Genetic determinants of emotionality and stress response in AcB/BcA recombinant congenic mice and *in silico* evidence of convergence with cardiovascular candidate genes

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Genomic loci bearing stress-related phenotypes were dissected in recombinant congenic strains (RCS) of mice with C57BL/6J (B6) and A/J progenitors. Adult male mice from 14 A/J and 22 B6 background lines were evaluated for emotional reactivity in open-field (OF) and elevated plus-maze tests. Core temperature was monitored by radio telemetry during immobilization and on standard as well as salt-enriched diets. In addition, urinary electrolytes were measured. Genome-wide linkage analysis of the parameters revealed over 20 significant quantitative trait loci (QTL). The highest logarithm of odds (LOD) scores were within the previously-reported OF emotionality locus on Chr 1 (LOD = 4.6), in the dopa decarboxylase region on Chr 11 for the plus-maze (LOD = 4.7), and within a novel region of calmodulin 1 on Chr 12 for Ca²⁺ excretion after a 24-h salt load (LOD = 4.6). RCS stress QTL overlapped with several candidate loci for cardiovascular (CV) disease. *In silico* evidence of functional polymorphisms by comparative sequence analysis of progenitor strains assisted to ascertain this convergence. The anxious BcA70 strain showed down regulation of *Atp1a2* gene expression in the heart (*P* < 0.001) and brain (*P* < 0.05) compared with its parental B6 strain, compatible with the enhanced emotionality described in knock out animals for this gene, also involved in the salt-sensitive component of hypertension. Functional polymorphisms in regulatory elements of candidate genes of the CV/inflammatory/immune systems support the hypothesis of genetically-altered environmental susceptibility in CV disease development.

INTRODUCTION

Animal models provide valuable tools to dissect the ecogenomics of multifactorial traits (1) as they allow significant reduction of the complexity of interacting hereditary and environmental factors. The genomic determinants of stress-related phenotypes (2) and hypertension (3) have begun to be identified in several mouse models, including comprehensive sets of strains derived from A/J and B6. Challenging efforts are being made toward fine-mapping to the quantitative trait nucleotide level and translation into new knowledge for mental (4,5) and cardiovascular (CV) health (6).

The AxB/BxA recombinant inbred (RI) panel was formed by interbreeding B6 and A/J founders (7). When comparing parental strains, the B6 group had higher open-field (OF) activity than the A/J group (8–14), more transitions in the light/dark box (15,16), more hole-poking responses (12), higher open arm duration in the elevated plus-maze (EPM) (14), and a higher number of cued and contextual fear-conditioning responses (17). Since the timid behaviour of the A/J albino strain is apparent in the plus-maze under red light, it is assumed that heightened susceptibility to light is not the main reason for its high anxiety levels relative to other strains (18). Analysis of AxB/BxA RI strains revealed several quantitative trait loci (QTL) for baseline and

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diazepam-induced activity in OF and light/dark box tests (19) as well as ethanol- (20) and cocaine-induced activation (21). Ambulatory QTL in the OF on Chr 1 (101 cM) and 10 (74 cM) were confirmed in F2 progeny derived from A/J and B6 lines (11).

The AcB/BcA recombinant congenic strains (RCS) used in our study differ from the RI panel in that each line carries a fraction (12.5%) of the genome on the complementary background (87.5%) of the other (7). This model system was designed for genetic dissection of complex diseases characterized by multiple susceptibility factors such as those involved in colon cancer (22). With A/J and B6 as progenitors, QTL were identified for host-immune responses (23,24), the acoustic startle response (25), alcohol intake (26), amphetamine-induced (27), cocaine-induced (28), nicotine-induced (29) and stress-induced activation (2).

The present study complements our previous QTL analyses of the stress response, metabolism and gene expression in mice and RI rat strains (30–34), indicating the pleiotropism of heightened stress susceptibility in genetic hypertension (35–37), which can be amplified and revealed with a high-salt diet as in some human populations (6). Hypothermia in mice corresponds to the initial stage of an acute stress response, evident after high doses of lipopolysaccharide (LPS) (38), similar to the rat hypothemic response (36,39). In seven inbred mouse strains (NM, C3H, Bl/6, Bl/10, Balb/c, A/J and SHM), body temperature changes (hypothermia followed by a recovery phase of thermogenesis) after injection of a high-LPS dose (500 µg/kg) were highly correlated \( r = +0.95, P < 0.01 \) with 15-min immobilization stress (unpublished data).

Here, the strain distribution and genomic determinants associated with behavioural profile (exploration of the OF and EPM) were assessed along with stress-induced physiological measures of body temperature changes, urinary volume and electrolyte excretion on a normal diet and after salt loading. Given the availability of complete genome sequences of the B6 and A/J parental lines, we performed detailed analyses of the identified QTL at the single nucleotide polymorphism (SNP) level, utilizing both private and public databases. Multiple bioinformatic tools were deployed to determine the putative functional implications of individual SNPs. This screening covered interference with functional motifs for both coding and regulatory regions, and changes in mRNA secondary structure or in microRNA target sequences in the 3′ untranslated region (3′-UTR).

RESULTS

Exploration of environmental stimuli

For emotionality (Emo) in the OF, broad sense heritability (H2) was 60.9% on the B6 and 50.1% on the A/J background. Polarity between parental strains was highly significant \( P < 0.0001 \), with the B6 lines showing less Emo. The introduction of A/J alleles into the genomic background of the informative strains was associated with increased Emo in the BcA70 and BcA72 lines relative to B6 \( P < 0.002 \) as presented in Figure 1A; conversely, line AcB54 was less emotional than its A/J parental strain \( P < 0.004 \). A similar strain distribution pattern (SDP) was obtained for fecal boli (data not presented), with the same informative BcA70 and AcB54 strains being significant in their respective B6 \( H2 = 61.0\% \) and A/J \( H2 = 46.0\% \) backgrounds. BcA70, BcA72 and AcB54 were also close to significance \( P < 0.01 \) for segment-crossed measurement \( H2 = 61.0\% \) in the B6 set; 60% in the A/J background. Significant Emo QTL were found for the B6 set on Chr 1, 3, 4 (2 loci), and 7 (distal) regions, with their respective logarithm of odds (LOD) scores of 4.6, 3.9, (3.5, 3.2) and 3.5 (Table 1), and where albino anxiogenic alleles were detected on BcA70 and BcA72 informative strains. Similar but only suggestive QTL on Chr 1, 3 and 4 were obtained for fecal boli in the B6 set, and no significant QTL were observed with crossed segments.

In the EPM, emergence latencies from the initial enclosed arm displayed a heritability estimate of 59.8% on the B6 and 57.6% on the A/J background, with huge polarity between parental strains \( P < 0.0001 \). Figure 1B illustrates that the BcA72, BcA85 and BcA87 lines took more time before starting to explore than B6 \( P < 0.002 \); conversely, AcB51 mice emerged faster than A/J \( P < 0.004 \). The SDP followed a similar pattern for enclosed arm visit duration, but intrastain differences were more prominent in the B6 set \( H2 = 53.5 \) versus 39.5% in the A/J set). Significant linkage signals were detected in the B6 background on Chr 9, 11 and 19 for emergence latencies \( LOD = 3.8, 4.7 \) and 3.9). These QTL corresponded to shorter emergence latencies in lines bearing A/J alleles. However, shorter emergence latencies were associated with the B6 allele in the AcB51 strain contributing to QTL on the upstream region of Chr 1 \( LOD = 3.4 \) found in the A/J set. Finally, for 1 QTL detected on proximal Chr 7 \( LOD = 3.8 \), the introduction of A/J alleles on the BcA69, BcA72 and BcA87 lines was associated with increased enclosed arm duration relative to B6 \( P < 0.01 \).

Body temperature stress response under normal and increased salt diet

In the 30-min immobilization period, two highly intercorrelated phases of response \( r = -0.84, P < 0.0001 \) from the means of all 36 strains) were noted: initial hypothermia, defined as the delta between minimum and baseline temperatures, followed by thermogenesis, measured as the temperature at the last minute of immobilization. Relevantly, each phase of the response was distinct in terms of the genetic determinants. Heritability estimates, which reflect the genetic influence on core temperature interstrain variability, appeared elevated on the A/J background for both hypothermic \( H2 = 51.7\% \) and thermogenesis \( H2 = 66.1\% \) responses compared with the pre-stress period \( H2 = 45.8\% \). The A/J line manifested much less thermogenesis than B6 \( P < 0.01 \), but the parental strains did not differ in terms of hypothermia \( P > 0.8 \). The AcB56 strain differed from A/J for both hypothermic \( P < 0.01 \) and thermogenesis \( P < 0.05 \) responses (Fig. 1C and D). Two significant QTL were found on Chr 2 and 15 in the A/J set (Table 1), where the presence of the B6 differential segment was associated with a reduced hypothermic response. Conversely, in the A/J set, near significant pro-thermogenesis QTL were detected on Chr 3 and 10 (Table 1). In the B6 background, the BcA77, BcA82 and...
BcA83 lines manifested significantly less thermogenesis than B6 ($P < 0.002$; Fig. 1D).

Salt loading did not change the polarity of the progenitors, with the A/J line showing less thermogenesis than B6 ($P < 0.01$); neither was there an ancestor difference on hypothermia ($P > 0.7$). However, higher intra-strain variability compared to standard diet conditions reflected the environmental component of the hypothermic phase. This was less the case in the B6 background, in which distinct QTL were detected for the hypothermic response, showing a phenotype-specific interaction between salt-loading and genetic background (Table 2).

The BcA66 strain was the most sensitive, experiencing even deeper hypothermia than the parental B6 line ($P < 0.05$, Fig. 2D), and contributing to the QTL revealed on Chr 1, 4 (2 loci), 15 and 18 (LOD = 3.5, 3.8, 3.6, 3.7 and 3.4), where A/J alleles increased the hypothermic response to stress. The BcA66, BcA73 and BcA77 lines displayed less thermogenesis than B6 ($P < 0.002$, Fig. 2E).

**Circadian rhythm of baseline temperature before and during a high-salt diet**

Significant main effects of diet and strain factors in 2-way ANOVA were found as a result of lower temperature under the high-salt condition [$F (1,15) = 35.35; P < 0.0001$] and for the A/J strains [$F (1,15) = 79.03; P < 0.0001$]. Nevertheless, an interaction between strain and circadian period effects on 24-h baseline temperatures was seen only under the high-salt condition [$F (1,15) = 8.69; P < 0.01$], in which strain differences were deeper during the dark phase. In concordance with the parental lines, significant interactions between all RCS and circadian period factors were observed for body temperature [$F (1,35) = 2.47; P < 0.0001$].

**Urinary volume and electrolytes after 24 h of a high-salt diet**

The A/J strain was characterized by lower urinary volume than B6 ($P < 0.01$). A QTL for urinary volume ($H_2 = 59.8\%$ in the B set) was detected at a single site on Chr 11, corresponding to A/J alleles that decreased urinary volume in the BcA80 and BcA85 strains ($P < 0.01$) relative to B6 (Fig. 2A). Among electrolytes ($Na^+$, $K^+$, $Ca^{++}$ and creatinine) that were measured, only $Ca^{++}$ excretion in the A/J set and creatinine excretion in the B6 set ($H_2 = 51$ and 62%, respectively) showed significant QTL, on Chr 1, 2, 4, 8, 11, 12, 13 and 17 for the former, and on Chr 15 for the latter (Table 2). All QTL for $Ca^{++}$ caused decreased excretion in the already low A/J set, with no concordant parental strain differences ($P > 0.8$, Fig. 2B). However, caution is warranted concerning $Ca^{++}$ excretion in the A/J set, because of numerous missing values in some contributing strains as a result of insufficient volumes. The QTL for creatinine excretion corresponded to an increase in the already high-excreting B6 strain ($P < 0.05$, A/J versus B6).

**In silico exploration of putative candidate genes**

The OF emotionality QTL interval is presented in greater detail because of high statistical significance, spatial resolution and concordance with the literature. Starting from 83 known genes in this region, 38 of these had some SNPs in between parental strains; 5 of these contained major putative changes and could be classified into 2 categories. The CV system-related genes $Atp1a2$ and $Nhlh1$ as well as the immune/inflammatory genes $Slamf1$, $Fcgr3$ and $Crp$ shared mRNA secondary structure changes with significant impact.
Table 1. Significant stress-related QTL with relevant candidate genes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Locus</th>
<th>Background</th>
<th>$P$-value (t-test)</th>
<th>LOD</th>
<th>Candidate genes and products</th>
<th>Human homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open-field</td>
<td>D1Mit113--D1Mit356</td>
<td>B6</td>
<td>2.46 x 10^{-10}</td>
<td>4.6</td>
<td>Emo1, emotionality 1; Bpq2, blood pressure QTL 2; Kcnj9, potassium channel, subfamily J, member 9; Atp1a2, Na+/K+ ATPase alpha2 polypeptide</td>
<td>1q24.2</td>
</tr>
<tr>
<td>Emotionality (defecation-activity)</td>
<td>D3Mit216--D3Mit199</td>
<td>B6 (BcA70)</td>
<td>(7.64 x 10^{-5})</td>
<td>3.9</td>
<td>Fabp2, fatty acid-binding protein 2, intestinal; Pitx2, paired-like homeodomain transcription factor 2</td>
<td>4q25–q31</td>
</tr>
<tr>
<td></td>
<td>D4Mit181--D4Mit291</td>
<td>B6</td>
<td>2.57 x 10^{-2}</td>
<td>3.5</td>
<td>Gem, GTP-binding protein expressed in skeletal muscle Htr1d, SHIT 1D receptor</td>
<td>8q13–q21</td>
</tr>
<tr>
<td></td>
<td>D4Mit336--D4Mit16</td>
<td>B6</td>
<td>1.63 x 10^{-2}</td>
<td>3.2</td>
<td>Gem</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D7Mit187--D7Mit71</td>
<td>B6</td>
<td>1.71 x 10^{-2}</td>
<td>3.5</td>
<td>Bpq7, blood pressure QTL 7; Th, tyrosine hydroxylase; Fgfr2, fibroblast growth factor receptor 2</td>
<td>11q14–q21; 11p15.5; 10q26</td>
</tr>
<tr>
<td>Elevated plus-maze</td>
<td>D7Mit247--D7Mit270</td>
<td>B6</td>
<td>4.12 x 10^{-2}</td>
<td>3.8</td>
<td>CcnE1, cyclin E1; Abbp3, AJ &amp; B6 blood pressure 3 Abbp1, AJ &amp; B6 blood pressure 1; Bpq1, blood pressure QTL 1; Igfbp2, insulin-like growth factor-binding protein 2</td>
<td>19q12</td>
</tr>
<tr>
<td>Enclosed arm duration</td>
<td>D1Mit156--D1Mit435</td>
<td>A/J</td>
<td>7.66 x 10^{-6}</td>
<td>3.4</td>
<td>Abbp1, AJ &amp; B6 blood pressure 1; Bpq1, blood pressure QTL 1; Igfbp2, insulin-like growth factor-binding protein 2</td>
<td>2q33–q34</td>
</tr>
<tr>
<td>Emergence latencies (s)</td>
<td>D9Mit67--D9Mit254</td>
<td>B6*</td>
<td>4.62 x 10^{-2}</td>
<td>3.8</td>
<td>Nrgn, neurogranin (hippocampal plasticity); Grik4, glutamate kainate receptor 4</td>
<td>11q22.3–11q24</td>
</tr>
<tr>
<td></td>
<td>D11Mit74--D11mit135</td>
<td>B6*</td>
<td>1.24 x 10^{-2}</td>
<td>4.7</td>
<td>Ddc, dopa decarboxylase; Nehf, heavy neurofilament polypeptide</td>
<td>7p11; 22q12.2</td>
</tr>
<tr>
<td></td>
<td>D19Mit10</td>
<td>B6*</td>
<td>2.33 x 10^{-2}</td>
<td>3.9</td>
<td>Cnnm2, cyclin M2; Pdcd11, programmed cell death protein 11</td>
<td>10q24.2–q24.3</td>
</tr>
<tr>
<td>Immobilization (normal diet)</td>
<td>D2Mit401--D2Mit280</td>
<td>A/J*</td>
<td>3.61 x 10^{-5}</td>
<td>4.0</td>
<td>Insmid, insulinoma-associated 1</td>
<td>20p11.2</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>D15Mit175--D15Mit115</td>
<td>A/J (AcB56)*</td>
<td>4.97 x 10^{-4}</td>
<td>3.9</td>
<td>Piger4, prostaglandin E receptor 4; Bpq6, blood pressure QTL 6</td>
<td>5p13.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bdfn2, body length QTL 2</td>
<td>8q22.3 (BAALC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hspa4lf, heat shock protein 4-like</td>
<td>13q14.1 (FKHR)</td>
</tr>
<tr>
<td>Thermogenesis</td>
<td>D3nMit224</td>
<td>A/J</td>
<td>1.7 x 10^{-3}</td>
<td>3.3</td>
<td>Cirbp, cold-inducible RNA-binding protein; Igfl, insulin-like growth factor-I</td>
<td>12q22–q23</td>
</tr>
<tr>
<td></td>
<td>D10Mit42--D10Mit231</td>
<td>A/J</td>
<td>5.84 x 10^{-7}</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Suggestive loci with LOD ≥ 3.2 were considered only in the albino background in which resolution was lower (14 strains). QTL regions in bold corresponded to convergent haplotype distribution between A/J and B6 sets of RCS: then, all the RCS means were used in t-test calculations. Statistics in parentheses were analysed on individual values relative to a unique informative strain. *The QTL effect of opposite direction to that predicted from the ancestor A/J and B6 difference.
Table 2. Significant diet-induced QTL after 24 h on a high-salt diet, with relevant candidate genes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Locus</th>
<th>Background</th>
<th>P-value (t-test)</th>
<th>LOD</th>
<th>Human homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary volume</td>
<td>D11Mit245</td>
<td>A/J (A5B7)</td>
<td>1.09 x 10^-3</td>
<td>3.2</td>
<td>Myo1c, myosin 1C; Nos2, inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Creatinine excretion</td>
<td>D1Mit83</td>
<td>A/J</td>
<td>1.58 x 10^-3</td>
<td>3.8</td>
<td>Apoa2, apolipoprotein AII; Slc30a1, zinc transporter 1</td>
</tr>
<tr>
<td>Creatinine excretion</td>
<td>D2Mit156</td>
<td>A/J (A5B7)</td>
<td>2.11 x 10^-2</td>
<td>3.4</td>
<td>Cacnb4, L-type calcium channel subunit beta 3</td>
</tr>
<tr>
<td>Creatinine excretion</td>
<td>D8Mit124</td>
<td>A/J</td>
<td>0.51 x 10^-2</td>
<td>3.8</td>
<td>Irs2, insulin receptor substrate 2</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>D4Mit2 – D4Mit361</td>
<td>B6</td>
<td>1.58 x 10^-2</td>
<td>3.7</td>
<td>Htr1d, 5HT 1D receptor</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>D13Mit77 – D13Mit78</td>
<td>A/J</td>
<td>2.11 x 10^-2</td>
<td>3.4</td>
<td>Itga5, integrin alpha 5; Cacnb3, L-type calcium channel subunit beta 3</td>
</tr>
</tbody>
</table>

**Quantitative real-time (RT)-PCR validation of differential gene expression**

Differential expression between progenitors and informative strains was observed only for one gene out of the five candidates. The anxious BcA70 strain showed down regulation of the *Atp1a2* gene in the brain ($P < 0.05$, Fig. 4A), and heart ($P < 0.001$, Fig. 4B) compared to its parental B6 strain.

**DISCUSSION**

We have previously reviewed the origin of stress research as a central theme not only for its impact on behaviour but also on CV outcomes. We recalled that ‘Hans Selye came to reflect on human adaptation when he was told that ‘those boys who did not make it in the trenches had unusually large adrenals’ as observed in autopsies. The time was the First Great War and the place was Charles University in Prague where as a young medical student, he overheard this conversation between assistant professors of anatomy and pathology’ (37). Using a model of recombinant congenic mice originating from a cross between two phenotypically distinct strains, we are reporting an extensive set of genomic determinants of stress/emotionality and potentially related immune components that we believe can serve for future exploration of novel patho-genetic, therapeutic and preventive targets in CV diseases with hypertension as its leading risk and global burden (40). We have narrowed down several previously-described larger genomic regions and uncovered novel ones contributing to the mammalian response to environmental disturbances.

Three Chr 1 markers located between 93.3 and 95.8 cM were highly associated with emotional reactivity in the OF for AcB/BcA RCS. Our fine mapping was consistent with a report on intercrosses of chromosome substitution strains derived from A/J and B6 (41). These QTL were near the highest LOD score obtained for the same measure with a F2 intercross from DeFries high- and low-activity strains (42). The same region was associated with haloperidol-induced cata-lypse in BxD/DxB RI strains (43). Further analyses of the F2 intercross from DeFries strains revealed the position of the emotional reactivity locus (*Emo1*) at 95 cM on Chr 1 (44). A similar association on a nearby chromosomal region was obtained with open arm entries and duration as well as enclosed arm entries in the EPM together with latencies before entering the lit side of the light/dark box (45). The same region was suggestively associated with hypoactivity induced by an injection of the anticholinesterase inhibitor paraoxon in BxD/DxB RI strains (46). Likewise, provisional
QTL were detected for circadian activity in the home-cage at 92.6 and 94.2 cM with the BxC/CxB RI strain (47).

Many QTL on Chr 1 seem to define a stress-related cluster involved in CV disease development, since QTL for blood pressure (BP) (48,49), atherosclerosis (50) and oxidative stress susceptibility (51) have been described in the vicinity of the Emo1 region. Table 1 illustrates the overlap with BP QTL in A/J and B6 backcrosses known as salt-induced bpq (48), and with BP QTL in A/J X B6 F2, named abhp (3). Because BP measurements were made under immobilization, one could consider these BP QTL as stress-related markers, since under radiotelemetry, mice did not habituate BP and heart rate responses, even after 10 days of exposure to immobilization (52).

Moreover, our most significant OF QTL on mouse Chr 1 was synthenic to a cluster of metabolic phenotypes of hypertension in our studies of French–Canadian families (53) and hypertensive dyslipidemic rats (54–56). Figure 5 presents homology maps related to the Chr 1 QTL. Functional polymorphisms were described in candidate genes of the CV/inflammatory/immune systems, supporting the hypothesis that an altered stress response plays a role in CV disease development (37). Validation by RT-PCR confirmed a down-regulation of the Atp1a2 gene in the informative strain for anxiety (Fig. 4). Brain differential expression of Atp1a2 may thus explain the observed enhanced emotionality (57), since heterogeneous knock out for this gene displays similar behaviour (58,59), while A/J mice show fear memory impairments with deficits in amygdalar long-term potentiation (60). Interestingly, this gene is also involved in the salt-sensitive component of hypertension, exerting a major action on cardiac output and peripheral resistance (61–63). Finally, heterozygous mutation of that gene in humans (Leu764Pro) will cause familial hemiplegic migraine 2 (64). Crp expression was not affected in our adult group. This observation was not unexpected (65), even in the presence of substantial differences between RCS progenitors revealed in our in silico analysis (Fig. 3). Nevertheless, further investigation is warranted, particularly in aged animals in which systolic hypertension can be caused by Crp-related nitric oxide and renin–angiotensin deregulation (66,67) that could possibly be amplified by chronic stress (68,69).

Rgs2 fell out of our significant locus boundaries, but nevertheless has already been described as a quantitative trait gene (QTG) for the Emo1 locus on Chr 1 (70,71). Interestingly, this gene was also linked to hypertension development (49,72) and was recently reported to be differentially inherited and expressed in AJ and B6 (73).

In addition to Chr 1 (Table 1), we noted significant linkages for emotional reactivity in the OF on Chr 3 (58.8 cM), 4 (2.5 and 59.1 cM) and 7 (64 cM). A suggestive QTL for paraoxon-induced hypoactivity were detected at the 22.7 cM locus on Chr 3 (46). For Chr 4, a provisional QTL for OF activity was identified with BxD/DxB RI strains at approximately the same site (59 cM) as one of our markers (74).
<table>
<thead>
<tr>
<th>Genes</th>
<th>2 kb promoter region, motifs (similarity score %)</th>
<th>3' UTR secondary structure modification</th>
<th>Coding region (amino acid description and functional domain position)</th>
<th>Details (Gene ID in NCBI; Pubmed ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atp1a2 (Na+/K+ ATPase alpha2)</td>
<td>rs32414724-Sp1 (85) in AJ</td>
<td>rs31561579–GATA-1 (91), MZF1 (90) in AJ; rs30544949—stem/loop within AAUAAA motif; structure changes within the coding domain pfam00702 haloacid dehalo-genaselike hydrolase like hydrolase (581–735)</td>
<td>GeneID 98660; PMID 12805306 (amygdala degeneration); PMID 15458945 (ouabain-induced hypertension)</td>
<td></td>
</tr>
<tr>
<td>Nhlh1 (nescient helix loop helix)</td>
<td>rs3191032–CdxA (85) in B6; cap (87) in AJ; rs31994294 (5'-UTR) deltaEF1 (86) in B6 only</td>
<td>AJ rs32512926–Sp1 (91); rs32005043–c-Rel (90), NF-kappaB p65 (90) stem (loop n=7 in B6)—changes interfere in coding region.19 nucleotides loop—GATA-2 (98)</td>
<td>GeneID 18071; PMID 12077327 (arrhythmia, autonomic dysfunction in knock out animals)</td>
<td></td>
</tr>
<tr>
<td>Slamf1 (signalling lymphocyte activation molecule)</td>
<td>rs30704710–AP-4 (89) in B6; MZF1 (86) in AJ</td>
<td>rs31529853–B6 only GATA-2 (91); 4 nucleotides loop with an 8-mer motif</td>
<td>GeneID 27218; PMID 11477403 (Regulation of SLAM-mediated signal transduction by SAP)</td>
<td></td>
</tr>
<tr>
<td>Crp (C-reactive protein)</td>
<td>rs32226283–SRY (86) in B6, Lyf-1 (85) in AJ</td>
<td>A/J 36 nucl. loop Phox-1 (100); GATA-1 (93); GATA-2 (91) rs31557028–TCF11/KCR-F1/Nrf1 homodimers (96), Octamer factor 1 (91) in B6 mutant only. Interference with the coding region secondary structure within the pentraxin domain (20–220)</td>
<td>Ala134Pro; Asn191Ser cd00152: Pentraxin domain (20–220)</td>
<td></td>
</tr>
<tr>
<td>Fegr3 (IgG Fc receptor low affinity III)</td>
<td>rs31781215–v-Myb (89) in A/J rs8242844–GATA-1 (90) in B6</td>
<td>Gly73Trp, Arg74Ser; Ala82Ser Signal peptide coding sequence (64–153)</td>
<td>GeneID 14131; PMID 17053192 (regulate chemokine expression and leukocyte invasion of the vessel wall in atherosclerosis)</td>
<td></td>
</tr>
</tbody>
</table>

Summary of the genes having SNP in the promoter region within functional motifs detected by TRANSFAC, in the 3'-UTR region that change mRNA secondary structure, or in the coding region with amino acid modification in a functional domain of the protein. Secondary structure analysis in the 3'-UTR was done only for genes of interest.
Moreover, QTL on Chr 4 were found in the OF and plus-maze (45) and confirmed in the rat synthetic region on Chr 5 (75–77). From the CV perspective, our QTL on Chr 3 and 7 were near homologous regions of rat Chr 2 for QTL associated with early-onset bradycardia and later-onset tachycardia in response to an airpuff stimulus (78).

In the EPM, QTL were found on Chr 1 (32.8–36.9 cM), 7 (16–18 cM), 9 (17–25 cM), 11 (0–17 cM) and 19 (47 cM). The Chr 9 locus containing \textit{Grik4} (glutamate kainate receptor 4) and Chr 19 QTL correspond to those obtained in the OF after saline injection in AxB/BxA RI and AcB/BcA RCS (2). The Chr 9 region is also near a homologous region of rat Chr 8 associated with early-onset bradycardia and later-onset tachycardia in response to an airpuff stimulus (78).

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The most significant QTL for the EPM were revealed on Chr 11 bearing \textit{Ddc} (7.0 cM) that encodes dopa decarboxylase, the monoaminergic-synthesizing enzyme for dopamine, noradrenaline, and 5-hydroxytryptamine synthesis and a relevant candidate gene for affective and attention deficit disorders (82).

With the standard diet, significant QTL for stress-induced hypothermia were identified on Chr 2 (79.7–81.7 cM) and Chr 15 (9.9–24 cM). Two other QTL were associated with thermogenesis on Chr 3 (22 cM) and Chr 10 (44–52 cM). The same Chr 10 region was also detected in the A/J set for emergence latencies in the plus-maze at a lower significance level (LOD = 1.7). The common region might explain the significant correlation between these two variables ($r = -0.59$, $P < 0.0001$) and suggest a shared biological pathway. The Chr 10 region contains the \textit{Igf1} gene encoding insulin-like growth factor 1 that is likely to be linked to BP regulation (83).

On the salt-diet challenge, QTL for stress-induced hypothermia were elicited on Chr 1 (8.4 cM), 4 (6.5 and 66 cM), 15 (59.2 cM) and 18 (20 cM). The most significant QTL on Chr 4 are a locus for triglyceride level at 6.5 cM (84) near \textit{Bpq3} (48) or \textit{Abhp2} (3), and within brain cannabinoid receptor 1 gene (\textit{Cnr1}) involved in both emotional processing (85), metabolic regulation (86) and, more recently, even in BP regulation (87). Both loci on Chr 4 are close to those detected in the OF; the one downstream contains \textit{Htr1d} receptors that were shown to regulate redox equilibrium and the tumour
necrosis factor-alpha cascade in the nervous system (88). Upstream Chr 4 caspase 8-associated protein 2 (Casp8ap2) gives another possible link to the immune response (89). Our Chr 18 QTL (22 cm) is located at the locus of Nr3c1 encoding for the glucocorticoid receptor, providing a possible link between stress and metabolic syndrome (90). Finally, Chr 15 QTL is identical to the one revealed for the acoustic startle response in the same RCS (25). This region is close to the Emo2 QTL on Chr 15 at 43.3 cm (11); Smoller et al. (91) provided evidence of linkage of human homolog Chr 12q13 with panic disorder/agoraphobia. Moreover, this region is syntenic to the rat Hsf1 gene region (http://gapp.gen.gu.se) associated with stress gene expression which we have previously identified through mapping of several Hsp mRNA as the first transcriptomic-based mapping approach reported (33).

The QTL for urinary volume were detected at a single Chr 11 site (44.8 cm). These QTL were concordant with a homologous region of rat Chr 10 associated with stress susceptibility (30,92), salt retention (34) and hypertension (93). The region also encodes Nos2 (45.6 cm), the inducible form of nitric oxide synthase near Abbp4 (58 cm).

The QTL for creatinine excretion on Chr 15 (51.1–54.5 cm) are near the one for stress-induced hypothermia under a high-salt load. The most significant QTL for Ca++ excretion (LOD = 4.6) on Chr 12 (48–50 cm) bear Calml encoding calmodulin 1, the overexpression of which induces cardiac hypertrophy (94).

In the context of genetic hypertension, we have also suggested that stress may be considered as a special case of gene/environment interaction in which some gene expression manifests susceptibility to the environment (32,33,39). In this perspective, we report phenotype-specific interactions between salt-loading and genetic background that may recall the marked heterogeneity of neuroendocrine responses to various stressors (95). In contrast, the overlapping linkage of Emo, stress susceptibility and hypertension to homologous genomic regions may be explained by pleiotropy or common pathways related to stress susceptibility; some of them might be grouped in gene clusters (53,96).

In silico sequence analysis (97–99) of selected candidate genes in A/J and B6 strains led us to hypothesis-driven functional genomics which can be a filter to transcriptome-based systematic screening studies (100), and confirms the relevance of considering polymorphisms in regulatory elements. Clearly, the loci determined in the current study represent an important stepping stone for further detailed analyses, as the actual relevance of particular polymorphisms within genes and intergenic regions for the resulting phenotypes is yet to be fully
The inbred progeny contained \( \sim 12.5\% \) of the genome of one parental line introduced in the other. The mice were group-housed in cages with wood shavings and in a temperature- and humidity-controlled room with a 12 h light–dark cycle (lights on at 0700 h). After at least 1 week of adaptation to their new surroundings and to handling, the mice were placed in their home-cage, the mice were immobilized for 30 min in a restraining device (IITC Life Science Inc., Woodland Hills, CA, USA). Two to 3 days later, they were placed in metabolic cages and fed an 8% salt-rich diet (Purina Modified Lab. Rodent Testdiet 5001C-2). The procedure was then repeated. The number of deaths associated with the surgical and restraint procedures was near zero in this cohort. There was no apparent relationship between survival and genotype after each procedure.

**Urinary volume and electrolytes with a salt diet**

Twenty-four-hour urine samples were collected from each mouse placed in metabolic cages under the high-salt diet. Urinary volume and electrolyte excretion (\( \text{Na}^+ \), \( \text{K}^+ \), \( \text{Ca}^{++} \) and creatinine) were measured by spectrophotometry (102,103), with values corrected for body weight (34).

**Statistical analyses**

The SDP of each phenotype was determined by standard descriptive statistics and heritability estimates (interstrain/intrastrain variance ratio), as described by Owen et al. (104). Informative strains, differing from their recurrent parental strain, were identified by an unpaired \( t \)-test or with Sattertwaite-Welsh correction for heterogeneous variances meeting Bonferroni correction criteria for multiple comparisons, i.e. with \( P < 0.002 \) for the B6 background (0.05 divided by 22 strains) and \( P < 0.004 \) for the A/J background.
Only phenotypes with heritability estimates over 50% for either background were retained for further analyses. QTL were first determined by computing single marker regressions with MapManager QTX software, using strain means and variances (105). The map distances were defined for all loci with Mouse Genome Informatics (http://www.informatics.jax.org) positions instead of calculated positions, and redundant markers were removed for the best fit to a self-RI model, which implicitly considers each background (albino or B6) as an independent RI set. Permutation tests (5,000 for single marker additive contributions) allowed us to calculate significant thresholds for each phenotype, adjusted for multiple testing (106). Interval mapping was then performed to obtain a likelihood ratio statistic divided by 4.61 to provide a LOD score. After reinserting the removed markers into the map, fine mapping of QTL intervals was appraised by visual tracking of haplotypes in contributing and non-contributing strains (45,107). In addition, cross confirmation of the ‘direction of effect’ between the two RCS sets was assessed (108). Significant QTL were confirmed by parametric t-tests, with genomic markers serving as the independent variable, and strain means as observations (25,109,110), controlling for equality of variances with a F test ratio (Statview 5.0).

In silico exploration of putative candidate genes

Figure 3 describes our whole approach for narrowing down QTL to QTG. For the Chr 1 QTL interval (171–174 Mb), candidate genes having SNPs polymorphic between A/J and B6 were listed with the help of the Celera Discovery System (http://cds.celera.com). QTL interval boundaries were translated in base pair location according to the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). For all other QTL, candidate genes of interest were selected from evidence-based physiological relevance, starting with the Entrez Gene NCBI database. A/J and B6 progenitors were then screened with the public inbred strain SNP database from Genome Build 36.1 (http://www.ncbi.nlm.nih.gov/SNP/MouseSNP.cgi), to find genes having SNPs within coding and untranslated mRNA sequences, or within 2 kb in the promoter region. The SNPs exact location was validated with the variation map option of NCBI Map Viewer and Blast 2 sequence tools. Depending on their location in the coding or regulatory regions (promoter or UTRs), the functional significance of the SNPs was respectively assessed by looking for protein domains (PROSITE patterns) or DNA motifs (TRANSFAC vertebrates motif Library) in the relevant database (http://motif.genome.jp/). Moreover, the impact of the SNPs in the 3’-UTR was further estimated with the Vienna RNA-fold program web interface (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi/). This program allows for the prediction and visualization of occurrence of mRNA secondary structure changes between A/J and B6 haplotypes, covering all SNPs within the gene sequence. In addition, a home-made pattern recognition program was built from 8-mers 3’-UTR target consensus motifs for microRNAs (111), particularly at the location of secondary structure changes. AUUUUA (112,113) and AAUAUA (112) motifs were also added to our pattern library. Stem-loop secondary structure, mRNA and regulatory-binding protein are all working as a structural barrier to translation (112,114); we therefore expect these features to potentially affect gene expression.

Preparation of total RNA and RT–PCR

According to haplotype distribution (top right table in Figure 3), BcA76 and AcB58 strains were chosen as negative controls and BcA70 as the informative strain for Emo, in addition to A/J and B6 progenitors. Mouse brain, heart, liver and kidneys were removed and kept at −80°C until RNA extraction. Target organs were selected for a detectable level of gene expression according to NCBI Entrez Gene profiles. Total RNA was extracted with TRizol reagent (Invitrogen Canada Inc., Burlington, Ontario). Total RNA (1 μg) was reverse-transcribed with random hexamer primer and M-MLV reverse transcriptase (Invitrogen). The Ap1a2 (forward primer 5’-ggctatggtgtgcgccg-3’, reverse primer 5’-gtgctcttcagccatgctc-3’), Slamf1 (forward primer 5’-cctcctcaagagcagctc-3’, reverse primer 5’-ggtgctcacgatgttggc-3’), Cbr (forward primer 5’-gatgtggcttcgctgctc-3’, reverse primer 5’-cattatgaaagaagacaggtcc-3’) genes and a housekeeping gene (18S) were selected and run in parallel for quantitative RT–PCR analysis. The level of expression of Nhh1 was insufficient to perform any comparison. Primers were designed with Primer3 (115) and synthesized by Invitrogen. RT–PCRs were performed with Platinum SYBR Green qPCR SuperMix-UDG kits (Invitrogen) in strips of 0.1 ml optical tubes (Corbett Research Pty Ltd., Sydney, Australia) according to the manufacturer’s protocol, using the Rotor-Gene 3000 system (Corbett Research). The results were analysed by the Pfaffl method (116). The expression of a given gene in the A/J ancestor strain was chosen as the control, to which each strain was then compared (n = 4 per strain).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

Methodological discussions with A.E. Boyle (from K.J. Gill’s laboratory), K. Morgan and R. Joober (McGill University) were very valuable and helpful. The statistical advice obtained from the SEMQ (Service d’Evaluation en Methodes Quantitatives, Université de Montréal, is much appreciated, and the editorial assistance of Ovid Da Silva, Research Support Office, Research Centre, CHUM, is acknowledged. RCS were kindly provided by Emerillon Therapeutics Inc.

Conflict of Interest statement. None declared.

FUNDING

S.T. received a doctoral scholarship from the Canadian Heart and Stroke Foundation. This study was supported by grants from the Canadian Institutes of Health Research to P.H. and to J.T. (CIHR MT-14654, Cardiogene).
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