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Transcriptome Analysis of Genes and Gene Networks Involved in Aggressive Behavior in Mouse and Zebrafish

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Despite moderate heritability estimates, the molecular architecture of aggressive behavior remains poorly characterized. This study compared gene expression profiles from a genetic mouse model of aggression with Zebrafish, an animal model traditionally used to study aggression. A meta-analytic, cross-species approach was used to identify genomic variants associated with aggressive behavior. The Rankprod algorithm was used to evaluated mRNA differences from prefrontal cortex tissues of three sets of mouse lines (N = 18) selectively bred for low and high aggressive behavior (SAL/LAL, TA/TNA, and NC900/NC100). The same approach was used to evaluate mRNA differences in Zebrafish (N = 12) exposed to aggressive or non-aggressive social encounters. Results were compared to uncover genes consistently implicated in aggression across both studies. Seventy-six genes were differentially expressed (PFP < 0.05) in aggressive compared to non-aggressive mice. Seventy genes were differentially expressed in zebrafish exposed to a fight encounter compared to isolated zebrafish. Seven genes (*Fos*, *Dusp1*, *Hdac4*, *Ier2*, *Bdnf*, *Btg2*, and *Nr4a1*) were differentially expressed across both species 5 of which belonging to a gene-network centred on the *c-Fos* gene hub. Network analysis revealed an association with the MAPK signaling cascade. In human studies *HDAC4* haploinsufficiency is a key genetic mechanism associated with brachydactyly mental retardation syndrome (BDMR), which is associated with aggressive behaviors. Moreover, the HDAC4 receptor is a drug target for valproic acid, which is being employed as an effective pharmacological treatment for aggressive behavior in geriatric, psychiatric, and brain-injury patients.

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INTRODUCTION

Aggression is a leading cause of mortality and morbidity. The World Health Organization (WHO) estimated that almost 1.5 million deaths each year are caused by violence, either inflicted by oneself or others (excluding armed conflict), with nonfatal victims of violence reaching even higher numbers [World Health Organization, 2007].

Aggressive behavior and conflict are ubiquitous in the animal kingdom and are both pre-programmed by phylogenetic adaptation and modulated by environmental exposures. Aggression can be vital for survival but can equally have life threatening consequences in addition to being energetically costly. In humans, impulsive aggression can reach pathological levels and is a major component in antisocial personality disorders, which is highly prevalent in populations of violent offenders (47% men and 21% women) [Fazel and Danesh, 2002], comorbid with several psychopathologies including ADHD, and can constitute a pathology in itself, for example in intermittent explosive disorder (IED) [Coccaro et al., 1998; Monuteaux et al., 2009].

Aggressive behavior is an evolutionarily well-conserved trait. Research in animal models has been motivated by the reasoning that molecular correlates of aggressive behaviors in animals may resemble the biological mechanisms relevant in human pathological aggression [Blanchard and Blanchard, 2003]. The identification of genetic markers consistently implicated in aggression across species may therefore point at the core biological components underlying aggressive behavior. Advancements in understanding the aetiology of aggression are crucial in order to improve diagnosis/prognosis and intervention strategies, which currently lack in effectiveness [McGuire, 2008].

Behavioral-genetic studies have reported moderate to high heritability estimates for aggression, in particular impulsive aggression as opposed to premeditated aggression, with genetic factors accounting for approximately 50% of individual variance [Miles and Carey, 1997; Seroczynski et al., 1999].

Several candidate-gene studies on human aggression have explored genes associated with different psychopathologies that often co-occur with aggressive behaviors, such as borderline personality disorder. These studies have mainly targeted serotonergic and catecholaminergic neurotransmitter systems. Associations have been reported between impulsive behavior and variants in genes coding for monoamine oxidase A (*MAOA*) [Brunner et al., 1993; Ni et al., 2007], catechol-O-methyl-transferase (*COMT*) [Flory et al., 2007], and the serotonin transporter (*5-HTT*) [Davidge et al., 2004]. However, these findings often show small effect sizes and fail to replicate [Vassos et al., 2014]. Genome-wide association studies (GWAS), which examine the association between genome-wide markers and traits, have also failed to identify common genetic variants that underpin aggressive behavior [Tielbeek et al., 2012].

Animal models can be useful in genetic research due to the availability of brain tissue, tight control of the environment and homogenous measures of the aggressive phenotype. Several mouse (*Mus musculus*) models have been highly informative in the aggression literature. For example, both *5-HT1B*- [Saudou et al., 1994] and *MAOA*- [Cases et al., 1995] knockout mice have been shown to display increased aggressive behavior compared to control mice. Transcriptomic characterisation of three pairs of mouse lines selectively bred for aggression found that the *NF-kB* and *MAPK* gene pathways (redox-signaling pathways) in the prefrontal cortex (PFC) are implicated in aggressive behavior and identified several novel candidate genes [Malki et al., 2014, 2016; Freudenberg et al., 2015; Veroude et al., 2016].

The zebrafish (*Danio rerio*) is a species where both females and males exhibit aggressive behavioual patterns [Spence et al., 2008; Panula et al., 2010; Jones and Norton, 2015]. A number of zebrafish genes have human orthologues, including several previously associated with aggressive behaviors [Barbazuk et al., 2000; Woods et al., 2000; Lieschke and Currie, 2007; Oliveira et al., 2011]. Studies suggest that similar neurotransmitters, hormones, and neuropeptides that regulate aggression in zebrafish may also regulate aggression in other vertebrates [Jones and Norton, 2015]. These mechanisms include histamine [Norton et al., 2011] arginine vasotocin [Larson et al., 2006], and oestrogen [Colman et al., 2009]. Pharmacologically-induced increases of histamine in the brain of zebrafish have been associated with increased aggressive and bold behavior [Nuutinen and Panula, 2011], whereas short-term exposure to synthetic oestrogen (17α-ethinylestradiol) reduces aggression during male-male dyadic encounters [Colman et al., 2009].

In this study, we evaluated mRNA expression differences in a genetic mouse model of aggression and in zebrafish exposed to different aggressive encounters. The hypothesis driving our approach is that there may be an overlap in genes related to aggression across the two animal species, which may help inform gene identification and further our understanding of the molecular mechanisms that may be relevant in aggressive behavior in humans.

METHODS

Design

A meta-analysis approach was used to uncover genes involved in aggression-related behaviors in two animal species. The mouse model of aggression used 3 different sets of mouse lines selected for high and low aggression from three different origins (Netherlands, Finland, USA). The zebrafish model consisted of animals that differed by experimental manipulations of social experience: experiencing a fight or not. In the current design, the zebrafish were divided into aggression groups with two levels (high and low aggression), where isolated fish were in the low aggression group and those who had fought (with an opponent or their own mirror image) were in the high aggression group. Gene expression data collected from the PFC of the aggressive and non-aggressive mice and from brain tissue of the aggressive and non-aggressive zebrafish was used to investigate genes expressed differentially in the aggressive groups. A meta-analytic approach, employing the RankProd non-parametric algorithm, was used to identify differences in gene expression across the aggressive mouse lines as well as the zebrafish, and cross-species analyses investigated whether there were common genes differentially expressed in both species.

A small subset of fish, specifically in the group that experienced an actual fight, also have an additional confounding factor of

fight-outcome (not present in the social isolation and mirror groups). It has been shown that fight outcome may lead to neurobiological differences referred to as the winner effect. We therefore performed a confirmatory, pairwise analysis on convergent genes carried forward from the primary analysis, comparing animals in the social isolation group (no fight experience, control group) with fish that were victorious in a real fight. Given that victorious fish have the highest level of aggression and isolated fish have no fight experience at all, these groups represent the extremes of the high and low aggression groups.

Animals

The mouse model consisted of 18 male mice from three sets of lines selectively bred for high and low aggressive behavior; short attack latency (SAL) and long attack latency (LAL) mice [Vanoortmerssen and Bakker, 1981], Turku Aggressive (TA) and Turku Non-Aggressive (TNA) mice [Lagerspetz, 1961] and the North Carolina Aggressive (NC900) and North Carolina Non-Aggressive (NC100) mice [Sandnabba, 1996]. The mice were bred in the laboratory of Sietse de Boer (Groningen, The Netherlands) and were housed in unisexual groups, in Perspex cages ($17 \times 11 \times 13$ cm) until weaning (~3/4 weeks of age). Each male mouse was then paired with a female mouse originating from the same line (~6–8 weeks old), in order to avoid social isolation and inter-male competition. The housing of each male-female pair was standardised and consisted of a Makrolon Type II cage (375 cm^2) with sawdust bedding, shredded paper as nesting material, and a cardboard tube as cage enrichment.

The SAL/LAL strains were originally selectively bred from a small randomly bred wild-type *Mus musculus domesticus* population found in Groningen, The Netherlands. The mice were tested for aggression level at the age of 14 weeks through a resident-intruder test. The experimental animal was the resident and was tested with a naïve albino intruder. Attack latency (the time taken for the resident to first attack) was recorded and averaged over three consecutive days to give the average attack latency score (ALS), which was used to determine SAL and LAL mice [Vanoortmerssen and Bakker, 1981].

The TA/TNA strains were selectively bred from a colony of Swiss albino mice in Turku, Finland. Males were tested at 60 days of age in a standard 7-min dyadic test in a neutral container against mice pretested for low aggression. Aggression level was rated on a 7-point scale. Males with high aggression levels were bred with the sisters of other high aggression mice, in order to avoid inbreeding and similarly for the low-aggression line [Lagerspetz, 1961].

The NC900/NC100 strains were selectively bred from a population of out-bred NCR mice in North Carolina, USA. Attack frequency was recorded using a standard 10-min dyadic test carried out in a neutral Plexiglas box, after 5 min of adaptation in which no physical contact was allowed. Attack frequency together with 31 other variables testing aggression and reactivity to stimulation provided a scoring system, where a maximum score of 900 represented highly aggressive mice [Hood and Cairns, 1988; Sandnabba, 1996].

The zebrafish data was obtained from the Gene Expression Omnibus (GEO) repository (GEO accession number: GSE56549) from a study conducted previously at the Oliveira lab [Oliveira et al., 2016]. All animals were wild-type (AB) male zebrafish (*Danio rerio*) and were acquired from the Zebrafish International Resource Center (ZIRC). They differed on experimental manipulations regarding social experience on four experimental conditions (three in each group). The four groups were; mirror fighters (M), winners of a real opponent fight (W), losers of a real opponent fight (L) and socially isolated fish (I). In the current study, zebrafish in the high aggression group had experienced a fight; either with their own mirror images or with opponents (M, W, L), and the zebrafish in the low aggression group were isolated (I). Mirror image exposure has been shown to elicit aggressive responses in fish, which do not recognize their own image. The behavior matches that shown in the presence of an intruder and aggressive behaviors are not limited to simply a reflex of heightened aggressive motivation[Teles and Oliveira, 2015]. Fish were isolated for five days prior to experimental tests. Experimental manipulations lasted for 30 min, and the fish were then anesthetized with a lethal dose of MS-222 (1,000–1,500 mg/L). They were subsequently decapitated and brain tissue was collected.

mRNA Extraction

Brains of the mice were dissected and tissues were snap frozen. The mRNA was extracted from the prefrontal tissue using the Trizol RNA isolation method and was quality assessed using gel electrophoresis. Three microgram mRNA was processed using the Affymetrix One-Cycle Target labeling protocol. The mRNA of each mouse was hybridised to an individual mouse 430 MOE v2 Gene Expression Array (Affymetrix, Santa Clara, CA), which was used because it offered the most widespread coverage of the mouse genome at the time of the study. The array comprises 45,101 probe sets, measuring expression levels of around 39,000 transcripts and variants covering over 19,000 mouse genes. The housing and experimental procedures were in accordance with the UK Home Office Animal (Scientific Procedures) Act 1986.

Brain samples of the zebrafish were rapidly collected and homogenized after decapitation. RNA was extracted following standard methods of RNeasy Lipid Tissue Mini Kit, Qiagen. The RNA was treated with DNase (RNase-free DNase set, Qiagen) in order to remove contaminations with genomic DNA. Concentration and purity was estimated using spectrophotometric absorbance in a NanoDrop ND-1000 UV-Vis Spectrophotometer (Nano-Drop Technologies).

Statistical Analysis

Raw probe intensity files from the Affymetrix chips were normalised and summarised using Robust Multi-array Averaging (RMA) [Irizarry et al., 2003], separately for each animal model. The RMA function was used within the Affymetrix package from Bioconductor, which provides outputs of base 2 log-transformed intensity data. MAS 5.0 was used to correct for sequence biases in present/absent detection calls. As the arrays contain multiple probe sets tagging the expression of each gene, probe sets were annotated using Panther (http://www.Panterdb.org). Data sets were matched on the basis of gene symbols in order to make the probe level data

between the mouse and zebrafish animal models comparable. The mouse and zebrafish transcriptomic data sets were then matched so they only contained intensity level data on genes that were present in both data sets. The Mouse Genome 430 2.0 Array contains 45,101 probes and the Affymetrix Zebrafish Genome Array contains 15,617 probes. The sampling procedure identified 4,535 orthologous genes tagged by the two arrays.

Differential Gene Expression

In order to examine differentially expressed genes between the low and high aggressive groups in each data set, the non-parametric rank product test [Breitling et al., 2004] was applied using the Bioconductor package RankProd [Hong et al., 2006] to the mouse and zebrafish data, separately. The RankProd package allows integration of microarray data from different laboratories with different experimental conditions by utilising a meta-analytic method. The Rank Product (RP) is a statistic that identifies genes consistently found among the most strongly expressed up- or down-regulated genes across experiments. Because the method combines gene ranks from different experiments, that is, origins, instead of using raw expression values, it is possible to identify top genes that are consistently differentially expressed across conditions. We report results for genes that show consistency in directionality of fold change across the three sets of mouse lines as differences in directionality points at interaction effects that are not explored as part of this study. A smaller RP value indicates a small probability of the gene being at the top of list, across conditions, simply by chance. The analysis was only conducted on probe sets tagging the expression of genes orthologous across both species.

The RankProd package assesses the significance of the detection and provides associated *P* values in the output. In the current study, *P* values were calculated with 100,000 permutations. Multiple testing was controlled for by using the proportion of false positives (PFP) [Fernando et al., 2004] at a threshold of PFP < 0.001. False discovery rate (FDR) is another multiple testing correction method and often provides similar outputs as the PFP method. However, PFP is preferred when there may be relationships between variables, as in RNA data. Prior to analysis we used the ComBat function in the Surrogate Variable Analysis (SVA) package for R, available from bioconductor (www.bioconductor.org), to control for non-biological experimental variation [Johnson et al., 2007].

Gene Network Analysis

Genes uncovered across both species were mapped to KEGG pathway maps in order to obtain further biological interpretation of higher-level systemic functions (http://www.genome.jp/). The gene set was uploaded to MetaCore^{TM} knowledge base in order to build gene networks and explore potential gene interactions. MetaCore^{TM} uses different algorithms using uploaded reference gene lists as seeds and known interactions from the database and edges to generate gene-content specific networks. The software further allows the option to explore the association between the network generated with diseases and processes. MetaCore^{TM} ranks networks based on *P*-value and gScore. A *P*-value is obtained by looking at the hypergeometric mean based on the intersection between the uploaded reference gene list and the prebuilt pathway maps and networks on MetaCore^{TM}. The statistics considers a numbers of variables and the *P*-value indexes the probability that the network occurred by chance. The software also returns a modified *z*-score (gScore) which effectively indexes the degree to which the network contains any fragments of canonical signaling pathways. A higher g-score suggests that the network is saturated with reference (seed) genes and contains more canonical pathway fragments.

RESULTS

In the first part of the analysis, we evaluated differences between low and high aggressive lines within each of the three mouse sets. RankProd allows the combination of results across all sets circumventing any issue driven by the predominant strain effects. The RankProd analysis returned 76 (PFP< 0.05) differentially expressed genes in the aggressive compared to the non-aggressive mouse lines; 44 genes were significantly up-regulated and 32 were significantly down-regulated. The same analysis was conducted in Zebrafish but here all animals were assigned to the same origin. The analysis returned 70 (PFP< 0.05) genes as differentially regulated between the zebrafish that experienced fighting compared to zebrafish in social isolation. 36 genes were significantly up-regulated and 34 genes were significantly down-regulated. A summary of the 10 top ranking genes based on highest Fold Change (FC), in the mouse and zebrafish models is presented in Tables I and II. FC refers to the ratio of change between the average initial value (expression level in the non-aggressive group) and average final value (expression level in the aggressive group). Hence, if the FC is 2, the expression level of a gene in the aggressive group is on average two times larger than in the non-aggressive group. We then identified those genes that were differentially expressed across both studies. A total of seven genes were differentially expressed

TABLE I. Top Differentially Expressed Genes Between Aggressive and Non-Aggressive Mice

Gene symbol	RP	Log_2 (FC)	PFP
Up-regulated			
Efhd2	8.377	3.399	<0.001
Rnf13	287.664	2.172	<0.001
Cap1	213.734	1.618	<0.001
Gins4	208.497	1.442	<0.001
Fos	121.160	1.429	<0.001
Sdc4	172.329	1.412	<0.001
Cdkl1	125.997	1.408	<0.001
Down-regulated			
Tmem69	87.720	−1.498	<0.001
Casp9	252.784	−1.486	0.003
Tmem14c	192.708	−1.467	<0.001

Table shows the gene symbol, rank product (RP), log_2 transformed fold change (FC), and PFP values for the top 10 differentially expressed genes in mice.

TABLE II. Top Differentially Expressed Genes Between Zebrafish in the Aggressive and Non-Aggressive Conditions

Gene symbol	RP	Log_2 (FC)	PFP
Up-regulated			
fos	2.855	5.025	<0.001
klf9	4.811	5.005	<0.001
fkbp5	10.263	3.860	<0.001
nr4a1	7.404	3.671	<0.001
acox1	57.167	2.889	<0.001
ier2	12.414	2.796	<0.001
btg2	15.774	2.573	<0.001
Down-regulated			
ighm	15.996	4.906	<0.001

Table shows the gene symbol, rank product (RP), log_2 transformed fold change (FC), and PFP values for the top 10 differentially expressed genes in zebrafish.

in the aggressive groups across the two species; *Fos*, *Dusp1*, *Hdac4*, *Ier2*, *Bdnf*, *Btg2*, and *Nr4a1* (see Table III) belonging to four gene families *Clic*, *Dusp*, *Rnf*, and *Npc*.

A confirmatory analysis tested mean expression levels in the seven genes carried forward from the above analysis between the extremes of the aggression groups in fish (no fight exposure and winner of an actual fight). This analysis was conducted to test whether differences were associated with aggression level rather than a winner effect. The results show that the seven genes remain significantly expressed even when grouping animals using this design configuration (Table IV). Moreover, the genes show consistency in the directionality of the effect, which is in the hypothesised direction: higher in the victorious fish group compared to the control group (Supplementary Fig. S1). These results add corroborating evidence for an association between the genes reported and aggression and that these effects are independent of the winner effect.

In order to gain further molecular insight into the potential relationship between the genes uncovered, we performed gene enrichment and pathway analysis using KEGG and MetaCore™. The results need to be interpreted with caution given the low number of reference genes entered but may nonetheless provide important clues on networks implicated in aggression. A significant KEGG pathway ($P < 0.00039$) containing four of the seven reference molecules uploaded was found to be associated with the MAPK signaling cascade (Fig. 1). The genes include *BDNF*, *Fos*, *Nr4a1*, and *Dusp1*. The network returned is plausible as the MAPK signaling cascade has previously been associated with aggressive phenotypes in mouse and zebrafish [Oliveira-Dos-Santos et al., 2000; Malki et al., 2014; Norton et al., 2011]. We further conducted an induced network analysis using MetaCore. The top ranking network based on gScore (gScore = 64.53, $P = 4.01 \times 10^{-12}$) includes five out the seven references molecules uploaded: *c-Fos*, *Dusp12*, *Hdac*, *Nr4a1* (*NUR77*), and *Bdnf*. The inclusion of the majority of the molecules uncovered from our analysis within the same gene-network suggests that these are likely to be functionally related. The network is centred on the *c-Fos* gene hub, which is one of the reference molecules uploaded. The *FOS* gene family consists of four members: *FOS*, *FOSB*, *FOSL1*, and *FOSL2* and encodes leucine zipper proteins. These can dimerize with JUN proteins forming the transcription factor complex AP-1. FOS proteins have previously been association with modulation of cell proliferation, differentiation, and apoptotic cell death. Several of the genes uploaded including *Bdnf*, *Nr4a1* and *Hdac*4, are one interaction away from the c-Fos (Fig. 2).

DISCUSSION

In this study we explored overlap in the molecular signature of two animal models of aggression in order to derive a set of candidate genes with higher prior probability of being associated with aggressive related behavior and to gain further understanding into neurobiological mechanisms of this complex trait. The results from this study identified seven genes that were differentially expressed across mouse and zebrafish models of aggression and could help to inform the identification of candidate genes and potential molecular mechanisms that may be involved in the aetiology and regulation of aggressive behavior in humans.

TABLE III. Genes Significantly Differentially Expressed Between Aggressive and Non-Aggressive Groups Identified in Both the Mice and Zebrafish

		Mouse (*Mus musculus*)				Zebrafish (*Danio rerio*)		
Gene symbol	Transcript ID	RP	Log_2 (FC)	PFP	Transcript ID	RP	Log_2 (FC)	PFP
Fos	NM_010234	121.160	1.429	<0.001	NM_205569	2.855	5.025	<0.001
Dusp1	NM_013642	195.962	1.382	0.003	NM_213067	168.942	1.491	0.013
Hdac4	NM_207225	211.260	1.390	0.002	NM_001039358	229.980	1.423	0.043
Ier2	NM_010499	258.132	1.288	0.004	NM_001142583	12.414	2.796	<0.001
Bdnf	NM_001048139	300.992	1.214	0.007	NM_131595	192.040	1.487	0.02
Btg2	NM_007570	304.883	1.280	0.007	NM_130922	15.774	2.573	<0.001
Nr4a1	NM_010444	342.626	1.261	0.013	NM_001002173	7.404	3.672	<0.001

Table contains the gene symbol, rank product (RP), log_2 transformed fold change (FC), and PFP values for the genes. All genes were up-regulated in both species.

TABLE IV. The Table Shows the Results From the Comparison Between Fish in Isolation Versus Fish How Have Fought and Won on the Same Seven Genes Reported From the Integrative Analysis

Probeset ID	Gene symbol	adj.P.Val	*P*-value	t	B	FC	Gene title
Dr.12986.3.S1_a_at	*fosab*	0.0000406	0.0000058	12.975355	3.54792	Positive	v-fos FBJ murine osteosarcoma viral oncogene homolog
Dr.2413.1.S1_at	*dusp1*	0.0020465	0.0017541	5.037648	−0.73891	Positive	Dual specificity phosphatase 1
Dr.13084.1.A1_at	*hdac4*	0.0016397	0.0011712	5.422503	−0.3694	Positive	Histone deacetylase 4
Dr.1137.1.A1_at	*ier2*	0.0012269	0.0007011	5.939751	0.08972	Positive	Immediate early response 2
Dr.8195.1.A1_at	*bdnf*	0.0012269	0.0005277	6.240922	0.33839	Positive	Brain-derived neurotrophic factor
Dr.6511.1.S1_at	*btg2*	0.0679000	0.0000224	10.497850	2.74976	Positive	B-cell translocation gene 2
Dr.9243.1.A1_at	*nr4a1*	0.0000914	0.0000261	10.242546	2.65341	Positive	Nuclear receptor subfamily 4, group A, member 1

Probe set ID, gene symbol, FDR adjusted *P*-value, *t*-statistics, beta, directionality of fold change and gene title. All genes are significantly expressed differentially between the two groups and positively expressed in the high aggressive group (fish that fought an actual opponent and won) compare to the controls (fish who never fought).

Convergent Molecular Targets

Several of the genes uncovered appear to be functionally related and have previously been associated with aggression-related traits. The KEGG pathway showed that several of the genes uncovered are associated with the MAPK signaling cascade. Perturbation of the MAPK signaling cascade has previously been associated with aggression and a number of psychopathologies including MDD and response to pharmacotherapy [Malki et al., 2013]. The results from the pathway analysis show that five out of the seven reference molecules belong to the same gene network with three of the genes interacting directly with the *c-Fos* gene-hub. A role for *c-Fos* has previously been reported in animal models of aggression [Gammie and Nelson, 2001; Davis and Marler, 2004; Haller et al., 2006]. In rodent models, an increase in c-FOS activation following aggressive encounters was found for several brain regions including prefrontal cortex [Davis and Marler, 2004]. The protein c-FOS forms a transcription complex with the JUN/AP-1 transcription factor. *Ap-1* regulates major physiological processes including cell proliferation, differentiation, and response to stress [Shaulian and Karin, 2002]. The *Fos* gene has systematically been implicated in aggressive behavior in animal models [Haller et al., 2006; Kabelik et al., 2010].

The results from our pathway analysis show that *c-Fos* interacts directly with the *Hdac4* gene. The *Hdac4* (Histone deacetylase 4) gene represents a potentially relevant drug target. The gene is a member of Histone Deacetylases (HDACs), a group of enzymes that repress transcription by catalyzing removal of acetyl groups from lysine residues in histones and non-histone proteins. The protein encoded by *Hdac4* is a repressor of Mef2C activity, a regulated transcription factor, which in turn acts as an effector of neurogenesis [Li et al., 2008]. A recent study found that both *Hdac4* and *Mef2C* are involved in neurogenesis [Davila et al., 2014]. Further, mouse models of Huntington disease have shown that *HDAC4* reduction delays cytoplasmic aggregate formation, restores *Bdnf* transcription, and can restore synaptic function, hence HDAC4 reduction may be a therapeutic strategy against neurodegeneration [Mielcarek et al., 2013]. The gene is associated with a number of disorders including 2q37 deletion syndrome which can include outbursts of uncontrollable aggression and tantrums. HDAC4 haploinsufficiency and de novo intragenic mutation have been identified as playing a role in the pathology.

Interestingly, *HDAC4* is a drug target of valproic acid (VPA), a mood stabiliser with potential application to a broad range of central nervous system related disorders [Gottlicher et al., 2001; Kramer et al., 2003]. Several studies have suggested the potential of valproic acid as a pharmacological treatment for aggression in geriatrics and in patients with acquired brain-injury [Horne and Lindley, 1995; Wilcox, 1994; Wroblewski et al., 1997; Ruedrich et al., 1999; Hollander et al., 2003; DelBello et al., 2004; Barzman et al., 2005; Barzman et al., 2006; Blader et al., 2009; Bidzan et al., 2012]. Studies have also shown that VA is effective in controlling disruptive behavior in adolescents with conduct disorder and adjunctive therapy to reduce aggression in children with ADHD [Donovan et al., 2000; Wozniak, 2005; MacMillan et al., 2006; Blader et al., 2009].

c-Fos also interacts directly with the *Bdnf* (Brain-derived neurotrophic factor) gene. This gene is a member of the neurotrophin

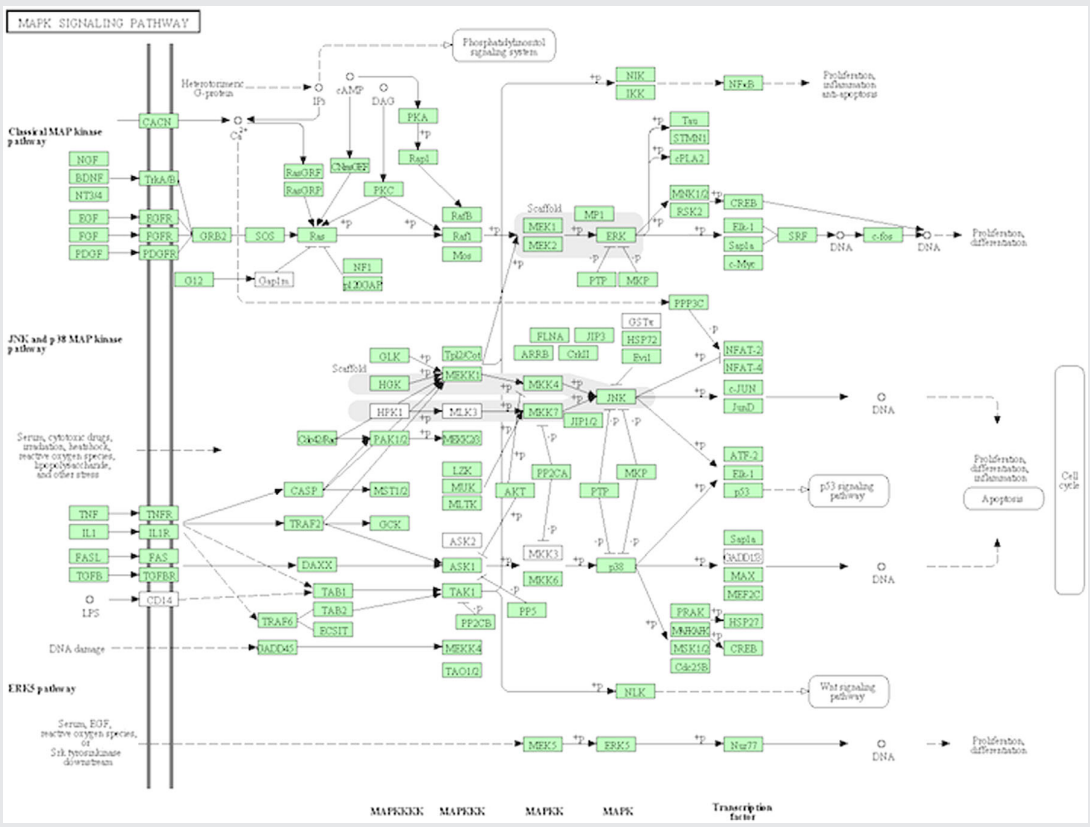

FIG. 1. Reference pathway with species-specific genes coloured in green. The pathway returned includes four of the seven references molecules uploaded and it is cantered on the MAPK signaling cascading. The pathway is functionally relevant and has previously been implicated in aggressive phenotypes in mouse. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

family, consisting of small secreted proteins that play an important role in nervous system development in vertebrates [Chao et al., 2006]. *Bdnf* interacts with the *NF-kB* gene complex, which has previously been associated with aggression in mice and suggests a role for redox signaling and inflammatory pathways [Malki et al., 2014]. *Bdnf* is thought to modulate release of neurotransmitters, such as dopamine and serotonin [Tyler and Pozzo-Miller, 2001], which are implicated in impulsive aggression [Seo et al., 2008]. *Bdnf*-restricted knockout mice display elevated levels of aggression and social dominance, and show increases in depression-like behavior [Chan et al., 2006; Ito et al., 2011], likely due to abnormalities in the serotonergic and monoaminergic systems [Lyons et al., 1999; Sakata and Duke, 2014]. In humans, the BDNF Val66Met polymorphism has been associated with aggressive behavior in schizophrenic patients [Spalletta et al., 2010] and hyperactivity (Attention-deficit hyperactivity disorder; ADHD) using both candidate gene studies [Kent et al., 2005; Lanktree et al., 2008] and GWAS [Lasky-Su et al., 2008; Neale et al., 2008], although the finding does not always replicate [Forero et al., 2009; Sanchez-Mora et al., 2010]. Expression of *Bdnf* critically relies on a potent *CREB* coactivator, CREB-regulated transcription coactivator 1 (CRTC1) [Kovacs et al., 2007]. *CREB* has previously been associated with pathological aggression, depression-like symptoms, and neuroplasticity in knockout mice [Breuillaud et al., 2012]. *CREB* also shows a direct interaction with c-FOS.

Several of the other genes uncovered in our analyses have also been associated with aggressive traits. The *Btg2* gene is a member of the BTG family of genes (member 2), which encode for anti-proliferative proteins involved in cell cycle regulation and modulate transcription regulation mediated by ESR1 (Estrogen receptor 1). Recently the *Btg2* gene has been associated with hypertension in female rats, likely mediated by estrogen factors [Hoffman et al., 2013]. Estrogen is also implicated in the regulation of the *Fos* gene by binding to the AP-1 DNA sequence that alters *Fos* transcription, which fits well with previous research showing that BTG2 promoter expression is regulated by estrogen receptor response elements [Karmakar et al., 2009; Paruthiyil et al., 2011]. Estrogens have repeatedly been shown to be modulators of aggression in rodent studies [Ogawa et al., 1997; Scordalakes and Rissman, 2003; Trainor et al., 2006]. *Fos* expression is likely to be

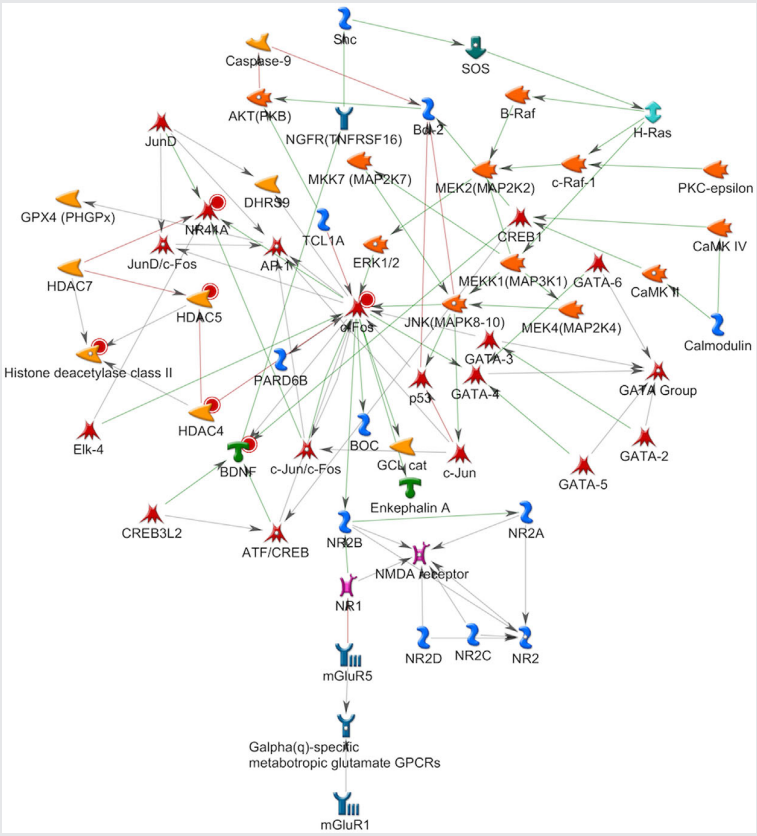

FIG. 2. Gene network returned from MetaCore. Uploaded (seed) genes are tagged with a red circle. The top ranking pathway uncovered includes five references molecules centred around the c-FOS gene hub. Several of the genes show a direct interaction with the c-FOS gene hub including the Hdac4 gene. This gene has been identified as a drug target for valproic acid which has been suggested may be used for the pharmacological treatment of aggression and conduct disorders. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

sexually dimorphic and animals of both sexes ought to be included in further studies to control for potential sex effects. Literature also suggests that BTG2 is involved in cell cycle arrest in response to DNA damage and other cellular stress, and is thought to be involved in neurogenesis processes as mediator of the growth arrest before differentiation [Tirone, 2001].

Dusp1 (dual specificity phosphatase 1) is an inducible immediate-early gene. It is expressed in response to stressors, such as oxidative damage and may be upregulated by activation of neurotransmitter receptors. The DUSP1 protein has intrinsic phosphatase activity and is an inactivator of mitogen-activated protein kinase (MAPK). DUSP1 is thought to play a role in human cellular response to environmental stress and is involved in neuroprotective mechanisms [Taylor et al., 2013].

Nr4a1 (Nuclear receptor subfamily 4, group A, member 1) encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily, which acts as a nuclear transcription factor. NR4A1 expression is induced by glucose and oxygen deprivation, acting as a protector for neurons, enhancing neural survival [Xiao et al., 2013]. NR4A1 also plays a role in immune pathways by repressing activation of the IL-2 (Interleukin 2) promoter, which is involved in white blood cell activity regulation, mediated by inhibition of the NF-kB [Harant and Lindley, 2004].

Strengths and Limitations

One of the strengths of this study is the use of two independent animal models of aggression. This design allowed to replicate findings both across studies and species and to uncover plausible candidate genes with higher probability of being implicated in the genetic aetiology of aggressive behavior. Animal studies have traditionally been successful in identifying genes implicated in human pathologies and the genes reported could inform candidate gene selection in future human studies [Malki et al., 2015]. The statistical approach used erred on the side of caution by using multiple, stringent statistical thresholds. However, the study also presents a number of important limitations, which ought to be considered when interpreting the results.

The zebrafish were not bred for aggressive behavior, although the experimental manipulation was carefully controlled for and represents a paradigm to study aggression; differences in gene expression between the zebrafish in the aggressive and non-aggressive conditions are likely to be dependent on mechanisms regulating aggressive behaviors. The zebrafish paradigm may be suboptimal, as the different types of aggression tests may have triggered activation of different genes. Studies have shown that experience-induced changes in neurotransmitter activities have an impact on later agonistic behaviors but that these may impact neurobiological mechanisms differently depending on whether the animal was victorious or a subordinate [Hsu et al., 2006]. However, for the purpose of this study we had two sub-optimal choices: group animals by test or group them by class (winner/loser). Given the low number of animals, it was unavoidable but to group animals by test as the only animals that fought an opponent were in just one of the groups. Mixing winners and losers may not be optimal but these were only a small subset of the animals. However, to test if the genes reported are associated with aggression rather than the possible confound of the winner effect, we performed a confirmatory case-control analysis. In the analysis, we compared the extremes of the groups available: fish in isolation that never fought were treated as controls and fish that were victorious against an opponent were treated as high aggression. Given the number of available animals, this grouping is suitable to test a few probe sets but it was not a suitable design for the entire array as the analysis would then have been underpowered. All genes were significantly altered in the high aggression group (showing consistency in directionality of expression) further suggesting an association between the genes reported and aggression. The evidence for association with aggression remains strong, as genes uncovered in the fish study had to replicate in the genetic mouse study, where aggression levels were independent of either test or winning outcome. Genes showing consistency of expression across studies and aggression-paradigms increase the chances that the results are true positives although at the probable cost of Type-II errors.

Due to the complex connectivity of different brain regions, the analysis of additional brain regions from these model organisms could have provided further insight into the mechanisms underlying variation in aggressive behavior. However, as with all studies that make use of publically available data, choices on brain tissues were restricted to what was available. The study is therefore more likely to reveal genes that are expressed across multiple brain tissues. Moreover, given that we were interested in uncovering gene orthologues across the two species, it is likely that genes and genetic pathways underlying aggressive behavior may have been missed when exploring differences within each study separately. However, this limitation is intrinsic in the cross-species design of the study.

It is also possible that other factors that were not controlled for, including stress and reaction to novelty, could be associated with expression differences, particularly in the zebrafish study. However, given the stringent statistical approach, these would have to cause a substantial biological insult to cause expression changes that could be detected above those relating to aggression. Moreover, these changes would still have to overlap between the fish study and the genetic model of aggression in mouse which is less likely to be susceptible to environmental factors.

Lastly, although animal models are useful in elucidating the genetic basis of human pathophysiology, results still need to be translated to human studies, as there are characteristics of the trait in humans that simply cannot be modelled in animals.

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REFERENCES

Barbazuk WB, Korf I, Kadavi C, Heyen J, Tate S, Wun E, Bedell JA, McPherson JD, Johnson SL. 2000. The syntenic relationship of the zebrafish and human genomes. Genome Res 10(9):1351–1358.

Barzman DH, DelBello MP, Adler CM, Stanford KE, Strakowski SM. 2006. The efficacy and tolerability of quetiapine versus divalproex for the treatment of impulsivity and reactive aggression in adolescents with co-occurring bipolar disorder and disruptive behavior disorder(s). J Child Adolesc Psychopharmacol 16(6):665–670.

Barzman DH, McConville BJ, Masterson B, McElroy S, Sethuraman G, Moore K, Kahwaty AM, Nelson D. 2005. Impulsive aggression with irritability and responsive to divalproex: A pediatric bipolar spectrum disorder phenotype? J Affect Disord 88(3):279–285.

Bidzan L, Grabowski J, Dutczak B, Bidzan M. 2012. [Impact of treatment with acetylcholinesterase inhibitors, valproic acid and antipsychotics on aggressive behaviour in Alzheimer's type dementia]. Psychiatr Pol 46(3):361–372.

Blader JC, Schooler NR, Jensen PS, Pliszka SR, Kafantaris V. 2009. Adjunctive divalproex versus placebo for children with ADHD and aggression refractory to stimulant monotherapy. Am J Psychiatry 166(12):1392–1401.

Blanchard DC, Blanchard RJ. 2003. What can animal aggression research tell us about human aggression? Horm Behav 44(3):171–177.

Breitling R, Armengaud P, Amtmann A, Herzyk P. 2004. Rank products: A simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 573(1–3):83–92.

Breuillaud L, Rossetti C, Meylan EM, Merinat C, Halfon O, Magistretti PJ, Cardinaux JR. 2012. Deletion of CREB-Regulated transcription coactivator 1 induces pathological aggression, depression-related behaviors, and neuroplasticity genes dysregulation in mice. Biol Psychiatry 72(7):528–536.

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.

Cases O, Seif I, Grimsby J, Gaspar P, Chen K, Pournin S, Müller U, Aguet M, Babinet C, Shih JC, De Maeyer E. 1995. Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA. Science 268(5218):1763–1766.

Chan JP, Unger TJ, Byrnes J, Rios M. 2006. Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. Neuroscience 142(1):49–58.

Chao MV, Rajagopal R, Lee FS. 2006. Neurotrophin signalling in health and disease. Clinical science 110(2):167–173.

Coccaro EF, Kavoussi RJ, Berman ME, Lish JD. 1998. Intermittent explosive disorder-revised: Development, reliability, and validity of research criteria. Compr Psychiatry 39(6):368–376.

Colman JR, Baldwin D, Johnson LL, Scholz NL. 2009. Effects of the synthetic estrogen, 17alpha-ethinylestradiol, on aggression and court-

ship behavior in male zebrafish (Danio rerio). Aquat Toxicol 91(4):346–354.

Davidge KM, Atkinson L, Douglas L, Lee V, Shapiro S, Kennedy JL, Beitchman JH. 2004. Association of the serotonin transporter and 5HT1Dbeta receptor genes with extreme, persistent and pervasive aggressive behaviour in children. Psychiatr Genet 14(3):143–146.

Davila JL, Goff LA, Ricupero CL, Camarillo C, Oni EN, Swerdel MR, Toro-Ramos AJ, Li J, Hart RP. 2014. A positive feedback mechanism that regulates expression of miR-9 during neurogenesis. PLoS ONE 9(4): e94348.

Davis ES, Marler CA. 2004. C-fos Changes following an aggressive encounter in female California mice: A synthesis of behavior, hormone changes and neural activity. Neuroscience 127(3):611–624.

DelBello MP, Adler C, Strakowski SM. 2004. Divalproex for the treatment of aggression associated with adolescent mania. J Child Adolesc Psychopharmacol 14(2):325–328.

Donovan SJ, Stewart JW, Nunes EV, Quitkin FM, Parides M, Daniel W, Susser E, Klein DF. 2000. Divalproex treatment for youth with explosive temper and mood lability: A double-blind, placebo-controlled crossover design. Am J Psychiatry 157(5):818–820.

Fazel S, Danesh J. 2002. Serious mental disorder in 23000 prisoners: A systematic review of 62 surveys. Lancet 359(9306):545–550.

Fernando RL, Nettleton D, Southey BR, Dekkers JC, Rothschild MF, Soller M. 2004. Controlling the proportion of false positives in multiple dependent tests. Genetics 166(1):611–619.

Flory JD, Xu K, New AS, Finch T, Goldman D, Siever LJ. 2007. Irritable assault and variation in the COMT gene. Psychiatr Genet 17(6):344–346.

Forero DA, Arboleda GH, Vasquez R, Arboleda H. 2009. Candidate genes involved in neural plasticity and the risk for attention-deficit hyperactivity disorder: A meta-analysis of 8 common variants. JPN 34(5):361–366.

Freudenberg F, Carreno Gutierrez H, Post AM, Reif A, Norton WH. 2015. Aggression in non-human vertebrates: Genetic mechanisms and molecular pathways. Am J Med Genet B, Neuropsychiatr Genet DOI: 10.1002/ajmg.b.32358.

Gammie SC, Nelson RJ. 2001. CFOS and pCREB activation and maternal aggression in mice. Brain Res 898(2):232–241.

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T. 2001. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20(24):6969–6978.

Haller J, Toth M, Halasz J, De Boer SF. 2006. Patterns of violent aggression-induced brain c-fos expression in male mice selected for aggressiveness. Physiol Behav 88(1–2):173–182.

Harant H, Lindley IJ. 2004. Negative cross-talk between the human orphan nuclear receptor Nur77/NAK-1/TR3 and nuclear factor-kappaB. Nucleic Acids Res 32(17):5280–5290.

Hoffman MJ, Flister MJ, Nunez L, Xiao B, Greene AS, Jacob HJ, Moreno C. 2013. Female-specific hypertension loci on rat chromosome 13. Hypertension 62(3):557–563.

Hollander E, Tracy KA, Swann AC, Coccaro EF, McElroy SL, Wozniak P, Sommerville KW, Nemeroff CB. 2003. Divalproex in the treatment of impulsive aggression: Efficacy in cluster B personality disorders. Neuropsychopharmacology 28(6):1186–1197.

Hong F, Breitling R, McEntee CW, Wittner BS, Nemhauser JL, Chory J. 2006. RankProd: A bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics 22(22):2825–2827.

Hood KE, Cairns RB. 1988. A developmental-genetic analysis of aggressive behavior in mice. II. Cross-sex inheritance. Behav Genet 18(5):605–619.

Horne M, Lindley SE. 1995. Divalproex sodium in the treatment of aggressive behavior and dysphoria in patients with organic brain syndromes. J Clin Psychiatry 56(9):430–431.

Hsu Y, Earley RL, Wolf LL. 2006. Modulation of aggressive behaviour by fighting experience: Mechanisms and contest outcomes. Biol Rev Camb Philos Soc 81(1):33–74.

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.

Ito W, Chehab M, Thakur S, Li J, Morozov A. 2011. BDNF-restricted knockout mice as an animal model for aggression. Genes Brain Behav 10(3):365–374.

Johnson WE, Li C, Rabinovic A. 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8(1):118–127.

Jones LJ, Norton WH. 2015. Using zebrafish to uncover the genetic and neural basis of aggression, a frequent comorbid symptom of psychiatric disorders. Behav Brain Res 276:171–180.

Kabelik D, Kelly AM, Goodson JL. 2010. Dopaminergic regulation of mate competition aggression and aromatase-Fos colocalization in vasotocin neurons. Neuropharmacology 58(1):117–125.

Karmakar S, Foster EA, Smith CL. 2009. Estradiol downregulation of the tumor suppressor gene BTG2 requires estrogen receptor-alpha and the REA corepressor. Int J Cancer 124(8):1841–1851.

Kent L, Green E, Hawi Z, Kirley A, Dudbridge F, Lowe N, Raybould R, Langley K, Bray N, Fitzgerald M, Owen MJ, O'Donovan MC, Gill M, Thapar A, Craddock N. 2005. Association of the paternally transmitted copy of common Valine allele of the Val66Met polymorphism of the brain-derived neurotrophic factor (BDNF) gene with susceptibility to ADHD. Mol Psychiatry 10(10):939–943.

Kovacs KA, Steullet P, Steinmann M, Do KQ, Magistretti PJ, Halfon O, Cardinaux JR. 2007. TORC1 is a calcium- and cAMP-sensitive coincidence detector involved in hippocampal long-term synaptic plasticity. Proc Natl Acad Sci USA 104(11):4700–4705.

Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, Brill B, Groner B, Bach I, Heinzel T, Gottlicher M. 2003. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J 22(13):3411–3420.

Lagerspetz K. 1961. Genetic and social causes of aggressive-Behavior in mice. Scand J Psychol 2(4):167–173.

Lanktree M, Squassina A, Krinsky M, Strauss J, Jain U, Macciardi F, Kennedy JL, Muglia P. 2008. Association study of brain-derived neurotrophic factor (BDNF) and LIN-7 homolog (LIN-7) genes with adult attention-deficit/hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet 147B(6):945–951.

Larson ET, O'Malley DM, Melloni RH, Jr. 2006. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. Behav Brain Res 167(1):94–102.

Lasky-Su J, Neale BM, Franke B, Anney RJ, Zhou K, Maller JB, Vasquez AA, Chen W, Asherson P, Buitelaar J, Banaschewski T, Ebstein R, Gill M, Miranda A, Mulas F, Oades RD, Roeyers H, Rothenberger A, Sergeant J, Sonuga-Barke E, Steinhausen HC, Taylor E, Daly M, Laird N, Lange C, Faraone SV. 2008. Genome-wide association scan of quantitative traits for attention deficit hyperactivity disorder identifies novel associations and confirms candidate gene associations. Am J Med Genet B Neuropsychiatr Genet 147B(8):1345–1354.