could indicate an important, but not obvious, means for regulating aggression In all cases (except for Gabra1) the standard gene accession number was used to load the gene sequence on which the D-LUX Designer created the probe. For Gabra1, the mouse RNA transcript, AK141596, was used as this allowed for targeting near the distal end of the gene identified as having altered expression in S mice. However, these primers used still were not able to cover the region identified by the Affymetrix probe. HPRT1 is commonly used as a reference gene in Real-Time PCR. Primers labeled with either the fluorogenic reporter dyes 6-carboxy-fluorescein (FAM) or 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethyoxyfluorescein (JOE) were multiplexed in the same tube during the Real-Time PCR reaction. This allowed for the amplification of the target and control gene within the same reaction tube. Kcna1, neurotensin, Nos1, Npy2r, Crhbp, and Gabra1 were labeled with FAM, which is read at 492 -516 nm wavelengths. HPRT1 was labeled with JOE, which is read at 535 -555nm wavelengths. In order to more tightly control for variation in the amplification procedure, we performed a dilution curve using the HPRT1 primers so that the range of amplification more closely resembled that of the gene of interest.

Quantitative Real-Time PCR was carried out in a Stratagene Mx3000P Real-time PCR system. Each sample was run in triplicate. The amplification protocol is as follows: an initial annealing step at 50°C for 2 min and an initial melting step at 95°C for 2 min, followed by 35 cycles of a 95°C melting step for 15 sec, a 55°C annealing step for 30 sec, and a 72°C elongation step for 30 sec. Following amplification, a dissociation curve analysis was performed to insure purity of PCR products. Data were analyzed under the following program term settings: (a) threshold fluorescence-amplification-based, (b) baseline correction-adaptive baseline with Mx4000 v 1.00 to v 3.00 algorithm, and (c) smoothing- moving average with amplification averaging 3 points. Relative mRNA levels were calculated using the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001). Briefly, the average C_T of the reference gene (HPRT1) was subtracted from the average C_T of the gene of interest (i.e., neurotensin or Npy2r) to determine the ΔC_T for each sample. The ΔC_T of the calibrator (an untreated control) is then subtracted from the ΔC_T of each of the samples to determine the $\Delta\Delta C_T$. This number is then used to determine the amount of mRNA relative to the calibrator and normalized by HPRT1, or the n-fold difference. The n-fold difference was calculated by the equation $2^{(-\Delta\Delta CT)}$. Statistical comparisons were determined using Sigma Stat statistical analysis software for Windows v 3.11 (SPSS Inc, Chicago, IL). One-tailed *t* tests were used, as we were able to predict direction based on data from the microarrays.

Results

Aggression differences between S and C mice

As expected, S mice were highly aggressive relative to the C mice. The 6 S mice and 6 C mice used for microarray analysis differed significantly in terms of total duration of attacks H(1,11) = 8.6, p = 0.002 (one-way ANOVA on Ranks) (Fig. 2). In terms of sites of attack, no differences statistical differences were found between groups (data not shown). On average for both groups, 77% of attacks were to the back/flank or belly (considered offensive attacks) and 23% of attacks were to the head/neck region (considered defensive attacks).

Gene expression in the preoptic area/hypothalamus

Using an intersection of genes showing the greatest significant differences for RMA and PLIER techniques we identified ∼200 genes with a mean (intersected) p value less than 0.019. That is, for 40,000 genes only ∼200 (or less than 0.5%) were identified as significant. Of those, the known genes are shown in Table 2. The full list of all 40,000 genes, their relative expression, and p-value ranking using both statistical techniques is presented in Supplemental Table 1. The genes that displayed significant differences between S and C mice were distributed across a number of categories (Table 2). The function/category of each gene was determined individually using PubMed and GenBank databases.

Real-time PCR analysis

Confirming the high-density oligonucleotide array results, we found that Kcna1, neurotensin, Nos1, and Npy2r were significantly altered in S relative to C mice (Fig. 2). For Crhbp, the differences between groups were just above significance (p = 0.051) and for Gabra1 there was a trend towards increased expression in S mice, but this did not reach significance.

Discussion

This study used high density oligonucleotide arrays in conjunction with an on-going selection study to identify genes in the CNS that may support maternal aggression. The portion of CNS examined (a continuous region including preoptic and hypothalamic regions) was chosen for examination because it had previously been implicated in the regulation of maternal aggression (see above). Some of genes identified are consistent with previous studies on maternal

aggression using different approaches. This work also identified new candidate genes in the regulation of maternal aggression that can be examined in subsequent hypothesis directed studies.

Gene expression differences between S and C mice that could underlie aggression differences

Three neurotransmitter receptors exhibit differential expression in S mice and each could contribute to elevations in maternal aggression. The decrease in neuropeptide Y (NPY) receptor 2 (Npy2r) in S mice is interesting because the knockout of Npy2r results in decreased fear and anxiety (Redrobe *et al.* 2003;Tschenett *et al.* 2003) and decreased anxiety has been linked to elevated maternal aggression (Lonstein & Gammie 2002). NPY has anxiolytic effects (Heilig 2004;Karlsson *et al.* 2005) in addition to its role in regulating feeding behavior (Kalra & Kalra 2003). Because Npy2r is an autoreceptor, it is thought that antagonizing this receptor leads to elevated NPY release and hence decreased anxiety (Heilig 2004). The increase in adenosine A1 receptor (Adora1) in S mice is intriguing because deletion of this gene elevates anxiety in some tests (Gimenez-Llort *et al.* 2002;Lang *et al.* 2003). This would suggest that increases in S mice of this receptor could decrease anxiety and thereby elevate aggression. Neither Npy2r nor Adora1 has previously been implicated in the regulation of maternal aggression.

The increases in Gabra1 in S mice were not confirmed by real-time PCR, but high quality PCR probes could not be developed for the specific site of the Gabra1 gene (the distal end) identified by the Affymetrix probes as showing increased expression. The PCR probes used here were upstream of the target, but did not cover it. Increased GABAergic neurotransmission has been shown to facilitate maternal aggression (Hansen *et al.* 1985;Mos & Olivier 1989), so increased expression of a GABA receptor subunit would not be unexpected in the regulation of maternal aggression. In would be valuable in future work to examine whether the transcript identified by the Affymetrix probe is of biological relevance to maternal aggression or not.

Elevated CRF binding protein (Crhbp) in S mice (almost confirmed by PCR, p=0.051) is interesting because recent work has shown an inhibitory role for CRF (and related peptides) in maternal aggression (D'Anna *et al.* 2005;Gammie *et al.* 2004). CRF binding protein acts to blunt CRF action (Seasholtz *et al.* 2001) and mice missing this gene exhibit elevated anxiety (Karolyi *et al.* 1999). Thus, in S mice increased production of CRF binding protein could both decrease anxiety and elevate aggression by suppressing CRF action.

The elevation of neuronal nitric oxide (NO) synthase 1 (Nos1) (confirmed by real-time PCR) in S mice suggests increased production of the signaling molecule, NO, could facilitate maternal aggression. This result is consistent with previous work showing that deletion of Nos1 results in dramatic reductions in maternal aggression (Gammie & Nelson 1999) and that pharmacological disruption of NO production decreases maternal aggression in prairie voles (Gammie *et al.* 2000) and rats (Popeski & Woodside 2004). Additionally, increased production of Nos1 occurs during lactation (Popeski *et al.* 1999) and one possibility for this change is to support maternal aggression. Thus, increased expression of Nos1 in S mice may have contributed to elevated aggression in these mice.

Decreased expression of neurotensin in S mice is intriguing and somewhat unexpected. Neurotensin is known for its possible role in schizophrenia and action of antipsychotic drugs (Kinkead & Nemeroff 2006) as well as stress induced analgesia (Dobner 2005), but a possible role for this neuropeptide in maternal aggression has not previously been suggested. Neurotensin can modulate CRF activity (Rostene & Alexander 1997;Rowe *et al.* 1995), a known modulator of maternal aggression (Gammie *et al.* 2004), so neurotensin may alter aggression through this pathway.

Another unexpected finding was the elevation in S mice of 5 different genes that are part of K + channel activation. The increase in Kcna1 in S mice was confirmed by real-time PCR. The loss of Kcna1 in mice causes elevated seizure-like activity (Smart *et al.* 1998), so elevated production of Kcna1 could act to dampen overexcitability. However, a clearer understanding of how changes in K+ conductance supports maternal aggression would require that the identity of the neurons containing these channels be determined before function could be properly tested.

The consistent increase in expression in S mice of genes involved in metabolism (24 out of 24) (Table 2) suggests that the S mice have an altered metabolism that could support increased aggressive output. Metabolism alters during the life-history of a number of animals and an increase in metabolism occurs during lactation in mice (as for other mammals) (Speakman *et al.* 2004). Thus, the ability to exhibit elevated maternal aggression in S mice may be bootstrapped in part to the ability to exhibit an elevation of metabolism.

The finding of elevated Fos and Egr1 in S mice is consistent with the elevated expression of metabolism genes described above. Further, increases in expression of both Fos and Egr1 occur with lactation and sensory input from pups (Li *et al.* 1999;Numan & Insel 2003;Numan & Numan 1994;Numan *et al.* 1998) and it is thought that this activity reflects increased neuronal activity that supports maternal care. One explanation for the elevation of both Fos and Egr1, then, is that baseline neuronal activity is higher in S mice and that this may support the ability to produce higher levels of maternal aggression.

As indicated above, S and C mice show differences in levels of aggression, but not in terms of sites of attacks on the male. The breakdown of sites of attacks in S and C mice seen here is similar to that found in a previous examination of these mice (Gammie *et al.* 2006). Traditionally, attacks to the back/flank region, including belly, in males, especially rats, has been termed offensive aggression, whereas attacks to the face/neck region have been termed defensive attacks (Blanchard & Blanchard 1981). Hence, maternal aggression contains elements of both forms of aggression. It is not clear to what extent any of the findings here may be relevant to male offensive aggression because both forms of aggression contain overlapping (Parmigiani *et al.* 1998) and differing (Del Punta *et al.* 2002;Gammie *et al.* 2005b;Gammie & Lonstein 2005;Gammie & Nelson 1999;Parmigiani *et al.* 1998) signaling components. For example, elevated Nos1 in S mice would likely not support intermale aggression because as indicated above NO is positively associated with maternal aggression, but negatively associated with intermale aggression (Demas *et al.* 1997;Nelson *et al.* 1995). For other genes, such as Crhbp and Npy2r, the change in expression in S mice would be expected to decrease anxiety and in some cases lower anxiety can alter offensive intermale aggression, for review, see (Blanchard & Blanchard 2005), so these gene changes could support enhanced intermale aggression. To date, we have not finished analysis of intermale aggression in S and C mice, but preliminary results indicate there are no overt differences in aggression between genotype (S.C. Gammie and S.A. Stevenson, unpublished observations). Whether or how gene expression changes found here relate to intermale aggression will have to be addressed in subsequent studies.

Methodological considerations

In this study, we chose to examine a continuous region of the CNS (that included both preoptic and hypothalamic regions) in order to observe gene expression difference in S versus C mice that could support altered aggressive output. As indicated above, this brain area was chosen for analysis because it contains brain regions previously implicated in the regulation of maternal aggression. Additional regions that support maternal aggression, including amygdale, were also collected, but have not been analyzed. These could provide further insights into the regulation of aggression. Although this dissection was focused on preoptic areas and a selected

portion of hypothalamus, one drawback could be a dilution effect such that small RNA changes are more difficult to detect as the amount of sampled tissue increases. Another possible drawback is that if changes in gene expression occur in opposite directions within different subregions collected, then changes may cancel each other out. In other studies, array examinations of larger brain regions have proven to be useful. The WebQTL database maintained by the GeneNetwork, www.genenetwork.org, uses array analysis of large forebrain regions for detecting gene expression among recombinant inbred mouse strains and this database has already been used to make important biological insights (Scott *et al.* 2005). Further, using microarrays and a larger dissected region than used here, we recently confirmed a number of known changes in gene expression that occur with lactation (Gammie *et al.* 2005a).

In this study we used a brief test (4 min) immediately before tissue collection. Although it is possible that brief exposure to the intruder male differentially altered gene expression in S and C mice, we think this likely had minimal impact on our results for the following reasons. 1) The time-course for onset of expression differences of even the earliest responding genes to stimulus, such as Fos, is at least 10 minutes (Ginsberg *et al.* 2006) and brains were collected prior to that interval. 2) Many genes that show altered expression in response to stimulus occur in the hours long range, such as genes that responds to elevated glucocorticoids (Morsink *et al.* 2006), and the stimulus in this study is well short of that range. 3) Although the S mice showed on average 50 more seconds of aggression than the C mice, this still only represented ~20% of the test time, so for the majority of the 4 min test mice from both groups were in similar non-fighting conditions.

As with any study examining just gene expression, it is not known to what extent mRNA differences are translated into functional protein that then contributes to phenotypic differences. Recent work suggests that in many cases, RNA alterations identified by gene arrays can be highly consistent with changes in protein expression (Kern *et al.* 2003) or show similarities with varying degrees of concordance (Bianchi *et al.* 2005;Cham *et al.* 2003;Li *et al.* 2004). For two of the genes identified by this study as having altered expression with selection, Nts and Crhbp, a good concordance of RNA levels with protein expression has been found (Chatzaki *et al.* 2002;Smits *et al.* 2004). Hypothesis directed studies based on information provided by this study along with examinations of protein expression, then, will be critical steps in understanding whether or how genes identified here contribute to maternal aggression behavior.

Because in this study we compared separated populations, the fixation of gene alleles due to random genetic drift could have contributed to some of our expression differences. However, we think the contributions from random genetic drift are low in this study for a few reasons. 1) The number of generations of separation between S and C populations was relatively low (5 generations of selection). 2) The technique of within-family selection we employed in this study was designed to maximize the effective population size, minimize inbreeding, and hence slow down random fixation (Swallow *et al.* 1998). 3) 26 breeders were used to maintain each S and C population for each generation (with overall population reaching on average 150 mice each generation) and larger population sizes decrease the rate of fixation (Falconer 1989). As a comparison, a study in *Drosophila* using 16 breeders per generation (and 117 different populations) found the first fixation of a given gene occurred at generation 4 and that was in only one of the 117 lines (Buri 1956). In computer simulations using a breeding population size of 40, fixation occurs for the first time only after 35 generations (Freeman & Herron 1998). Further, recent work indicates that for selected traits for which there is a strong difference from the non-selected controls (as for us with only a few generations of selection), that most correlated traits are likely associated with the selection itself and not genetic drift (Konarzewski *et al.* 2005). An important point for this caveat of possible genetic drift as well

as the others described above, is that any gene identified in this study not be considered an end-point, but rather a new starting point for developing hypothesis directed studies aimed at understanding the genetic basis of maternal aggression.

Conclusions

In this study we examined gene expression differences between a line of mice selected for high maternal aggression relative to a non-selected control line. Using high density arrays, we were able to identify candidate genes that may regulate maternal aggression. A subset of the identified genes are consistent with work from previous studies using different approaches, but a new interesting set of candidate genes was also identified. One value of this study, then, was to use a unique mouse model to provide insights into the genetics of maternal aggression that can then be followed up with hypothesis directed studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

Agrell J, Wolff JO, Ylonen H. Counter-strategies to infanticide in mammals: costs and consequences. Oikos 1998;83:507–517.

Bianchi L, Canton C, Bini L, Orlandi R, Menard S, Armini A, Cattaneo M, Pallini V, Bernardi LR, Biunno I. Protein profile changes in the human breast cancer cell line MCF-7 in response to SEL1L gene induction. Proteomics 2005;5:2433–2442. [PubMed: 15880780]

Blanchard, DC.; Blanchard, RJ. Stress and aggressive behavior. In: Nelson, RJ., editor. Biology of Aggression. Oxford University Press; New York: 2005.

Blanchard, RJ.; Blanchard, DC. The organization and modeling of animal aggression. In: Brain, PF.; Benton, D., editors. The biology of aggression. Sijthoff and Noordhoff; Alphen aan den Rijn: 1981. p. 529-561.

Bronikowski AM, Rhodes JS, Garland T Jr, Prolla TA, Awad TA, Gammie SC. The evolution of gene expression in mouse hippocampus in response to selective breeding for increased locomotor activity. Evolution 2004;58:2079–2086. [PubMed: 15521463]

Buri P. Gene frequency in small populations of mutant *Drosophila*. Evolution 1956;10:367–402.

Cham CM, Xu H, O'Keefe JP, Rivas FV, Zagouras P, Gajewski TF. Gene array and protein expression profiles suggest post-transcriptional regulation during CD8+ T cell differentiation. J Biol Chem 2003;278:17044–17052. [PubMed: 12582156]

Chatzaki E, Margioris AN, Gravanis A. Expression and regulation of corticotropin-releasing hormone binding protein (CRH-BP) in rat adrenals. J Neurochem 2002;80:81–90. [PubMed: 11796746]

Consiglio AR, Lucion AB. Lesion of hypothalamic paraventricular nucleus and maternal aggressive behavior in female rats. Physiol Behav 1996;59:591–596. [PubMed: 8778839]

D'Anna KD, Gammie SC. Hypocretin-1 dose-dependently modulates maternal behaviour in mice. J Neuroendocrinol. 2006(in press)

D'Anna KD, Stevenson SA, Gammie SC. Urocortin 1 and 3 impair maternal defense behavior in mice. Behav Neurosci 2005:161–171.

Del Punta K, Leinders-Zufall T, Rodriguez I, Jukam D, Wysocki CJ, Ogawa S, Zufall F, Mombaerts P. Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. Nature 2002;419:70–74. [PubMed: 12214233]

Demas GE, Eliasson MJ, Dawson TM, Dawson VL, Kriegsfeld LJ, Nelson RJ, Snyder SH. Inhibition of neuronal nitric oxide synthase increases aggressive behavior in mice. Mol Med 1997;3:610–616. [PubMed: 9323712]

Dobner PR. Multitasking with neurotensin in the central nervous system. Cell Mol Life Sci 2005;62:1946–1963. [PubMed: 16003489]

Ebensperger L. The potential effects of protected nests and cage complexity on maternal aggression in house mice. Aggressive Behavior 1998;24:385–396.

Falconer, DS. Introduction to quantitative genetics. 3rd. Longman Scientific & Technical; Wiley, Burnt Mill, Harlow, Essex, England, New York: 1989.

Feldker DE, Datson NA, Veenema AH, Meulmeester E, de Kloet ER, Vreugdenhil E. Serial analysis of gene expression predicts structural differences in hippocampus of long attack latency and short attack latency mice. Eur J Neurosci 2003a;17:379–387. [PubMed: 12542675]

Feldker DE, Datson NA, Veenema AH, Proutski V, Lathouwers D, De Kloet ER, Vreugdenhil E. GeneChip analysis of hippocampal gene expression profiles of short- and long-attack-latency mice: technical and biological implications. J Neurosci Res 2003b;74:701–716. [PubMed: 14635221]

Freeman, S.; Herron, JC. Evolutionary analysis. Prentice Hall; Upper Saddle River, NJ: 1998.

Gammie SC, Garland T, Stevenson SA. Artificial selection for maternal defense behavior in mice. Behav Genet. 2006(in press)

Gammie SC, Hasen NS, Awad TA, Auger AP, Jessen HM, Panksepp JB, Bronikowski AM. Gene array profiling of large hypothalamic CNS Rregions in lactating and randomly cycling virgin mice. Brain Res Mol Brain Res 2005a;139:201–211. [PubMed: 15953654]

Gammie SC, Hasen NS, Stevenson SA, Bale TL, D'Anna KD. Elevated stress sensitivity in corticotropin-releasing factor receptor 2 deficient mice decreases maternal, but not intermale aggression. Behav Brain Res 2005b;160:169–177. [PubMed: 15836912]

Gammie, SC.; Lonstein, JS. Maternal aggression. In: Nelson, RJ., editor. Biology of Aggression. Oxford University Press; New York: 2005.

Gammie SC, Negron A, Newman SM, Rhodes JS. Corticotropin-releasing factor inhibits maternal aggression in mice. Behav Neurosci 2004;118:805–814. [PubMed: 15301606]

Gammie SC, Nelson RJ. Maternal aggression is reduced in neuronal nitric oxide synthase-deficient mice. J Neurosci 1999;19:8027–8035. [PubMed: 10479702]

Gammie SC, Nelson RJ. cFOS and pCREB activation and maternal aggression in mice. Brain Res 2001;898:232–241. [PubMed: 11306009]

Gammie SC, Olaghere-da Silva UB, Nelson RJ. 3-Bromo-7-nitroindazole, a neuronal nitric oxide synthase inhibitor, impairs maternal aggression and citrulline immunoreactivity in prairie voles. Brain Res 2000;870:80–86. [PubMed: 10869504]

Gimenez-Llort L, Fernandez-Teruel A, Escorihuela RM, Fredholm BB, Tobena A, Pekny M, Johansson B. Mice lacking the adenosine A1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur J Neurosci 2002;16:547–550. [PubMed: 12193199]

Ginsberg AB, Frank MG, Francis AB, Rubin BA, O'Connor KA, Spencer RL. Specific and time-dependent effects of glucocorticoid receptor agonist RU28362 on stress-induced pro-opiomelanocortin hnRNA, c-fos mRNA and zif268 mRNA in the pituitary. J Neuroendocrinol 2006;18:129–138. [PubMed: 16420282]

Giovenardi M, Padoin MJ, Cadore LP, Lucion AB. Hypothalamic paraventricular nucleus modulates maternal aggression in rats: effects of ibotenic acid lesion and oxytocin antisense. Physiol Behav 1998;63:351–359. [PubMed: 9469726]

Han ES, Wu YM, McCarter R, Nelson JF, Richardson A, Hilsenbeck SG. Reproducibility, sources of variability, pooling, and sample size: Important considerations for the design of high-density oligonucleotide array experiments. J Gerontol a-Biol 2004;59:306–315.

Hansen S, Ferreira A, Selart ME. Behavioural similarities between mother rats and benzodiazepine-treated non-maternal animals. Psychopharmacology (Berl) 1985;86:344–347. [PubMed: 2994146]

Hasen NS, Gammie SC. Differential fos activation in virgin and lactating mice in response to an intruder. Physiol Behav 2005;84:684–695.

Heilig M. The NPY system in stress, anxiety and depression. Neuropeptides 2004;38:213–224. [PubMed: 15337373]

Heise S, Lippke J. Role of female aggression in prevention of infanticidal behavior in male common voles, *Microtus arvalus* (Pallas, 1779). Aggress Behav 1997;23:293–298.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249–264. [PubMed: 12925520]

Kalra SP, Kalra PS. Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. Endocrine 2003;22:49–56. [PubMed: 14610298]

Karlsson RM, Holmes A, Heilig M, Crawley JN. Anxiolytic-like actions of centrally-administered neuropeptide Y, but not galanin, in C57BL/6J mice. Pharmacol Biochem Be 2005;80:427–436.

Karolyi IJ, Burrows HL, Ramesh TM, Nakajima M, Lesh JS, Seong E, Camper SA, Seasholtz AF. Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice. Proc Natl Acad Sci U S A 1999;96:11595–11600. [PubMed: 10500222]

Kern W, Kohlmann A, Wuchter C, Schnittger S, Schoch C, Mergenthaler S, Ratei R, Ludwig WD, Hiddemann W, Haferlach T. Correlation of protein expression and gene expression in acute leukemia. Cytometry Part B, Clinical Cytometry 2003;55:29–36.

Kinkead B, Nemeroff CB. Novel treatments of schizophrenia: targeting the neurotensin system. CNS & Neurol Disorders Drug Targets 2006;5:205–218.

Konarzewski M, Ksiazek A, Lapo IB. Artificial selection on metabolic rates and related traits in rodents. Integr Comp Biol 2005;45:416–425.

Lang UE, Lang F, Richter K, Vallon V, Lipp HP, Schnermann J, Wolfer DP. Emotional instability but intact spatial cognition in adenosine receptor 1 knock out mice. Behav Brain Res 2003;145:179–188. [PubMed: 14529816]

Leypold BG, Yu CR, Leinders-Zufall T, Kim MM, Zufall F, Axel R. Altered sexual and social behaviors in trp2 mutant mice. Proc Natl Acad Sci U S A 2002;99:6376–6381. [PubMed: 11972034]

Li C, Chen P, Smith MS. Neural populations in the rat forebrain and brainstem activated by the suckling stimulus as demonstrated by cFos expression. Neuroscience 1999;94:117–129. [PubMed: 10613502]

Li W, Amri H, Huang H, Wu C, Papadopoulos V. Gene and protein profiling of the response of MA-10 Leydig tumor cells to human chorionic gonadotropin. J Androl 2004;25:900–913. [PubMed: 15477362]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408. [PubMed: 11846609]

Lonstein JS, Gammie SC. Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. Neurosci Biobehav Rev 2002;26:869–888. [PubMed: 12667494]

Morsink MC, Steenbergen PJ, Vos JB, Karst H, Joels M, De Kloet ER, Datson NA. Acute activation of hippocampal glucocorticoid receptors results in different waves of gene expression throughout time. J Neuroendocrinol 2006;18:239–252. [PubMed: 16503919]

Mos J, Olivier B. Quantitative and comparative analyses of pro-aggressive actions of benzodiazepines in maternal aggression of rats. Psychopharmacology (Berl) 1989;97:152–153. [PubMed: 2567023]

Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson VL, Dawson TM, Snyder SH. Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. Nature 1995;378:383–386. [PubMed: 7477374]

Numan, M.; Insel, TR. The neurobiology of parental behavior. Springer; New York: 2003.

Numan M, Numan MJ. Expression of Fos-like immunoreactivity in the preoptic area of maternally behaving virgin and postpartum rats. Behav Neurosci 1994;108:379–394. [PubMed: 8037882]

Numan M, Numan MJ, Marzella SR, Palumbo A. Expression of c-fos, fos B, and egr-1 in the medial preoptic area and bed nucleus of the stria terminalis during maternal behavior in rats. Brain Res 1998;792:348–352. [PubMed: 9593990]

Ogawa, S.; Nomura, M.; Choleris, E.; Pfaff, DW. The role of estrogen receptors in the regulation of aggressive behaviors. In: Nelson, RJ., editor. Biology of Aggression. Oxford University Press; New York: 2005.

Parmigiani S, Ferrari PF, Palanza P. An evolutionary approach to behavioral pharmacology: using drugs to understand proximate and ultimate mechanisms of different forms of aggression in mice. Neurosci Biobehav Rev 1998;23:143–153. [PubMed: 9884108]

Parmigiani S, Palanza P, Rogers J, Ferrari PF. Selection, evolution of behavior and animal models in behavioral neuroscience. Neurosci Biobehav Rev 1999;23:957–969. [PubMed: 10580310]

Peripato AC, De Brito RA, Vaughn TT, Pletscher LS, Matioli SR, Cheverud JM. Quantitative trait loci for maternal performance for offspring survival in mice. Genetics 2002;162:1341–1353. [PubMed: 12454078]

Popeski N, Amir S, Woodside B. Changes in NADPH-d staining in the paraventricular and supraoptic nuclei during pregnancy and lactation in rats: role of ovarian steroids and oxytocin. J Neuroendocrinol 1999;11:53–61. [PubMed: 9918229]

Popeski N, Woodside B. Central nitric oxide synthase inhibition disrupts maternal behavior in the rat. Behav Neurosci 2004;118:1305–1316. [PubMed: 15598139]

Redrobe JP, Dumont Y, Herzog H, Quirion R. Neuropeptide Y (NPY) Y2 receptors mediate behaviour in two animal models of anxiety: evidence from Y2 receptor knockout mice. Behav Brain Res 2003;141:251–255. [PubMed: 12742262]

Rostene WH, Alexander MJ. Neurotensin and neuroendocrine regulation. Front Neuroendocrinol 1997;18:115–173. [PubMed: 9101258]

Rowe W, Viau V, Meaney MJ, Quirion R. Stimulation of CRH-mediated ACTH secretion by central administration of neurotensin: evidence for the participation of the paraventricular nucleus. J Neuroendocrinol 1995;7:109–117. [PubMed: 7767323]

Scott RE, White-Grindley E, Ruley HE, Chesler EJ, Williams RW. P2P-R expression is genetically coregulated with components of the translation machinery and with PUM2, a translational repressor that associates with the P2P-R mRNA. J Cell Physiol 2005;204:99–105. [PubMed: 15617101]

Seasholtz AF, Burrows HL, Karolyi IJ, Camper SA. Mouse models of altered CRH-binding protein expression. Peptides 2001;22:743–751. [PubMed: 11337087]

Smart SL, Lopantsev V, Zhang CL, Robbins CA, Wang H, Chiu SY, Schwartzkroin PA, Messing A, Tempel BL. Deletion of the K(V)1.1 potassium channel causes epilepsy in mice. Neuron 1998;20:809–819. [PubMed: 9581771]

Smits SM, Terwisscha van Scheltinga AF, van der Linden AJ, Burbach JP, Smidt MP. Species differences in brain pre-pro-neurotensin/neuromedin N mRNA distribution: the expression pattern in mice resembles more closely that of primates than rats. Brain Res Mol Brain Res 2004;125:22–28. [PubMed: 15193419]

Speakman JR, Krol E, Johnson MS. The functional significance of individual variation in basal metabolic rate. Physiological & Biochemical Zoology 2004;77:900–915. [PubMed: 15674765]

Svare, B. Maternal aggression: hormonal, genetic, and developmental determinants. In: Krasnegor, NA.; Bridges, RS., editors. Mammalian parenting : biochemical, neurobiological, and behavioral determinants. Oxford University Press; New York: 1990. p. 118-132.

Svare B, Betteridge C, Katz D, Samuels O. Some situational and experiential determinants of maternal aggression in mice. Physiol Behav 1981;26:253–258. [PubMed: 7195046]

Swallow JG, Carter PA, Garland T Jr. Artificial selection for increased wheel-running behavior in house mice. Behav Genet 1998;28:227–237. [PubMed: 9670598]

Tschenett A, Singewald N, Carli M, Balducci C, Salchner P, Vezzani A, Herzog H, Sperk G. Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. Eur J Neurosci 2003;18:143–148. [PubMed: 12859347]

Wolff JO. Maternal aggression as a deterrent to infanticide in *Peromyscus leucopus* and *P. maniculatus*. Anim Behav 1985;33:117–123.

Wolff JO. Why are female small mammals territorial. Oikos 1993;68:364–370.

Wolff JO, Peterson JA. An offspring-defense hypothesis for territoriality in female mammals. Ethol Ecol Evol 1998;10:227–239.

A
Bregma 0.26 mm

B
Bregma -0.22 mm

C
Bregma -0.70 mm

D
Bregma -1.58 mm

E
Bregma -2.06 mm

F
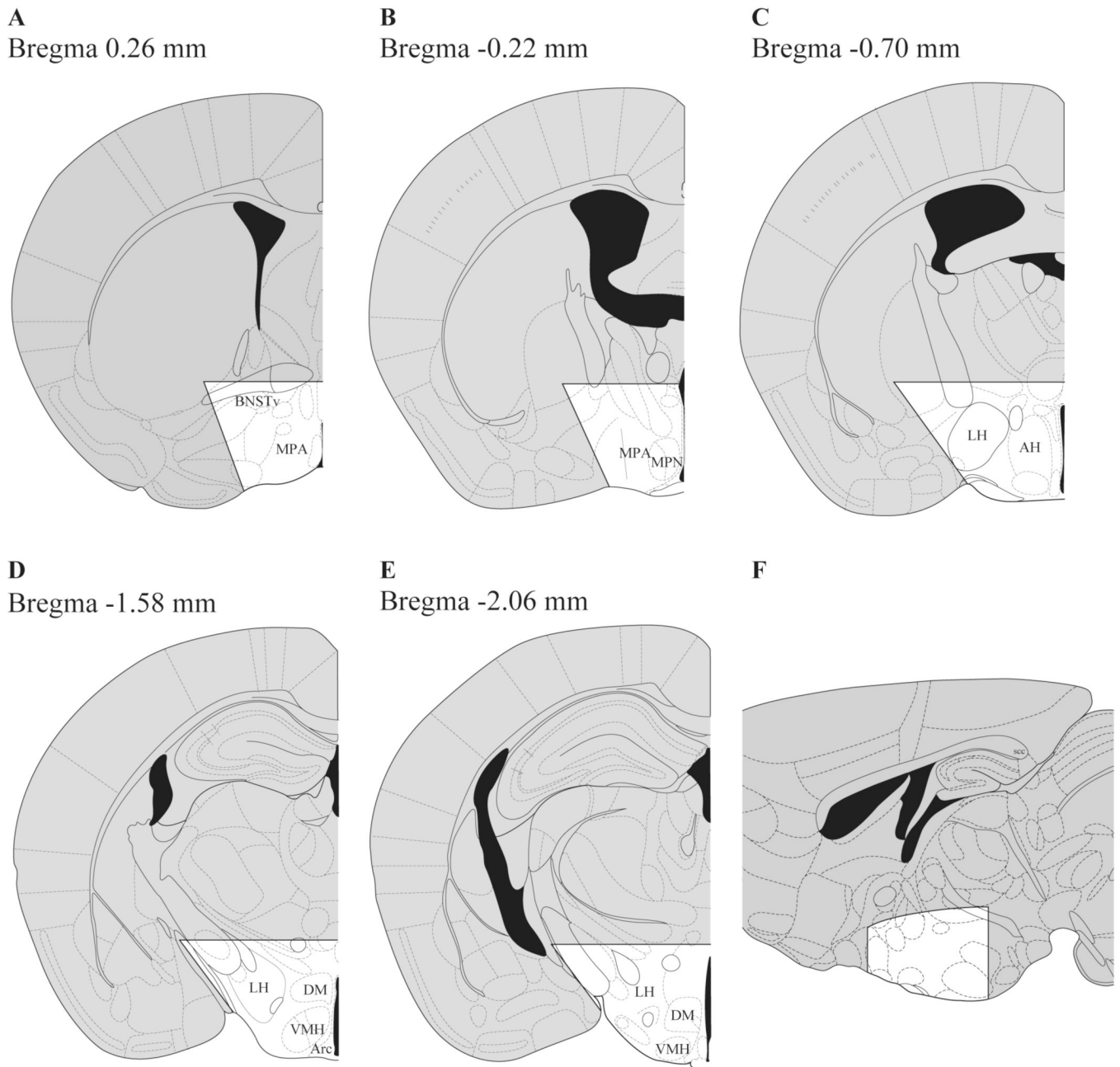
Fig 1.
Schematic representation of the brain regions (light, non-gray) dissected for gene array analysis. Abbreviation are: anterior hypothalamic area (AH); arcuate nucleus (Arc); bed nucleus of the stria terminalis, ventral (BNSTv); dorsomedial nucleus of the hypothalamus (DM); lateral hypothalamus (LH); medial preoptic area (MPA); medial preoptic nucleus (MPN); and ventromedial nucleus of the hypothalamus (VMH).
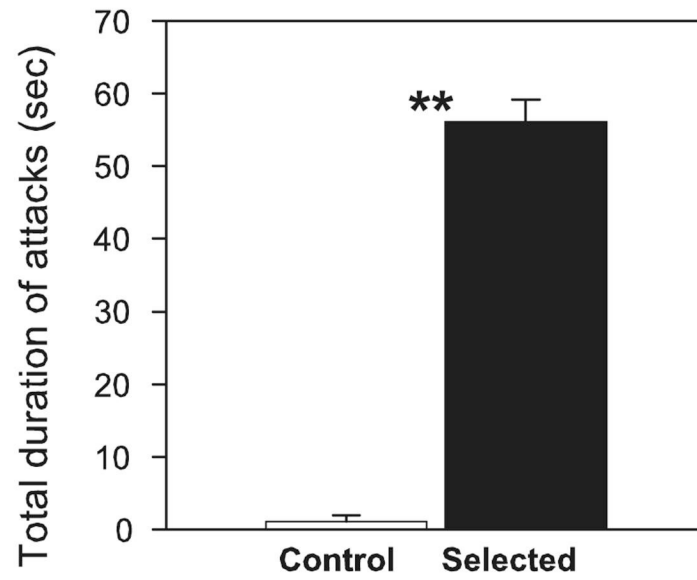
Fig 2.
Maternal aggression profile of the six C and six S mice used for microarray analysis in terms of total duration of attacks. ** = p < 0.01 (ANOVA on Ranks).
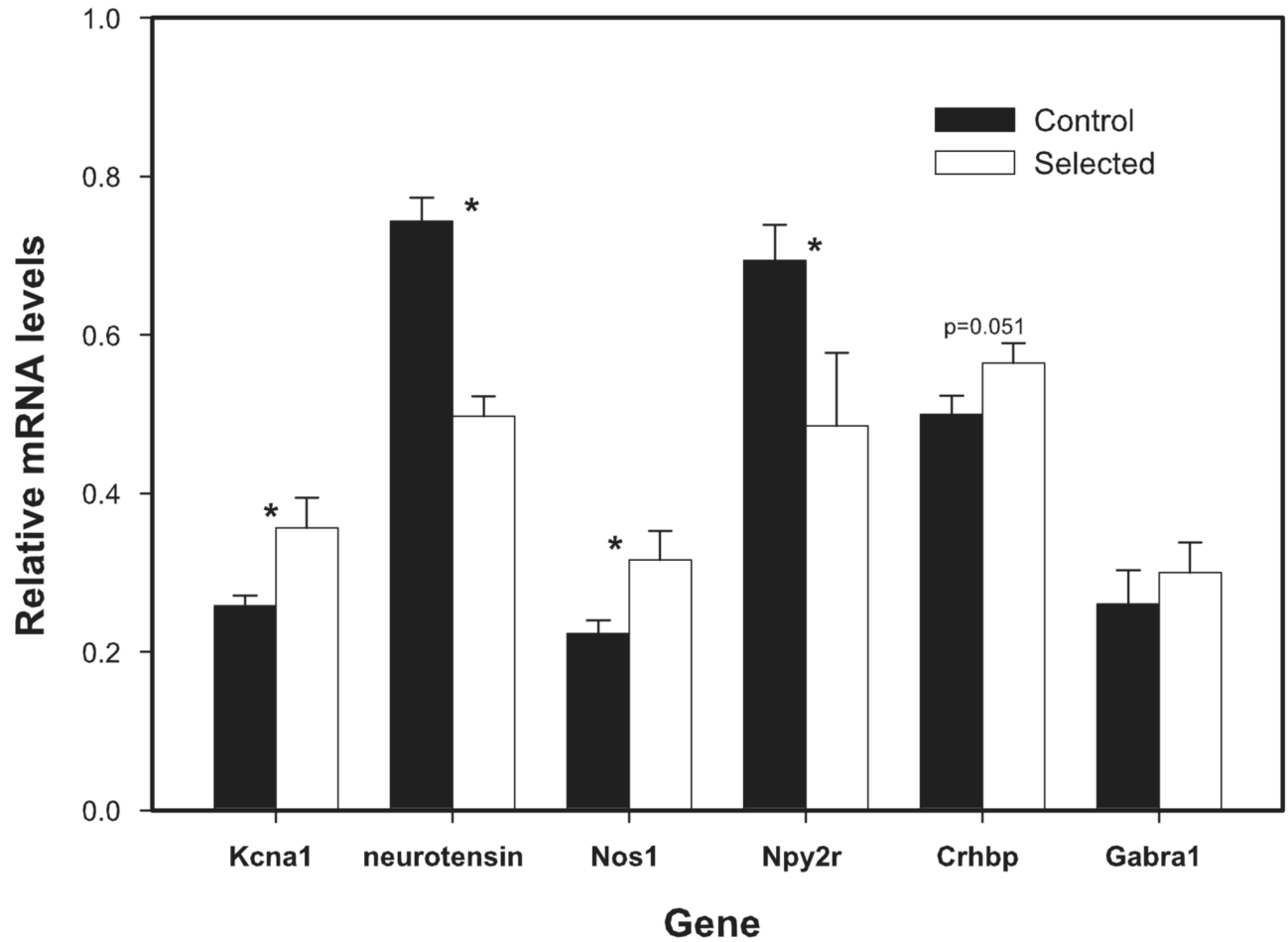
Fig 3.
Real-time PCR analysis of Kcna1, neurotensin, Nos1, Npy2r, Crhbp, and Gabra1 expression. Confirming array results, selection resulted in increased Kcna1 and Nos1 and decreased neurotensin and Npy2r mRNA levels relative to control mice. Increased expression of Crhbp in S mice was almost confirmed by PCR (p = 0.051). mRNA levels are expressed relative to the calibrator (see Methods for more details). * = p < 0.05, one-way ANOVA.

Table 1

List of genes and the primers used (along with primer position) for real-time PCR analysis.

Gene		Primer	Primer position
Kcna1	Forward	5′-CCA TGA CCA CTG TGG GAT ACG-3′	2198
	Reverse	5′-GCC TCC AAC TGT CAC AGG G-3′	2206
Neurotensin	Forward	5′-TAA ATA ACG TGA ACA GCC-3′	305
	Reverse	5′-CCA ACA AGG TCG TCA TCCA TGC-3′	326
Nos1	Forward	5′-TCA AGT ACG CCA CCA ACA AAG G-3′	1456
	Reverse	5′-TGA GGG AAT ATA GTG ATG GC-3′	1468
Npy2r	Forward	5′-CAT CAT ATC TTT CTC CTA CAC C -3′	1010
	Reverse	5′-GAC GTG GTT CCT CAG CTT ACT CC -3′	1016
Crhbp	Forward	5′-AGC TAG AAA CCT CGA CCG GAA AC-3′	1028
	Reverse	5′-CAT GTC AAT CAC TGA AGC-3′	1067
Gabra1	Forward	5′-CCT TAG TGC AGT GAA GTG GCA AT-3′	4417
	Reverse	5′-GTT TTG CTA AAC TCT GGA AAG-3′	4462

Table 2

List of genes showing highest significant differences in gene expression between S and C mice when using an intersection of RMA and PLIER statistics (intersected p<0.018). Fold change greater than 1.0 indicate increases in S relative to C mice.

Accession #	Fold change	Gene
Neuropeptide signaling		
AI854101	1.278535	corticotropin releasing hormone binding protein
NM_008731	0.750767	neuropeptide Y receptor Y2
NM_024435	0.740359	neurotensin
Receptor		
NM_053190	1.34706	endothelial differentiation sphingolipid G-protein-coupled receptor 8
AK013481	1.180269	Eph receptor A4
BE630294	1.141175	adenosine A1 receptor
BB279185	1.122029	progestin and adipoQ receptor family member IV
BE945884	1.118826	GABA A receptor subunit alpha 1
AW555641	1.116437	attractin like 1
AW121511(2)	1.081967	seizure related 6 homolog (mouse)-like 2
Ion channel		
L16912	1.978007	K+ large conductance calcium-activated channel subfamily M alpha member 1
BQ175978(3)	1.256841	K+ voltage-gated channel shaker-related subfamily member 1
BB750192	1.159445	K+ voltage gated channel shaw-related subfamily member 1
U31908	1.154564	K+ voltage-gated channel shaker-related subfamily beta member 2
BI408602	1.125937	K+ channel tetramerisation domain containing 17
Kinase/phosphatase		
AK017345	1.356632	pantothenate kinase 1
BB208212	1.156457	phosphatidylinositol 4-kinase type 2 alpha
NM_011049	1.15546	PCTAIRE-motif protein kinase 1
U35368	1.154432	protein tyrosine phosphatase receptor type E
L28176	1.144761	neurofibromatosis 2
BE136125	1.133364	dual specificity phosphatase 7
BG071931	1.123121	calcium/calmodulin-dependent protein kinase ID
Transcription factor		
BB579760	1.56199	zinc finger protein 191
AV026617	1.548904	FBJ osteosarcoma oncogene
NM_007913	1.335803	early growth response 1
BB322941	1.226015	nuclear receptor subfamily 4 group A member 2 (nurr1)
BC017622	1.187674	zinc finger FYVE domain containing 20
BE947440	1.169332	scratch homolog 1 zinc finger protein
AI875447	1.168121	zinc finger DHHC domain containing 9
AV322952	1.154524	forkhead box P2
BE993443	1.135146	POU domain class 3 transcription factor 3
D49658	0.491125	LIM homeobox protein 8
Signal transduction		
BM230524	1.379079	rap guanine nucleotide exchange factor 5
AV291679	1.263038	ras association domain family 4
BC027242	1.214817	vav 3 oncogene
BF453885	1.222874	CDC42 effector protein 2
NM_025531	1.210454	guanine nucleotide binding protein gamma 11
AV287690	1.209128	InaD-like
AF326561(2)	1.197196	SH3-domain GRB2-like 2
NM_019566	1.19588	ras homolog gene family member G
NM_009167	1.191388	src homology 2 domain-containing transforming protein C3
BC016250	1.185892	CDC42 effector protein 1
AK020972(2)	1.185843	SET binding factor 1
AV214969	1.160186	left-right determination factor 2
NM_009307	1.168606	synaptotagmin 2
NM_011838	1.15478	Ly6/neurotoxin 1

Accession #	Fold change	Gene
BC024864	1.147599	SH3 and cysteine rich domain 2
BC014803(2)	1.145801	complexin 1
AK014572	1.133278	solute carrier family 6 (glycine transporter) member 9
NM_008712	1.13129	nitric oxide synthase 1 neuronal
AV276781	0.842117	tripartite motif protein 23
BB025231	0.755404	nischarin
BE305862	0.712609	SH3 multiple domains 2
DNA-related function		
NM_007960	1.180149	ets variant gene 1
NM_025822	1.15719	arginine/serine-rich coiled-coil 1
BC027426	1.152873	cellular repressor of E1A-stimulated genes 1
NM_008671	1.064322	nucleosome assembly protein 1-like 2
BB409568	0.56779	euchromatic histone methyltransferase 1
Extracellular signaling		
BI452727	1.224902	follistatin-like 1
AA144045	1.178888	semaphorin 7A
BB444134	0.625103	follistatin
Metabolism		
NM_013467	2.067343	aldehyde dehydrogenase family 1 subfamily A1
AI596237	1.35663	lysosomal acid lipase 1
BC015253	1.269326	arachidonate 15-lipoxygenase second type
AI842353	1.256185	kazal-type serine protease inhibitor domain 1
BM951276	1.23124	fukutin related protein
BG067254	1.226121	coproporphyrinogen oxidase
BB048942	1.223529	polypeptide N-acetylgalactosaminyltransferase 9
BC003491	1.222964	haloacid dehalogenase-like hydrolase domain containing 3
AK014670	1.218375	CDP-diacylglycerol synthase 1
NM_011224	1.214416	muscle glycogen phosphorylase
BC019391	1.208962	glycerol-3-phosphate dehydrogenase 1
AA414485	1.200591	seven in absentia 2
BB707122	1.196928	TCDD-inducible poly(ADP-ribose) polymerase
AV231866	1.185986	N-acetylgalactosaminyltransferase T-6
NM_024229	1.178394	phosphate cytidylyltransferase 2 ethanolamine
NM_009752	1.173722	galactosidase beta 1
BB366634	1.173419	male sterility domain containing 1
BC026595	1.164121	cystathionine beta-synthase
BC017126	1.152518	neurochondrin
BC018179	1.150765	ubiquitin specific protease 1
BM944122	1.143801	ATPase type 13A2
NM_013759	1.133139	selenoprotein X 1
BC003329	1.127548	makorin ring finger protein 1
AK018159(2)	1.112904	proprotein convertase subtilisin/kexin type 2
Structure		
BC005679	1.891608	syndecan 4
AK011116	1.69255	hemoglobin alpha adult chain 1
NM_010758	1.337203	myelin-associated glycoprotein
NM_080454	1.336939	gap junction membrane channel protein alpha 12
BC026833(2)	1.328426	gap junction membrane channel protein beta 1
BC006045	1.257563	pleckstrin homology domain containing family H (with MyTH4 domain) member 1
BC016584	1.248811	glycolipid transfer protein
NM_019999	1.241163	brain protein 17
AB059644	1.235731	calmin
BC011482	1.219334	membrane bound C2 domain containing protein
AF291655	1.213598	tenomodulin
AK018783(2)	1.209132	vesicle-associated membrane protein 1
AV337421(2)	1.205978	R7 binding protein

Accession #	Fold change	Gene
M35131	1.190867	neurofilament heavy polypeptide
BI328541	1.186428	kinesin family member 5B
AK005096	1.170625	fibronectin type III domain containing 5
AV220161	1.167709	RAB6B member RAS oncogene family
NM_018790	1.165217	activity regulated cytoskeletal-associated protein
BB757269	1.164057	pleckstrin homology domain containing family M (with RUN domain) member 2
BB082407	1.16317	hyaluronan and proteoglycan link protein 4
AF041861	1.152035	synaptojanin 2
NM_133769	1.141642	cytoplasmic FMR1 interacting protein 2
M20480	1.137212	neurofilament light polypeptide
BC025568	1.110304	tetraspanin 14
AW556311	0.876037	ELOVL family member 5 elongation of long chain fatty acids (yeast)
BB369657	0.844514	pleckstrin homology domain containing family A member 5
NM_011261	0.834896	reelin
NM_023289	0.832813	CEA-related cell adhesion molecule 11
BM231698	0.830714	exostoses (multiple) 1
BC019745	0.815841	transmembrane protein 2
NM_019394	0.790876	melanoma inhibitory activity 1
Neurimmune		
AF010586	1.194147	chemokine (C-X3-C motif) ligand 1
NM_010701	0.785721	leukocyte cell derived chemotaxin 1
AV229143	0.762351	interferon activated gene 202B
NM_008332	0.723433	interferon-induced protein with tetratricopeptide repeats 2
Cell cycle/death		
NM_130859	1.346454	caspase recruitment domain family member 10
AA201054	1.235925	TNFRSF1A-associated via death domain