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Comparative mRNA Analysis of Behavioral and Genetic Mouse Models of Aggression

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Mouse models of aggression have traditionally compared strains, most notably BALB/cJ and C57BL/6. However, these strains were not designed to study aggression despite differences in aggression-related traits and distinct reactivity to stress. This study evaluated expression of genes differentially regulated in a stress (behavioral) mouse model of aggression with those from a recent genetic mouse model aggression. The study used a discovery-replication design using two independent mRNA studies from mouse brain tissue. The discovery study identified strain (BALB/cJ and C57BL/6J) $\times$ stress (chronic mild stress or control) interactions. Probe sets differentially regulated in the discovery set were intersected with those uncovered in the replication study, which evaluated differences between high and low aggressive animals from three strains specifically bred to study aggression. Network analysis was conducted on overlapping genes uncovered across both studies. A significant overlap was found with the genetic mouse study sharing 1,916 probe sets with the stress model. Fifty-one probe sets were found to be strongly dysregulated across both studies mapping to 50 known genes. Network analysis revealed two plausible pathways including one centered on the *UBC* gene hub which encodes ubiquitin, a protein well-known for protein degradation, and another on *P38 MAPK*. Findings from this study support the stress model of aggression, which showed remarkable molecular overlap with a genetic model. The study uncovered a set of candidate genes including the *Erg2* gene, which has previously been implicated in different psychopathologies. The gene networks uncovered points at a Redox pathway as potentially being implicated in aggressive related behaviors.

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Key words: aggression; mouse; BALB/c; transcriptomics

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INTRODUCTION

Aggression is a behavior towards another individual with the intention either to intimidate or cause harm, and is consequently associated with violence. This trait is a substantial cause of

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mortality and morbidity globally [Anderson and Bushman, 2002; Veroude et al., 2015].

Figures pertaining to aggression-related mortality continue to rise, with a major predicted increase over the next 20 years [Bartolomeos et al., 2007]. Aggression also constitutes the sixth highest cause of disability in young people worldwide and contributes to approximately 5% of all disability [Vassos et al., 2014]. A study of the burden of crime on USA services found that ~3% of expenses were secondary to violent crime driven by aggression. This highlights the social and economic costs incurred by aggressive behaviors. Hence, there is a need to understand the causes and develop potential management strategies of aggression, which currently lack the desired efficacy [McGuire, 2008].

Behavior-genetic studies have systematically reported moderate heritability estimates of aggression, with different measures of aggression resulting in estimates varying from 22% to 51% [Miles and Carey, 1997; Seroczynski et al., 1999; Vassos et al., 2014]. Consequently, current research aims to identify genetic variants associated with aggressive behaviors, which may help to inform candidate gene selection and the identification of gene pathways to gain further understanding of the genetic substrate of this complex trait [Kudryavtseva et al., 2015; Waltes et al., 2015; Zhang-James and Faraone, 2015]. Gene selection has predominantly been driven by inspecting genes and neurobiological pathways with an established association with aggression, including the serotonin (5-HT) gene, levels of which were found to be low in more aggressive individuals. Different polymorphisms of the gene encoding the 5-HTT-serotonin transporter have been associated with aggression in specific populations including children and cocaine-dependent individuals [Nelson and Trainor, 2007; Siever, 2008]. Other neurotransmitters also have been implicated, including excitatory neurotransmitters: catecholamines, vasopressin, cholecystokinin, and substance P, together with inhibitory neurotransmitters, including enkephalins, GABA, and serotonin acting via 5-HT₁ receptors [Siegel et al., 2007]. Studies have investigated candidate genes from further pathways including those responsible for the regulation and catabolism of catecholamines such as monoamine oxidase A (MAOA) and catechol-O-methyl-transferase (COMT) [Brunner et al., 1993; Caspi et al., 2002; Moffitt, 2005]. Increased aggression and neurotransmitter levels have been observed in mice with either the MAOA or COMT genes deleted [Volavka et al., 2004].

Deciphering the neurobiology of aggression has been supported not only by an increased understanding of neurophysiology and the complex interplay of neurotransmitters and substrates, but also by well-established relationships with other psychological phenomena such as stress, implying the involvement of variations in the hypothalamic-pituitary-adrenal (HPA) axis, serotonergic, and vasopressin systems. Indeed, it has been shown that aggressive behavior can be associated with stressors including early life trauma and childhood maltreatment [Widom, 1989; Lewis, 1992; Loeber and Stouthamer-Loeber, 1998; Hildyard and Wolfe, 2002; Ethier et al., 2004; Fonagy, 2004; Veenema et al., 2006]. Furthermore, these behavioral processes are also associated with numerous psychopathologies including major depressive disorder, schizophrenia, personality disorders, anxiety disorders, and post-traumatic stress disorder [Agid et al., 1999; Veenema, 2009]. Nevertheless, the aetiology of these pathologies is poorly understood.

Animal models offer intrinsic advantages for exploring the genetics of the molecular mechanisms underpinning complex disorders such as aggression and violent behavior. Importantly, environmental variables can be more precisely controlled in ways that is not possible in human studies. Furthermore, the creation of replicable inbred strains reduces the potentially confounding effects of genetic variation. Finally, genetic models can be created by means of artificial selection to display specific behaviors at easily observable extremes, which is not reproducible in human studies.

Several studies on mouse aggression have used the BALB/c and C57BL/6 strains to identify candidate genes involved in its aetiology [Potter, 1985]. BALB/c mice are very susceptible to stress, resulting in anhedonia and increased aggression [Mineur et al., 2003; Isingrini et al., 2010; Belzung and Griebel, 2001]. This contrasts with the C57BL/6C57BL/6 strain, which is resistant to stress [Mineur et al., 2003; Isingrini et al., 2010]. Both strains were among the earliest inbred strains developed, in a time when many doubted this could be even be done, but were not selectively bred to for their aggressive characteristics. Nevertheless, given the established relationship between stress and aggression, there is likely to be significant overlap between many of the genes involved in modulating aggressive behavior and the current pool of candidate genes for sensitivity to stress uncovered using the BALB/c and C57BL/6C57BL/6 strains.

A recent study reported the first transcriptomic characterisation three mouse models developed specifically for the study of aggression [Malki et al., 2014]. These models consisted of three pairs of lines: Turku Aggressive (TA) and Turku Non-Aggressive (TNA) mice; North Carolina Aggressive (NC900) and Non-Aggressive (NC100) mice, and short attack (SAL) and long attack latency (LAL) mice, in which one of each pair was bred for high aggression and the other for low aggression.

This study aims to identify genetic overlap between the stress study and the selection lines study by assessing the differential gene expression dependent on stress exposure, and subsequently comparing results with those obtained from an independent genetic mouse model of aggression. Meta-analysis of the hypothesized genetic overlap between the BALB/c/C57BL/6C57BL/6 model and three aggression specific mouse models will provide greater insight into the aetiology of aggressive behavior and identify a set of gene and gene networks implicated in aggression.

METHODS

Study Design

Two independent animal studies were used in a discovery-replication design. The discovery study was based on a behavioral model of aggression using 16 animals from two inbred mouse strains (BALB/cJ and C57BL/6J), half of which were exposed to a chronic mild stress protocol. The discovery took advantage of the stress sensitivity characteristic of the BALB/cJ strain and the resilience of the C57BL/6J strain to model individual variation in response to stress and associated aggressive behaviors in humans. The replication study was based on a genetic model of aggression and consisted of 18 animals from three pairs of lines, artificially selected for high or low aggressive behaviors (Turku Aggressive [TA] and Turku Non-Aggressive [TNA] mice, North Carolina

Aggressive [NC900] and North Carolina Non-Aggressive [NC100] mice, and the short attack latency [SAL] and long attack latency [LAL] mice). Within each mouse line, half the animals were in high aggression and half in the low aggression group. Probe sets differentially expressed in response to stress and strain interaction uncovered in the stress mouse model were intersected with mRNA differences uncovered by comparing high and low aggressive strain subtypes in an independent genetic mouse model of aggression.

Animals

Eighteen animals from three different pairs of lines bred specifically to exhibit high and low aggressive behaviors were used for the genetic mouse model of aggression. We used the Turku Aggressive (TA) and Turku Non-Aggressive (TNA) mice, North Carolina Aggressive (NC900) and North Carolina Non-Aggressive (NC100) mice, and the short attack latency (SAL) and long attack latency (LAL) mice. All animals were bred in the laboratory of Sietse de Boer in the Netherlands and separated by sex until weaning in Perspex cages. At 3 and 4 weeks of age, after the mice had been weaned, male mice were paired with a 6–8 week old female of the same line and each pair was housed in a Makrolon Type II cage with similar internal environments.

The TA/TNA strains were bred from Swiss albino mice in Turku, Finland. Aggressive traits were assessed in males at the age of 60 days using a seven-point scale, in a standard 7 min dyadic test against non-aggressive mice, for which they were placed in a neutral container. The male mice with high aggression scores were bred together with the sisters of other aggressive mice in order to avoid inbreeding.

The NC900/NC100 strains were bred from an outbred population of NCR mice in North Carolina. Aggressive mice were selected based on increased aggression and reactivity to stimulation, the former having been assessed via a standard 10 min dyadic test with attack frequency recorded.

The SAL/LAL strains were bred from a feral *Mus musculus domesticus* population captured in Groningen, The Netherlands. Mice were selected for breeding at the age of 14 weeks with the use of the resident-intruder test, in which the experimental mice were the residents and had their attack latencies (time taken for the first attack by the resident) recorded against the naïve albino intruders. The attack latencies determined the SAL and LAL mice.

The BALB/cJ and C57BL/6J strains were purchased from the Jackson Laboratory in Bar Harbor, ME, and bred at UMass Medical School, Worcester, for a minimum of two generations. Weaning occurred at 28 ± 1 days and mice were housed in same sex groups in groups of two to four until the age of 2 months ± 1 week, at which point they were assigned to the control or unpredictable chronic mild stress (UCMS) group. The UCMS treatment began at the same time, continuing during the testing phase, although precautions were taken to ensure that stressors were not applied just before a test. Throughout these phases, all mice were kept in similar rooms [Malki et al., 2015].

Unpredictable Chronic Mild Stress Protocol

The UCMS model leads to depressive traits in mice due to repeated administration of unavoidable stressors at unpredictable moments.

This model emulates well-documented research showing a strong association between stress and major depressive disorders. Stressors used here included: predator sounds, cage tilting, placement in an empty cage with and without water on the bottom, damp sawdust, reversal of the light/dark cycle, and switching on the lights transiently during the dark phase. Food or water deprivation was not included in the procedure. Two stressors on average were randomly applied on a daily basis for 2 weeks. This schedule was carried out for 4 weeks before animals were sacrificed. Animals used for this study did not undergo behavioral testing.

mRNA Extraction Protocol

Male mice from each of the two lines in the three artificially selected mouse models were sacrificed by cervical dislocation at the age of 13 and 14 weeks old. The prefrontal cortices (PFC) of all 18 mice were extracted via dissection and snap frozen. RNA was extracted using the Trizol RNA isolation method and RNA quality was measured via gel electrophoresis. After the Affymetrix One-Cycle Target labelling protocol, 3 μ g of RNA was processed and hybridized to Affymetrix MOE430v2 Gene Expression Arrays.

The stress-induced model of aggression using the BALB/cJ and C57BL/6J strains involved the extraction of RNA from 16 frozen dissected hippocampi using the guanidium thiocyanate method with TRIzol (Invitrogen Life Technologies, UK). With the use of reverse transcriptase and poly-T primer, the mRNA was subsequently converted to cDNA, which was amplified using T7 RNA polymerase with biotin-UTP and biotin-CTP. The cDNA was fragmented in a fragmentation buffer and then hybridized on the GeneChip Mouse Genome 430A 2.0 to allow scanning and data analysis.

Statistical Analysis

Mean probe set intensities from C57BL/6J and BALB/cJ animals obtained from Affymetrix MOE 430 arrays were normalized and summarized using Robust Multichip Average (RMA) method [Irizarry et al., 2003]. Probe sets that were systematically absent (based on the MAS 5.0 detection present/absent call) across all the arrays were removed leaving 22,690 probe sets. Raw intensity measures from the genetic mouse study were also processed using RMA based upon an additive background-multiplicative model, returning log₂-transformed intensities. RMA was performed using the Affymetrix package for R (<http://cran.r-project.org/>). The Affymetrix software package, Microarray Suite (MAS) 5.0, was used to identify missing values in the data using the detection function.

Principal component analysis (PCA) analysis was first performed on the BALB/c/C57BL/6 data using the “prcomp” function in R in order to identify main sources of variation. Prior to analysis, the data was scaled using Pareto transformation using the “prep” function available in the Bioconductor package “pcaMethods” for R.

General linear models were conducted in R, using strain and stress as fixed effects and normalized probe intensity as dependent variable. The model was used to uncover probe sets differentially expressed in response to stress $\times$ strain interaction. False Discovery Rate (FDR) adjusted *P*-values at a threshold of 0.05 were used to identify probe sets differentially expressed in response to stress $\times$

strain controlling for the number of multiple non-independent tests.

The RankProd package for R from Bioconductor was used to uncover genes differentially expressed between high and low aggressive lines in the three selected mouse models. RankProd is a non-parametric method derived from biological reasoning that identifies up and down-regulated genes across replicates dependent on different experimental conditions (origins). RankProd offers several advantages compared to more traditional parametric models [Malki et al., 2013]. In this study, RankProd was used to rank probe sets by fold change in expression between aggressive and non-aggressive sub-strains. The RankProd algorithm allowed the evaluation of mRNA expression differences between high and low groups within each strain independently and then to combine the results in a meta-analysis approach. This allowed circumventing the issue of the predominant strain effects given the use of mice of three different genetic backgrounds. A percentage of false positive predictions (pfp) of 0.05, determined using 100,000 permutations, was used to control for the number of multiple, non-independent tests. Lastly, only expression differences that showed consistency in the directionality of expression change across all strains were reported. Probe sets that varied in directionality across the three different strains point at interaction effects which were not looked at in the genetic mouse model; given that the genetic mouse model was used as a replication study, we were only interested in uncovering differences related to main effects of aggression.

Probe sets uncovered in the BALB/c/C57BL/6 model were intersected with those uncovered in the selected mouse models of aggression using nominal *P*-values. We then selected the most highly differentially regulated probes across both mouse models using FDR corrected values. These were then carried forward for networks analysis using the Ingenuity Pathway Analysis software (IPA): IPA provides integrated data from different experimental platforms to aid in the identification of key biological and molecular networks in “omics” data. IPA constructs pathways using previously identified associations between genes and proteins stored in the Ingenuity Pathways Knowledge Base (IPKB), independent of established canonical pathways. The parameters were determined using the probe set list uploaded to the IPA platform. IPA then returns generated networks ranked by order of mean significance according to *P*-value. Significance is determined using “right-tailed Fisher Exact Test” considering the number of focus genes (matched to probe-sets uploaded) that are involved in the process, and the total number of genes known to be associated with that process in the IPKB.

RESULTS

PCA was first used to explore strain and stress components in the stress model of aggression. A clear separation of both stress and strain was visible, suggesting that stress effects caused by the CMS paradigm are sufficient to cause a pervasive effects across the transcriptome. This is likely driven by the stress sensitivity of the BALB/c strain and replicates previously published findings [Tannenbaum et al., 2003; Priebe et al., 2005].

We then explored interaction effects in the behavioral model of aggression using strain (BALB/c and C57BL/6) as one factor and

stress (CMS and Control) as the other. The results from the linear model uncovered 2,375 probe-sets out of 22,690 as significantly dysregulated in response to stress-strain interaction at a nominal *P*-value of 0.05. We then evaluated differences in the genetic model of aggression. Probe sets in the genetic mouse model of aggression were analyzed using the RankProd algorithm, which evaluated differences between high and low aggressive sub strains for each of the three strains and combined the results using a meta-analysis approach. Probe sets showing consistency in the directionality of fold change across the three stains and reaching a nominal *P*-value of 0.05 were uncovered and intersected with those found in the behavioral model of aggression. Out of the 2,375 probe sets found in the stress model of aggression, 1,916 were also found in the genetic model. The results show an important overlap across the two models of aggression in mouse (81%). We then selected those probe sets showing the greatest expression difference across the two studies. In the stress model of aggression we adjusted for multiple testing using FDR < 0.05 while in the genetic model of aggression we used Pfp < 0.05 and consistency of directionality of fold change. Fifty-one highly implicated probe sets were identified across the two models, 30 of which were down-regulated (Table I) and 21 of which were up-regulated (Table II). Probe sets were uploaded to IPA for networks analysis. Fifty of these probe sets were found on Ingenuity's reference list and mapped to known genes. IPA returned two significant pathways. The first pathway, with a score of 40, is centred on the Ubiquitin C (UBC) gene hub (Fig. 1). The second pathway, with a score of 28, is centred on the P38 mitogen-activated protein kinase (MAPK) gene hub, which is a key redox-signaling pathway (Fig. 2). Both pathways are biologically plausible and consistent with literature on the neurobiology of aggression.

DISCUSSION

In this study, we presented a comparative analysis of mouse transcriptome from disease relevant brain regions, between a behavioral model of mouse aggression using a chronic stressor and a genetic mouse model of aggression. Over 81% of the probe sets uncovered by exploring stress x strain interaction were also differentially expressed across high and low aggressive sub strains. The results validate the use of the BALB/cJ and C57BL/6 as a good model to explore the neurobiology of aggression and reinforce the known association between stress and aggression. The most strongly differentially expressed probe sets across both the behavioral and genetic model of aggression, point at genes and gene networks with higher probability of association with aggressive behaviors and may provide additional insight into neuogenetic factors that may be modulating violent behavior in humans. Networks analyses of these genes revealed two networks with a plausible association with aggression; the first network is centred on the UBC gene hub while the second is a redox-associated network.

Ubiquitination Pathway

The association of ubiquitination and the *UBC* gene with aggression is poorly documented. However, there are features of ubiquitination which may apply to aggressive behavior and may help further understanding of its neurobiology.

TABLE I. Table Reports the Most Highly Up-Regulated Genes Common Across Both the Stress Study and Genetic Study

Down-regulated genes					
Probe set ID	Gene symbol	Gene title	Fold change	P-value	FDR
1417262_at	Ptgs2	prostaglandin-endoperoxide synthase 2	5.48	1.000E-06	1.000E-06
1423748_at	Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	3.78	1.000E-06	1.000E-06
1424607_a_at	Gm4354	predicted gene 4354	2.41	1.000E-06	1.000E-06
1425408_a_at	2610034M16Rik	RIKEN cDNA 2610034M16 gene	3.31	1.000E-06	1.000E-06
1427683_at	Egr2	early growth response 2	4.88	1.000E-06	1.000E-06
1428052_a_at	Zmym1	zinc finger, MYM domain containing 1	3.88	1.000E-06	1.000E-06
1449875_s_at	H2-T10 /// H2-T22 /// H2-T9	histocompatibility 2, T region locus 10 /// .. locus 22 /// .., locus 9	3.86	1.000E-06	1.000E-06
1450779_at	Fabp7	fatty acid binding protein 7, brain	2.88	1.000E-06	1.000E-06
1448793_a_at	Sdc4	syndecan 4	3.15	1.043E-06	2.222E-04
1421205_at	Atm	ataxia telangiectasia mutated homolog (human)	2.71	1.252E-05	2.400E-03
1420500_at	Dnajc1	DnaJ (Hsp40) homolog, subfamily C, member 1	2.43	1.000E-06	3.091E-03
1426896_at	Zfp191	zinc finger protein 191	2.49	4.799E-05	7.667E-03
1448839_at	Kank3	KN motif and ankyrin repeat domains 3	2.56	6.364E-05	8.714E-03
1448554_s_at	Myh6 /// Myh7	myosin, heavy polypeptide 6, cardiac muscle, alpha /// .., beta	2.32	6.051E-05	8.923E-03
1435755_at	1110001A16Rik	RIKEN cDNA 1110001A16 gene	2.33	8.555E-05	1.093E-02
1425263_a_at	Mbp	myelin basic protein	2.35	1.085E-04	1.300E-02
1452378_at	Malat1	metastasis associated lung adenocarcinoma transcript 1	2.03	2.890E-04	2.916E-02
1452234_s_at	Tmem191c	transmembrane protein 191C	2.43	2.619E-04	2.953E-02
1425964_x_at	Hspb1	heat shock protein 1	1.64	3.109E-04	2.980E-02
1426114_at	Hnrnpab	heterogeneous nuclear ribonucleoprotein A/B	1.98	2.848E-04	3.033E-02
1422444_at	Itgab	integrin alpha 6	2.21	3.965E-04	3.455E-02
1422943_a_at	Hspb1	heat shock protein 1	1.55	4.413E-04	3.525E-02
1423154_at	BC005537	cDNA sequence BC005537	1.88	3.933E-04	3.590E-02
1420982_at	Rbm39	RNA binding motif protein 39	1.93	4.340E-04	3.617E-02
1428381_a_at	Pdpf	pancreatic progenitor cell differentiation and proliferation factor	1.89	5.175E-04	3.968E-02
1426607_at	Gm7120 /// LOC101055764	predicted gene 7120 /// transmembrane protein C5orf28 homolog	1.93	5.561E-04	4.100E-02
1416632_at	Me1	malic enzyme 1, NADP(+)-dependent, cytosolic	2.31	5.822E-04	4.133E-02
1435559_at	Myo6	myosin VI	2.11	6.521E-04	4.464E-02
1424270_at	Dclk1	doublecortin-like kinase 1	2.04	7.470E-04	4.773E-02
1448529_at	Thbd	thrombomodulin	1.35	7.334E-04	4.848E-02

The table shows Affymetrix's probe set name, gene symbol, gene title, fold change, P-value, and fdr value.

UBC is one of the four genes which encodes ubiquitin, the other three being *UBB*, *UBA52*, and *RPS27A* [Board et al., 1992]. More specifically, *UBA52* and *RPS27a* encodes the single copy of ubiquitin while *UBB* and *UBC* encode for polyubiquitin. Ubiquitin is responsible for post-translational cellular protein ubiquitination, which is a reversible modification involving the regulation of several processes, including: protein degradation, protein trafficking, DNA repair, apoptosis, cell-cycle regulation, and signal transduction. Out of these functions, ubiquitin is best known for tagging proteins for proteolysis via the 26S proteasome.

Existing literature suggests that *UBC* is extremely susceptible to oxidative stress, which may provide some insight into the neurobiology of aggression as the significance of oxidative stress in aggression has also been documented [Coccaro et al., 2016; Kim et al., 2015]. Research has found that reduced antioxidant function and increased levels of oxidative stress markers are associated with

aggressive behavior. Given that *UBC* is central to one of the neurobiological pathways derived from the ingenuity pathway analysis and has multiple functions, it may be a possibility that oxidative stress to *UBC* impairs normal cellular processes involving other related substrates, thus contributing to aggressive traits. This may be plausible due to the aforementioned documentation of increased markers of oxidative stress, which could possibly be due to impaired functioning of proteolysis via the 26S proteasome. However, it is uncertain whether oxidative stress contributes to aggression or aggression contributes to oxidative stress and further research is needed to explore this.

P38 MAPK Redox Signaling Pathway

The MAPK signaling pathway can be activated by a series of stimuli, including cytokines and stress, which have an inflammatory

TABLE II. Table Reports the Most Highly Down-Regulated Genes Common Across Both the Stress Study and Genetic Study

Up-regulated genes					
Probe set ID	Gene symbol	Gene title	Fold change	P-value	FDR
1419034_at	Csnk2a1	casein kinase 2, alpha 1 polypeptide	6.97	1.000E-06	1.000E-06
1419035_s_at	Csnk2a1 /// LOC100047957	casein kinase 2, alpha 1 polypeptide /// casein kinase II subunit alpha-like	7.29	1.000E-06	1.000E-06
1419344_at	Tcte1	t-complex-associated testis expressed 1	3.94	1.000E-06	1.000E-06
1426548_a_at	Atpbd4	ATP binding domain 4	5.03	1.000E-06	1.000E-06
1433497_at	Aqr	aquarius	2.93	1.000E-06	1.000E-06
1434216_a_at	Nudt19	nudix [nucleoside diphosphate linked moiety X]-type motif 19	6.92	1.000E-06	1.000E-06
1449181_at	Fech	ferrochelatase	3.81	1.000E-06	1.000E-06
1428224_at	Hnrpd1	heterogeneous nuclear ribonucleoprotein D-like	2.65	4.173E-06	1.000E-03
1425495_at	Zfp62	zinc finger protein 62	2.34	8.346E-06	1.778E-03
1420357_s_at	Xlr3a /// Xlr3b /// Xlr3c	X-linked lymphocyte-regulated 3A /// X-linked lymphocyte-regulated 3B /// X-linked lymphocyte-regulated 3C processing of precursor 4, ribonuclease P/MRP family, [<i>S. cerevisiae</i>]	2.47	1.043E-05	2.000E-03
1448419_at	Pop4		2.42	1.252E-05	2.182E-03
1422564_at	Actl6b	actin-like 6B	2.14	7.512E-05	1.200E-02
1423875_at	Fam160b1	family with sequence similarity 160, member B1	1.73	9.181E-05	1.257E-02
1436684_a_at	RioK2	RIO kinase 2 [yeast]	2.26	8.659E-05	1.277E-02
1424680_at	Fam26e	family with sequence similarity 26, member E	2.09	1.075E-04	1.373E-02
1418822_a_at	Arf6	ADP-ribosylation factor 6	1.97	1.231E-04	1.475E-02
1426159_x_at	Tcrb-J	T cell receptor beta, joining region	2.14	1.315E-04	1.482E-02
1450483_at	Gjc2	gap junction protein, gamma 2	1.99	2.984E-04	2.860E-02
1423413_at	NdrG1	N-myc downstream regulated gene 1	1.44	2.963E-04	2.989E-02
1434329_s_at	Adipor2	adiponectin receptor 2	1.54	2.911E-04	3.100E-02
1451511_at	Hibch	3-hydroxyisobutyryl-Coenzyme A hydrolase	2.03	4.705E-04	4.295E-02

The table shows Affymetrix's probe set name, gene symbol, gene title, fold change, P-value, and fdr value.

component, and are involved in cellular processes such as cell differentiation and apoptosis [Wada and Penninger, 2004]. Interestingly, elevated levels of inflammatory markers in the plasma of individuals exhibiting aggressive behavior has been documented. These markers include C-reactive protein (CRP) and interleukin 6 [Coccaro et al., 2015]. Therefore, there may be an association between this pathway and aggression, a hypothesis, which is further backed up by the implication of interleukin-1 in this pathway derived from ingenuity. This is supported by previous research which has also shown that interleukin-1b increases the response to environmental stressors [Anisman and Merali, 2003]. Raised inflammatory cytokines have also been associated with major depressive disorder, which may provide another link with aggression as genetic, and behavior links have been previously made between the two, as well as with several other psychopathologies [Capuron and Miller, 2004].

The pathway itself involves a three-tiered phosphorylation cascade of MAP kinases, in which activated MAP3Ks (MAPK kinase kinases) phosphorylate and stimulate MAP2Ks (MAPK kinases) to phosphorylate MAPKs. The stimulated MAPKs subsequently regulate various cellular processes including those mentioned above, via methods such as the activation of gene transcription. The MAPKs include extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), ERK5, p38 kinase, and

c-Jun N-terminal kinase (JNK), which each play specific roles in this pathway [Seger and Krebs, 1995]. Interestingly, the ingenuity analysis has also implicated ERK1 and ERK2 in the aetiology of aggression, which further supports the involvement of the MAPK pathway.

Redox signaling may also be involved with other signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which has previously been implicated in aggression. Both pathways are postulated to be connected via p150, an *nf-kb1* gene product which has a role in regulating MAPK signalling. The NF-κB pathway is activated by phosphorylation of inhibitory κBα (IκBα) proteins which normally keep it quiescent, allowing the release of the NF-κB dimer and translocation to nuclei where it is able to regulate gene expression. This pathway is essential in regulating cellular processes such as apoptosis, differentiation, immunity, and stress responses. Activating stimuli of the NF-κB pathway include interleukin 1 and tumor necrosis factor (TNF), both of which are associated with aggression [Patel et al., 2010]. Previous studies on rat have reported an association between MAPK and increased aggression scores in rat [DeMar et al., 2006; Frey et al., 2006]. Given that NF-κB regulates a large variety of cells, the interaction of this pathway with MAPK is likely to be very complex and requires further research to fully understand it in the context of aggression.

FIG. 1. The most significant gene network returned by the Ingenuity Pathway Analysis is centered on the UBC (Ubiquitin C) gene complex. The grey molecules represent reference molecular uploaded to the IPA database. The UBC gene is susceptible to oxidative stress, a mechanism that has previously been hypothesized to be involved in the neurobiology of aggression. However, it is unclear whether oxidative stress may be a cause of consequence of aggressive behavior.

A Link Between Ubiquitin and the P38 MAPK Redox Signaling Pathway

An interesting finding is that ubiquitin and redox signaling appear to be connected, adding more layers of complexity to the neurobiology of aggression as it is likely that the two ingenuity pathways are

not only connected, but influenced by other factors such as the aforementioned NF- κ B pathway.

Ubiquitin and redox signaling appear to be linked through the phosphorylation of the ubiquitination pathway; therefore, the MAPK phosphorylation cascade may influence ubiquitination, hence protein degradation. This has implications regarding the

FIG. 2. The second most significant pathways returned by IPA with a score of 28 is centred on the P38 MAPK (p38 mitogen-activated protein kinases) gene complex. The grey molecules represent genes uploaded to the Ingenuity reference database. P38 MAPK regulates a number of different cellular processes including regulation of BDNF. In rat studies, P38 MAPK has been previously reported as being associated with increased aggression scores.

level of cellular proteins, which would presumably have a role in influencing aggressive behavior. The effect that these varying levels would have on aggressive traits is likely to depend on the specific proteins affected; hence, further unravelling and analysis of our results is the essential first step in understanding these interactions.

Although this study presents a number of strengths, including the discovery replication design using two independent mouse studies and accessibility to data from the first genetic mouse model of aggression, there are also a number of limitations. First, due to the complex connectivity of different brain regions, the analysis of the same brain regions from these model organisms could have provided further insight into the mechanisms underlying variation in aggressive behavior. Several genes may have been missed as identification of highly expressed genes across studies is further restricted by the correlation structure of gene

expression across two different brain regions: hippocampus and prefrontal cortex. However, choices on brain tissues were restricted to what was available. Only data from prefrontal cortex was available from the genetic mouse model of aggression. Second, mice are not miniature human beings and therefore there are number of behavioral characteristics in human that cannot be modelled in mouse. While the results can serve to inform the molecular mechanisms that may be relevant in aggression in human, these will have to be replicated in human studies. Moreover, the chronic mild stress protocol used is generally considered a mild stress; it is possible that a more severe stress protocol may cause stronger and more pervasive dysregulation at the level of the transcriptome. However, stress effects were visible simply through PCA, which highlights the stress sensitivity of the BALB/cJ and suitability of the CMS protocol for this study. Lastly, although the sample size was

sufficiently powered to detect a number of important differences, microarray data tends to be high dimensional by nature. It is possible that a meta-analysis approach using a clustering method could be used to gain additional into the neurobiology of this complex trait.

CONCLUSIONS

As hypothesized, significant overlap between genes differentially regulated in response to a stressor were also differentially regulated in a genetic mouse model of aggression, both validating the stress model of aggression and identifying genes with a higher probability of being implicated in modulating aggressive behaviors. These results are a promising start to the unravelling of the complex neurobiology behind aggression and provide clues on the candidate genes that may be relevant in human.

REFERENCES

- Agid O, Shapira B, Zislin J, Ritsner M, Hanin B, Murad H, Troudart T, Bloch M, Heresco-Levy U, Lerer B. 1999. Environment and vulnerability to major psychiatric illness: A case control study of early parental loss in major depression, bipolar disorder, and schizophrenia. *Mol Psychiatry* 4(2):163–172.
- Anderson CA, Bushman BJ. 2002. Human aggression. *Ann Rev Psychol* 53:27–51.
- Anisman H, Merali Z. 2003. Cytokines, stress and depressive illness: Brain-immune interactions. *Ann Med* 35(1):2–11.
- Bartolomeos K, Brown D, Butchart A, Phinney Harvey A, Meddings D, Sminkey L. 2007. Third milestones of a global campaign for violence prevention report, 2007: Scaling up. Geneva: World Health Organization.
- Belzung C, Griebel G. 2001. Measuring normal and pathological anxiety-like behavior in mice: A review. *Behav Brain Res* 125(2):141–149.
- Board PG, Coggan M, Baker RT, Vuust J, Webb GC. 1992. Localization of the human UBC polyubiquitin gene to chromosome band 12q24.3. *Genomics* 12(4):639–642.
- Brunner HG, Nelen M, Breakefield XO, Ropers HH, van Oost BA. 1993. Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. *Science* 262(5133):578–580.
- Capuron L, Miller AH. 2004. Cytokines and psychopathology: Lessons from interferon-alpha. *Biol Psychiatry* 56(11):819–824.
- Caspi A, McClay J, Moffitt TE, Mill J, Martin J, Craig IW, Taylor A, Poulton R. 2002. Role of genotype in the cycle of violence in maltreated children. *Science* 297(5582):851–854.
- Coccaro EF, Lee R, Coussons-Read M. 2015. Cerebrospinal fluid and plasma C-reactive protein and aggression in personality-disordered subjects: A pilot study. *J Neural Transm* 122(2):321–326.
- Coccaro EF, Lee R, Gozal D. 2016. Elevated plasma oxidative stress markers in individuals with intermittent explosive disorder and correlation with aggression in humans. *Biol Psychiatry* 79(2):127–135.
- Crawley JN, Belknap JK, Collins A, Crabbe JC, Frankel W, Henderson N, Hitzemann RJ, Maxson SC, Miner LL, Silva AJ, Wehner JM, Wynshaw-Boris A, Paylor R. 1997. Behavioral phenotypes of inbred mouse strains: Implications and recommendations for molecular studies. *Psychopharmacology* 132(2):107–124.
- DeMar JC Jr, Ma K, Bell JM, Igarashi M, Greenstein D, Rapoport SI. 2006. One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats. *J Lipid Res* 47(1):172–180.
- Ethier LS, Lemelin JP, Lacharite C. 2004. A longitudinal study of the effects of chronic maltreatment on children's behavioral and emotional problems. *Child Abuse Negl* 28(12):1265–1278.
- Fonagy P. 2004. Early life trauma and the psychogenesis and prevention of violence. *Ann N Y Acad Sci* 1036:181–200.
- Frey BN, Andrezza AC, Cereser KM, Martins MR, Valvassori SS, Reus GZ, Quevedo J, Kapczinski F. 2006. Effects of mood stabilizers on hippocampus BDNF levels in an animal model of mania. *Life Sci* 79(3):281–286.
- Hildyard KL, Wolfe DA. 2002. Child neglect: Developmental issues and outcomes. *Child Abuse Negl* 26(7):679–695.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4(2):249–264.
- Isingrini E, Camus V, Le Guisquet AM, Pingaud M, Devers S, Belzung C. 2010. Association between repeated unpredictable chronic mild stress (UCMS) procedures with a high fat diet: A model of fluoxetine resistance in mice. *PLoS ONE* 5(4):e10404.
- Kim MN, Choi J, Ryu HW, Ryu KY. 2015. Disruption of polyubiquitin gene Ubc leads to attenuated resistance against arsenite-induced toxicity in mouse embryonic fibroblasts. *Biochim Biophys Acta* 1853(5):996–1009.
- Kudryavtseva N, Markel A, Orlov YL. 2015. Aggressive behavior: Genetic and physiological mechanisms. *Russ J Genet* 5(4):413–429.
- Lewis DO. 1992. From abuse to violence: Psychophysiological consequences of maltreatment. *J Am Acad Child Adolesc Psychiatry* 31(3):383–391.
- Loeber R, Stouthamer-Loeber M. 1998. Development of juvenile aggression and violence. Some common misconceptions and controversies. *Am Psychol* 53(2):242–259.
- Malki K, Mineur YS, Tosto MG, Campbell J, Karia P, Jumabhoy I, Sluyter F, Crusio WE, Schalkwyk LC. 2015. Pervasive and opposing effects of unpredictable chronic mild stress (UCMS) on hippocampal gene expression in BALB/cJ and C57BL/6J mouse strains. *BMC Genomics* 16:262.
- Malki K, Pain O, Du Rietz E, Tosto MG, Paya-Cano J, Sandnabba KN, de Boer S, Schalkwyk LC, Sluyter F. 2014. Genes and gene networks implicated in aggression related behavior. *Neurogenetics* 15(4):255–266.
- Malki K, Tosto MG, Jumabhoy I, Lourdasamy A, Sluyter F, Craig I, Uher R, McGuffin P, Schalkwyk LC. 2013. Integrative mouse and human mRNA studies using WGCNA nominates novel candidate genes involved in the pathogenesis of major depressive disorder. *Pharmacogenomics* 14(16):1979–1990.
- McGuire J. 2008. A review of effective interventions for reducing aggression and violence. *Philos Trans R Soc Lond B Biol Sci* 363(1503):2577–2597.
- Miles DR, Carey G. 1997. Genetic and environmental architecture of human aggression. *J Pers Soc Psychol* 72(1):207–217.
- Mineur YS, Prasol DJ, Belzung C, Crusio WE. 2003. Agonistic behavior and unpredictable chronic mild stress in mice. *Behav Genet* 33(5):513–519.
- Moffitt TE. 2005. The new look of behavioral genetics in developmental psychopathology: Gene-environment interplay in antisocial behaviors. *Psychol Bull* 131(4):533–554.
- Nelson RJ, Trainor BC. 2007. Neural mechanisms of aggression. *Nat Rev Neurosci* 8(7):536–546.
- Patel A, Siegel A, Zalcman SS. 2010. Lack of aggression and anxiolytic-like behavior in TNF receptor (TNF-R1 and TNF-R2) deficient mice. *Brain Behav Immun* 24(8):1276–1280.

- Potter M. 1985. History of the BALB/c family. *Curr Top Microbiol Immunol* 122:1–5.
- Priebe K, Romeo RD, Francis DD, Sisti HM, Mueller A, McEwen BS, Brake WG. 2005. Maternal influences on adult stress and anxiety-like behavior in C57BL/6J and BALB/cJ mice: A cross-fostering study. *Dev Psychobiol* 47(4):398–407.
- Seger R, Krebs EG. 1995. The MAPK signaling cascade. *FASEB J* 9(9):726–735.
- Seroczynski AD, Bergeman CS, Coccaro EF. 1999. Etiology of the impulsivity/aggression relationship: Genes or environment? *Psychiatry Res* 86(1):41–57.
- Siegel A, Bhatt S, Bhatt R, Zalcman SS. 2007. The neurobiological bases for development of pharmacological treatments of aggressive disorders. *Curr Neuropsychopharmacol* 5(2):135–147.
- Siever LJ. 2008. Neurobiology of aggression and violence. *Am J Psychiatry* 165(4):429–442.
- Tannenbaum BM, Tannenbaum GS, Anisman H. 2003. Impact of life-long macronutrient choice on neuroendocrine and cognitive functioning in aged mice: Differential effects in stressor-reactive and stressor-resilient mouse strains. *Brain Res* 985(2):187–197.
- Vassos E, Collier DA, Fazel S. 2014. Systematic meta-analyses and field synopsis of genetic association studies of violence and aggression. *Mol Psychiatry* 19(4):471–477.
- Veenema AH. 2009. Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: What can we learn from animal models? *Front Neuroendocrinol* 30(4):497–518.
- Veenema AH, Blume A, Niederle D, Buwalda B, Neumann ID. 2006. Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. *Eur J Neurosci* 24(6):1711–1720.
- Veroude K, Zhang-James Y, Fernandez-Castillo N, Bakker MJ, Cormand B, Faraone SV. 2015. Genetics of aggressive behavior: An overview. *Am J Med Genet B Neuropsychiatr Genet* 171(1):3–43.
- Volavka J, Bilder R, Nolan K. 2004. Catecholamines and aggression: The role of COMT and MAO polymorphisms. *Ann N Y Acad Sci* 1036:393–398.
- Wada T, Penninger JM. 2004. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 23(16):2838–2849.
- Waltes R, Chiocchetti AG, Freitag CM. 2015. The neurobiological basis of human aggression: A review on genetic and epigenetic mechanisms. *Am J Med Genet B Neuropsychiatr Genet*, 22 Oct 2015, DOI: 10.1002/ajmg-b.32388 (in press).
- Widom CS. 1989. Child abuse, neglect, and adult behavior: Research design and findings on criminality, violence, and child abuse. *Am J Orthopsychiatry* 59(3):355–367.
- Zhang-James Y, Faraone SV. 2015. Genetic architecture for human aggression: A study of gene-phenotype relationship in OMIM. *Am J Med Genet B Neuropsychiatr Genet*, 19 Aug 2015, DOI: 10.1002/ajmg-b.32363 (in press).