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Research report

A candidate syntenic genetic locus is associated with voluntary exercise levels in mice and humans

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HIGHLIGHTS

- Identification of mouse QTL (Chr2) for voluntary running wheel activity (RWA).
- RWA is considered a model of voluntary exercise (VE) in humans.
- Human region 20q13.2 is syntenic to mouse QTL on Chr2.
- Genetic variants within 20q13.2 are associated with VE.
- This was demonstrated in two independent human cohorts.

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ABSTRACT

Individual levels of physical activity, and especially of voluntary physical exercise, highly contribute to the susceptibility for developing metabolic, cardiovascular diseases, and potentially to psychiatric disorders. Here, we applied a cross-species approach to explore a candidate genetic region for voluntary exercise levels. First, a panel of mouse chromosome substitution strains was used to map a genomic region on mouse chromosome 2 that contributes to voluntary wheel running levels – a behavioral readout considered a model of voluntary exercise in humans. Subsequently, we tested the syntenic region (HSA20: 51,212,545–55,212,986) in a human sample (Saint Thomas Twin Register; $n = 3038$) and found a significant association between voluntary exercise levels (categorized into excessive and non-excessive exercise) and an intergenic SNP *rs459465* (adjusted *P*-value of 0.001). Taking under consideration the methodological challenges embedded in this translational approach in the research of complex phenotypes, we wanted to further test the validity of this finding. Therefore, we repeated the analysis in an independent human population (ALSPAC data set; $n = 2557$). We found a significant association of excessive exercise with two SNPs in the same genomic region (*rs6022999*, adjusted *P*-value of $P = 0.011$ and *rs6092090*, adjusted *P*-value of 0.012). We explored the locus for possible candidate genes by means of literature search and bioinformatics analysis of gene function and of trans-regulatory elements. We propose three potential human candidate genes for voluntary physical exercise levels (*MC3R*, *CYP24A1*, and *GRM8*). To conclude, the identified genetic variance in the human locus 20q13.2 may affect voluntary exercise levels.

1. Introduction

Low level of voluntary exercise (one of types of physical activity) in modern societies is considered to be one of the most profound risk factors for the development of obesity and cardiovascular diseases, among other illnesses [1–3]. Conversely, appropriate level of exercise is believed to have broad beneficial effect on human health. Despite the accumulating evidence and common knowledge regarding benefits of physical activity, only a minority of the Western population engages in sufficient physical activity to experience its benefits [4,5]. Broadened understanding of the mechanisms that influence levels of physical activity could help in improving the existing health programs. However, participation in physical activity is influenced by an array of factors

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including biological, psychological, cultural and environmental ones. Furthermore, many of these factors influence various complex phenotypes which may be correlated (e.g. obesity and levels of physical activity). This complexity hampers efforts to understand biological processes underlying physical activity. One of the possible ways to unravel the biological pathways involved in physical activity is to investigate the genetic basis of this highly heritable phenotype [5–8] under controlled genetic and environmental conditions [9].

Voluntary exercise is a complex phenotype and various definitions [6,10] and operationalizations may be used in studies to examine it, e.g. direct observation, questionnaires (diaries and retrospective recall), surveys, calorimetry, heart rate monitors and motion sensors [10,11]. These assessment methods differ in terms of feasibility and reliability, therefore, the results obtained by the use of various methods do not necessarily correlate [6,10]. Use of animal models may, to some extent, help to standardize the complex conditions influencing the levels of physical activity and methods of measurement. In rodent studies, voluntary running wheel activity (RWA) in a home cage was proposed to be the most appropriate model for voluntary exercise in humans [5,12]. Indeed, if one defines voluntary exercise as locomotor activity "that is not directly required for survival or homeostasis and not directly motivated by any external factor" [10], than voluntary RWA is indeed the most suitable rodent behavior to model human voluntary exercise (although some researchers would not agree [13]). Studying RWA in various mouse lines which differ in expression of this phenotype allows systematic genetic studies on this complex trait.

Previous studies proved that genetic factors have an influence on the levels of physical activity [14]. Heritability of physical activity in humans was assessed repeatedly [6]. Multiple studies pointed to genes associated with physical activity levels in humans [7,15–17], and various linkage and genome wide association studies (GWAS) in humans were able to point to genetic regions associated with physical activity levels [6]. Furthermore, it is possible to selectively breed lines of mice based on their high or low physical activity [18]. Finally, numerous studies using RWA in mice as an animal model for physical activity, pointed to promising candidate genes and genetic regions [19–23]. Nevertheless, despite the relative (in comparison to other complex phenotypes) ease of operationalization of physical activity in humans and translation of this phenotype to an animal model, there is virtually no overlap between the results obtained from rodent and human genetic studies for voluntary activity [6]. Therefore, there is a need for research aiming at the translation of genetic findings from animal to human studies.

In the current study, we aimed at identifying a narrow candidate genetic region contributing to physical activity levels. For this purpose we used a cross-species approach. First, we used a panel of mouse chromosome substitution strains (CSS, also called consomic strains or lines) [24] that enable identification of candidate genetic regions for complex traits, such as voluntary RWA. Later, we tested this discovered candidate region for mouse RWA in two independent human populations. Based on the integrated mouse-human approach, we propose new candidate genes potentially contributing to the individual levels of physical activity.

2. Methods

2.1. Ethical statement

All animal experiments were approved by Animal Experiments Committee of the Academic Biomedical Centre, Utrecht-The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act'; 1996) and on the Dierproevenbesluit' (The Dutch 'Experiments on Animals Decision'; 1996). Every effort was made to minimize animal suffering.

The relevant institutional review boards or ethics committees approved the research protocol of the individual population based studies used in the current analysis. The study involving participants enrolled to TwinsUK was approved by the St Thomas' Hospital research ethics committee. Ethical approval for the study involving ALSPAC dataset was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. All human participants and also, in case of the minors, their first kin gave written informed consent.

2.2. Animal studies

2.2.1. Mice

Initial breeding pairs for CSS and their progenitors A/J (A) and C57BL/6J (B6) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The advantages of using CSS strains as well as the steps required to generate a CSS panel has been described previously [25]. All mice were bred in the Rudolf Magnus Institute of Neuroscience animal facility and were 11–13 weeks old at the start of the experiment (lights on at 2:00 a.m. off at 2:00 p.m., temperature $22.0 \pm 2.0\,^{\circ}C$, fed ad libitum). In total, 384 female mice were tested in the experimental procedure; B6 strain ($n = 36$), A strain ($n = 23$) and of the 19 tested CS strains ($n = 257$; median of 12 mice per CS strain), CSS2F2 (F_2-intercross between C57BL/6J-Chr 2^A/NaJ (CSS2) and B6) ($n = 68$). Mice from CSS16 were not tested due to low availability. The low number of mice in the F_2 population was adequate for proper analysis due to the used methodology. The consomic F_2 cross approach is a specifically designed to identify single QTLs on a single chromosome as there is a strong reduction in the amount of epistatic interactions with loci from the other 20 chromosomes. Because of this sensitivity, significant less F_2 mice are necessary to identify these QTLs when compared to the traditional whole genome intercross mapping approach (this has been indicated elegantly by the laboratory that generated the consomic mice [24]. In addition QTL analysis was performed by MQM-mapping (multiple-QTL-model or marker-QTL-marker) which is more powerful than the traditional interval mapping approach [26]. The levels for physical activity, such as voluntary RWA, are known to be very different in males and females. As we are interested in the genetics of physical activity levels in relation to eating disorders with a gender pre-dominance in females (such as in anorexia nervosa) [27], we decided to perform this genetic screen in females.

2.2.2. Running wheel activity measurement in mice

Mice were maintained in running wheel cages (wheel circumference: 43.96 cm; surface made of metal rods) for a week. We did not observe coasting of mice in the running wheels. Individual wheel running revolutions were automatically registered. The average of the RWA for the days 6 and 7 was used in the QTL analysis. This was done because mice require time to adapt to the running wheel cages and to develop stable RWA pattern. Patterns of the RWA for CSS2, A, B6 mice as well as for mice from the F_2 populations are present in Figure A1.

2.2.3. DNA samples, genetic marker analysis and map construction

Genomic DNA was isolated from spleen and/or tail from F_1-hybrids, F_2-intercross mice and CSS2 and B6 mice, using a phenol/chlorophorm/iso-amylalcohol protocol [28]. A total of 14 microsatellite markers (D2Mit117, D2Mit417, D2Mit370, D2Mit458, D2Mit156, D2Mit380, D2Mit94, D2Mit66, D2Mit206, D2Mit525, D2Mit493, D2Mit51, D2Mit113, D2Mit148), dispersed throughout mouse chromosome 2, was used to map a region on chromosome 2 associated with RWA in the F_2 population.

In addition nine single nucleotide polymorphisms (SNPs) were genotyped (Taqman Assay by Design, Applied Biosciences, Foster City, CA, USA): *rs27524348, rs27498297, rs27434812, rs28277299, rs13476894, rs27292002, rs27289000, rs27619825, rs27289254*. SNP analysis was performed as described in [29]. Segregation ratio of the genotypes of individual markers was checked by means of the Chi-squared goodness-of-fit-test. None of the markers showed ($P < 0.05$) segregation distortion.

2.2.4. QTL analysis

The quantitative trait loci (QTL) analysis was conducted on the females from CSS2 × B6 F_2 population. The purpose of this analysis was to fine-map genetic regions regulating levels of voluntary wheel running. This method was chosen because the consomic-F_2-cross approach is specifically designed to identify single QTLs on a single chromosome as there is a strong reduction in the amount of epistatic interactions with loci from the other 20 chromosomes. Because of this sensitivity, significant less F_2 mice are necessary to identify these QTLs when compared to the traditional whole genome intercross mapping approach [24].

The location of the QTLs affecting the measured quantitative trait and the variance explained by each locus were determined using the MapQTL® software package (version 4.0) [30]. RWA was normally distributed in the F_2 population (Figure A2). QTL analysis was performed by MQM-mapping (multiple-QTL-model or marker-QTL-marker) [31]. Results were expressed as LOD scores. Permutation tests (10,000 permutations) were done to assess the statistical significance of a QTL (5% significance level: LOD score ≥ 2.62).

2.3. Genetic association study on human participants

The coordinates of a QTL found in the mouse linkage study have been converted to a syntenic region in humans via the UCSC LiftOver tool (http://genome.ucsc.edu/cgi-bin/hgLiftOver). Mouse region chromosome 2: 169,276,294–172,557,293 bp, according to 37.2 mouse genome build, was syntenic to a region on human chromosome 20 (HSA20; 51,212,545–55,212,986 bp), according to hg18 genome build.

2.3.1. TwinsUK-sample description and physical activity measurements

The participants were 3038 females enrolled to the St. Thomas' UK Adult Twin Registry (TwinsUK) between 1994 and 2007. These participants were a portion of the total TwinsUK sample who provided data for physical activity. The composition of the TwinsUK was described in detail elsewhere [32,33]. The analysis was conducted in the age group between 30 and 55 years of age (mean age = 44.16, SD = 7.49) at the time of examination, which was considered the most representative age group with respect to the self-reported, voluntary physical activity. Participants were genotyped on Illumina 370k or Illumina 610k platform.

Physical activity of participants from TwinsUK study was assessed on the basis of self-reported questionnaires (so-called Q10 and Q17D). The questionnaire Q10 included the following questions: (1) *Currently, how many minutes per week do you spend in weight bearing activity? e.g. aerobics, running, dance, football, basketball, racquet sports, etc. (do not include walking or gardening)*; (2) Questionnaire Q10: *Currently, how many minutes per week do you spend in non-weight bearing activity? e.g. swimming, cycling, yoga, aqua aerobics, etc.* The questionnaire Q17D included the following questions: (1) *During the last week, how many hours did you spend on each of the following physical activities?/Physical exercise such as swimming, jogging, aerobics, football, tennis, gym workout, power walking etc.*; (2) *During the last week, how many hours did you spend on each of the following physical activities?/Cycling, including to work and during leisure time*; (3) *During the last week, how many hours did you spend on each of the following physical activities?/Gentler exercise, Yoga and Pilates.* Data of questionnaire Q10 were used preferentially as they were available for 3233 participants (in comparison to only 2683 in case of Q17D questionnaire). If data from questionnaire Q10 for a given participant were not available, the score from questionnaire Q17D was used. Finally, the amount of sport was categorized as excessive exercise (EE) if a participant spent 5 or more hours per week to exercise as proposed by Davis [34].

2.3.2. TwinsUK data – quality control

As mentioned, the TwinsUK individuals were genotyped on Illumina 370k or Illumina 610k platform. The data were quality controlled per each platform separately (Table 1). Illumina 610 and Illumina 370 datasets were merged by a standard merging procedure implemented in Plink v1.07 (–merge flag) [35]. After merging of both datasets, there were 496 males, 5150 females and 7422 SNPs. No individuals were overlapping between the datasets. The data were quality controlled (QC) per each genotyping platform separately (Table 1). The genotyping rate after QC was 99.9% in both Illumina 610 and Illumina 370 datasets. Further analyses were performed on the merged dataset, of 3038 females who had non-missing EE phenotype data. The QQ-plots (observed *P*-values plotted against the *P*-values expected under the null hypothesis) in the analyses did not suggest inflation of the *P*-values (Figure A3A, Lambda < 1).

2.3.3. ALSPAC – sample description and physical activity measurements

ALSPAC (Avon Longitudinal Study of Parents and Children) recruited 14,541 pregnant women resident in Avon, UK with expected dates of delivery 1st April 1991 to 31st December 1992. Of these *initial* pregnancies, there were a total of 14,676 fetuses, resulting in 14,062 live births and 13,988 children who were alive at 1 year of age. The enrolment was extended to include additional children eligible using the original enrolment definition up to the age of 18 years. This increased the number of pregnancies included to 15,247. Of this total sample of 15,458 fetuses, 14,775 were live births and 14,701 were alive at 1 year of age. The cohort profile describes the index children of these pregnancies. Follow-up includes 59 questionnaires (4 weeks to 18 years of age) and 9 clinical assessment visits (7–17 years of age) [36]. Please note, that the study website contains details of all the data that is available through a fully searchable data dictionary (http://www.bristol.ac.uk/alspac/researchers/data-access/data-dictionary/). We obtained data for 7220 girls from the total cohort. Out of those 6759 were not related, 2557 had data on physical activity measured by the questionnaire and 1312 had activity data measured by accelerometer. The assessment of activity levels took place at the age of 15. For 969 girls we had the data for both the questionnaire and the accelerometer measurements of physical activity. All participants were genotyped on Illumina Human Hap 550-quad.

Physical activity was assessed using: (1) self reported questionnaire and (2) accelerometers. For the research involving self-reported questionnaire, participants answered a following question: *Average number of times respondent participated in vigorous activity in the past month: (1) none, (2) less than once per week, (3) 1 to 3 times per week, (4) 4 to 6 times per week, (5) every day*. The threshold for EE was set for answer 4 or higher. For the research involving objective measurement of activity using accelerometer, participants wore an accelerometer for seven consecutive days. Data analysis was undertaken, as described previously [37], to determine the periods of time at varying intensity levels of physical activity. The ranges (in counts per minute) for the activity intensities were <200 for sedentary activity (SA), 200 to <1800 for light activity (LA), and ≥1800 for moderate-to-vigorous physical

Table 1
Quality control of the TwinsUK data.

STEP\Platform	Illumina 370k	Illumina 610k
Total number of individuals	2040	3614
Removed individuals with more than 5% missing genotypes	0 [a]	0 [a]
Removed for increased heterozygosity (± 3SD)	5	3
Remaining individuals	2035	3611
Total nr of SNPs	4158	7760
SNPs removed for more than 5% missed calls	170	157
SNPs removed for violation of Hardy–Weinberg equilibrium at $P < 0.001$	9	32
Removed SNPs which had minor allele frequency of less than 1%	2	275
Remaining SNPs	3977	7296

Quality control steps per each genotyping platform in the TwinsUK data.
[a] 99.9% total genotyping rate.

activity (MVPA), as validated by Puyau et al. [38] for the Actiwatch device worn on the lower right leg. Patients were subdivided into groups characterized by high levels of physical activity and low levels of physical activity (HLPA and LLPA groups, respectively) on the basis of their participation in moderate or vigorous physical activity measured by the accelerometer. Accelerometer calibration studies showed that MVPA readouts correspond to high levels of physical activity such as participation in sports [38]. Thus, participants were categorized as participating in EE if the total time spent on MVPA was equal or higher than 5 h during 7 days of measurement, which corresponds to the definition used in previous publications [39].

2.3.4. ALSPAC – quality control

We excluded the subjects with increased number of heterozygous genotypes (more or less than 3SD from the mean). Exclusion of outliers with increased or decreased number of heterozygous genotypes is a part of quality control procedures done in a number of genome-wide and smaller studies [40]. Because a largely increased number of heterozygotes may suggest a contamination of a sample (e.g. mixing of two samples) or inbreeding.

Furthermore, we excluded the subjects with more than 3% missing genotypes. This resulted in 3846 females remaining for the further analysis. The genetic data were available for 8393 SNPs located in two chromosomal regions, including the HSA20 syntenic region of interest. We removed 12 SNPs due to more than 3% unsuccessful calls and 16 SNPs based on violation of the Hardy–Weinberg equilibrium test at $P < 0.001$. No SNPs had minor allele frequency of less than 1%.

After quality control, there were 3162 SNPs in the HSA20 region of interest. The total genotyping rate in the remaining individuals was 99.9%. The QQ-plots (observed *P*-values plotted against the *P*-values expected under the null hypothesis) in the analyses did not suggest inflation of the *P*-values (Figure A3, panels B and C, lambda < 1 in both cases). Of the 3846 females who passed the QC, there were 1396 with non-missing physical activity questionnaire data and 987 with non-missing accelerometer data. Those individuals were included in the logistic regression analyses of the association between the genotypes and two phenotypes.

2.4. Statistical analysis

2.4.1. Mouse running wheel activity

Differences in RWA in the panel of CS-strains were assessed by a Kruskal–Wallis test (due to the heteroscedasticity of the data), with post hoc (unpaired Student's *t*-test) comparing all CSS with the B6 control strain (corrected $\alpha = 003$) [41].

2.4.2. QTL analysis

The Kolmogorov–Smirnov one-sample test was used to check normality of the data. All data within genotype groups were found to be normally distributed.

2.4.3. Human genetic data

The *P*-values were corrected for multiple testing by means of Bonferroni correction (reported as adjusted *P*-values). These adjusted *P*-values are reported through the manuscript. The significance threshold for the adjusted *P*-values was set at $\alpha = 05$. Data were handled and analyzed with SPSS v20 and Plink v1.07 [35]. The analyses of the human genetic data assumed an additive genetic model.

2.4.4. TwinsUK data

Genetic markers were tested for association with a dichotomous phenotype representing excessive vs. non-excessive exercise by means of the DFAM procedure implemented in Plink v1.07 [35]. (Bonferroni correction This procedure uses a clustered analysis (with the Cochran–Mantel–Haenszel test), which allows for inclusion of non-independent individuals (such as siblings). DFAM procedure in Plink does not allow the use of covariates (such as age). Therefore, we replicated the analysis using the GWAF procedure implemented in R-package (function gee.batch), and using age as a covariate. The results corroborated the findings from Plink, therefore we report only the results obtained using Plink method.

2.4.5. ALSPAC data

The phenotype (excessive vs. non-excessive exercise) was used as an outcome variable in the logistic regression models, with genotypes as the predictor variables and age as well as BMI as covariates.

3. Results

3.1. Running wheel activity in CSS panel

In order to determine which mouse chromosome contains genetic loci regulating a RWA, we tested baseline RWA of a panel of CSS (except CSS16 due to low availability) and both progenitor strains which are known to be low active (A/J, abbreviated to A) and high active (C57BL/6J, abbreviated to B6) [27] (Fig. 1A). The Kruskal–Wallis test showed a significant difference between the strains ($\chi^2(20) = 106.055$, $P < 0.001$). Post hoc analysis showed that progenitor lines were significantly different in respect to the RWA, with A mice being less active than B6 strain ($P < 0.001$). Three consomic lines run significantly less than the host strain (B6) (CSS2 ($P < 0.001$), CSS4 ($P < 0.001$) and CSS19 ($P < 0.001$)). These results suggested that QTLs for voluntary RWA could be found on one of the three chromosomes (Chr2, Chr4, Chr19). In the initial effort to find a chromosomal region associated with RWA we chose to further proceed with line CSS2 due to two reasons. First, CSS4 strain has severely impaired maternal care and therefore, any phenotype observed in adult animals may be contributed to the improper care and nutritional status during this important developmental period [42]. Second, females from CSS2 line do not differ from females of B6 host line for the latency to fall from accelerating

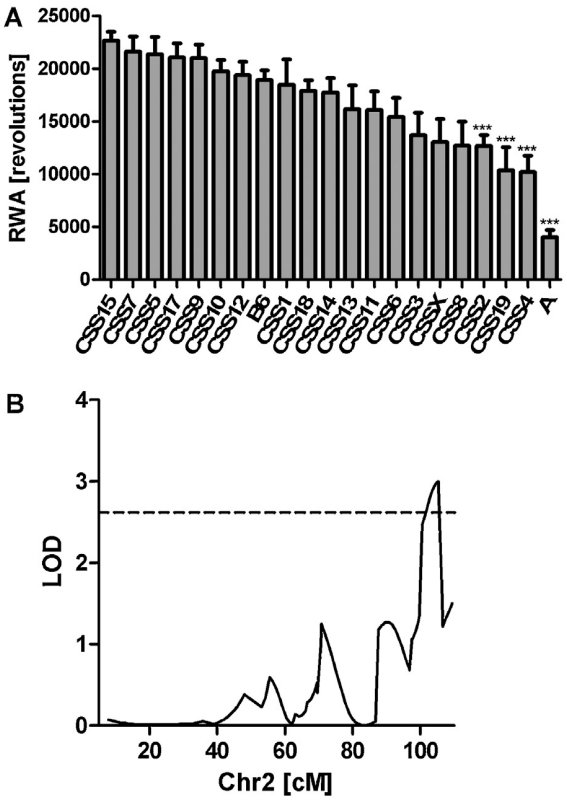

Fig. 1. Mouse chromosome 2 contains QTL for voluntary running wheel activity. (A) Running wheel activity (RWA) as average count of wheel revolutions on days 6 and 7 for the CSS mouse panel and two progenitor lines (A and B6). Data are expressed as means with SEM. Kruskal–Wallis test with post hoc test: ***$P < 0.001$. (B) Chromosomal mapping of locus associated with RWA for F_2 population between CSS2 and B6 strains. Dotted line indicates the significance cutoff for the LOD score (= 2.62).

rotarod (data presented on the Mouse Phenome Database: http://phenome.jax.org/db/q?rtn=projects/details&sym=Lake3; experiments conducted by Jeffrey Lake, Leah Rae Donahue and Muriel T Davisson from The Jackson Laboratory, Bar Harbor, ME USA and published as data set Lake3). This indicates that the differences in the RWA observed between B6 and CSS2 mice are not caused by sensory-motor coordination of CSS2 mice. Therefore, we considered CSS strain to be the most appropriate strain to search for candidate genes contributing to the voluntary exercise. Finally, mice from CSS19 line perform worse on the rotarod test than mice from CSS2 line, which may influence their ability to run in the running wheel (due to impaired sensory-motor coordination).

3.2. QTL mapping

To fine-map genetic regions regulating levels of voluntary wheel running, we conducted a quantitative trait loci (QTL) analysis using RWA and polymorphic DNA markers of females ($n = 68$) from a CSS2 × B6 F_2 population. The distributions of the measured RWA in the progenitor lines (A and B6), CSS2 parental lines as well as the F_2 population are shown in (Figure A2). A genetic recombination occurred in 67 out of 68 animals of the F_2 population. QTL analysis showed a locus on mouse chromosome 2 (Chr2) (Fig. 1B) associated with voluntary wheel running (MQM-MapQTL procedure). The significant peak of the QTL region (169,276,294 to 172,557,293 bp; estimated minus 1 LOD support confidence interval using linear interpolation calculations [43]) was positioned at SNP *rs27289254* (LOD score = 3.00) which is relatively close to *Mc3r* (MGI:96929), a gene known to influence RWA levels in mice [44]. The *rs27289254* is placed in the intergenic region between *Dok5* (MGI:1924079) and *Cbnl4* (MGI:2154433) genes. The QTL explained 16.6% of the variance in RWA in the F_2 population. The total QTL region contains 47 genes (according to Ensembl Database; Table A1 shows all the transcripts) out of which only 16 are validated protein-coding genes: *Tshz2* (MGI:2153084), *Zfp217* (MGI:2685408), *Bcas1* (MGI:1924210), *Cyp24a1* (MGI:88593), *Pfdn4* (MGI:1923512), *Dok5*, *Cbln4*, *Mc3r*, *Fam210b* (MGI:1914267), *Aurka* (MGI:894678), *Cstf1* (MGI:1914587), *Cass4* (MGI:2444482), *Rtdc1* (MGI:1913654), *Gcnt7* (MGI:3606143), *Fam209* (MGI:1923676) and *Tfap2c* (MGI:106032). Using Mouse SNP Query (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF) we assessed that, out of these 16 genes, only six (*Bcas1*, *Pfdn4*, *Fam210b*, *Cass4*, *Rtdc1*, *Fam209*) have a coding non-synonymous SNP between B6 and A mice.

3.3. Testing of genetic associations in syntenic human regions

In order to test the hypothesis that the QTL for physical activity found on mouse chromosome 2, contains a candidate gene or a regulatory element regulating levels of physical activity in humans, we translated the mouse QTL region and tested the associations in two different human populations (initial analysis: TwinsUK [32]; replication: ALSPAC [45]). The region on mouse Chr2 between 169,276,294 and 172,557,293 bp is syntenic to the human region on chromosome 20 (HSA20: 51,212,545–55,212,986).

3.3.1. Basic characteristics of the TwinsUK sample

Basic characteristics of the TwinsUK sample, divided into EE and nonEE groups, are shown in Table A2. There were no significant differences between the groups in terms of age, BMI and ethnicity.

3.3.2. Mapping of the QTL region in TwinsUK sample

In the adult population of females from TwinsUK we assessed if there is an association between participation in EE and the candidate genetic region. Gene association analysis of Chr20 region syntenic to the candidate region on mouse Chr2 revealed a significant association of EE with an SNP *rs459465* (unadjusted *P*-value = 5.23E−07; adjusted *P*-value of 0.001). *Rs459465* is located in an intergenic region, and a gene in the closest proximity to it is *DOK5*. The gene and the SNP are not in linkage disequilibrium. Fig. 2 panel A shows the SNP in the genetic context.

3.3.3. Basic characteristics of the ALSPAC sample

Basic characteristics of the ALSPAC sample divided in EE and nonEE groups are shown in Table A3. The division was made twice: (1) based on the questionnaire outcome; and, (2) based on accelerometer readouts. There were no significant differences between the groups in terms of age, BMI and ethnicity.

3.3.4. Mapping of the QTL region in ALSPAC sample

In order to replicate the finding from TwinsUK population, we repeated the analysis in adolescent population from ALSPAC longitudinal study. We conducted two association analyses between genotype and the EE measured with the use of: (1) the questionnaire, (2) accelerometer. For the analysis of EE defined on the basis of the questionnaire, gene association analysis of HSA20 region revealed a significant association of EE with two SNPs: (1) *rs6022999* (unadjusted *P*-value = 3.63E−03; adjusted *P*-value of *P* = 0.011) and (2) *rs6092090* (unadjusted *P*-value = 3.84E−03; adjusted *P*-value of 0.012). *Rs6022999* is placed in the intron region of *CYP24A1* gene whereas *rs6092090* is between the *DOK5* gene and the SNP found in the analysis of the TwinsUK sample. None

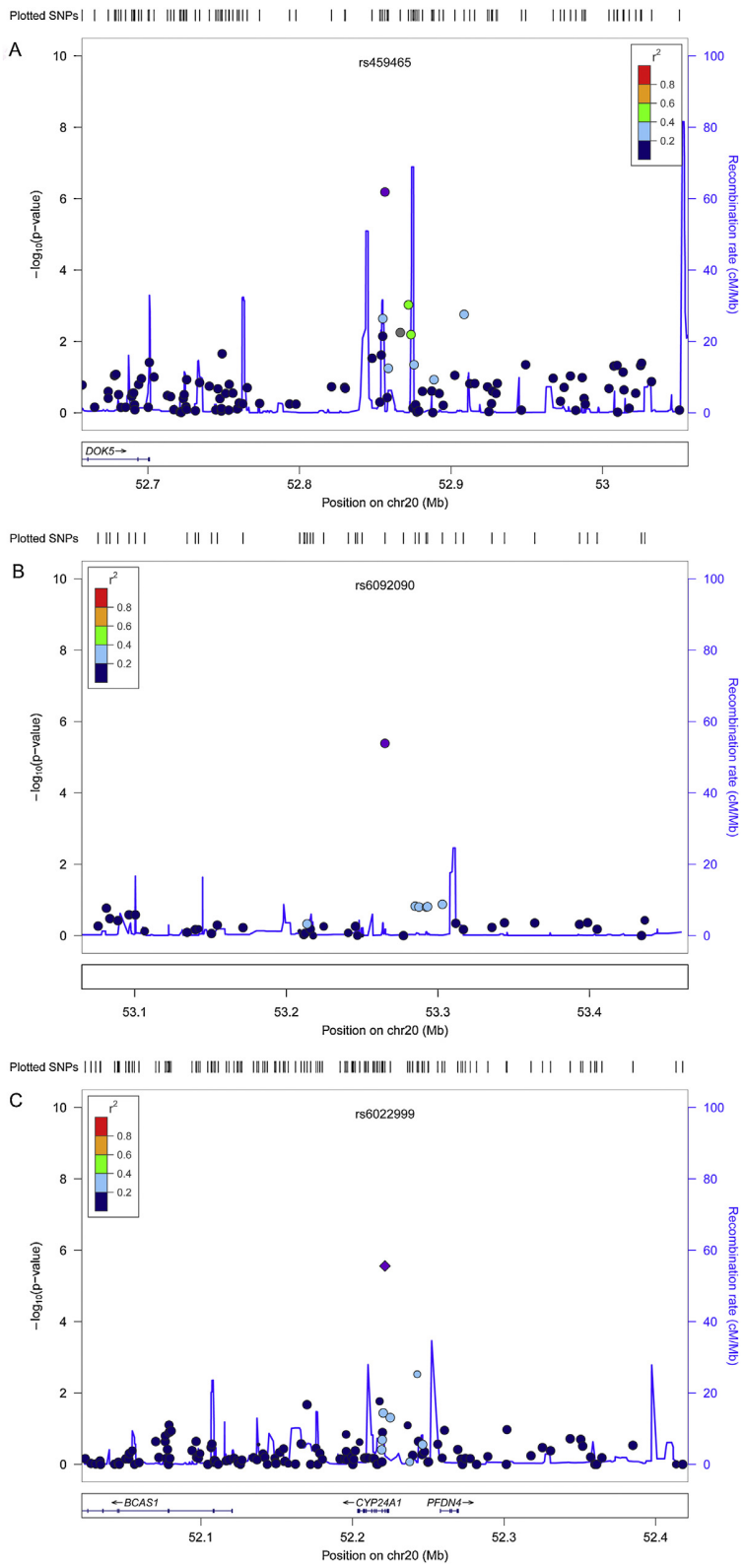

Fig. 2. Genetic context of SNPs associated with high physical activity in humans. The SNPs which were significantly associated with physical activity levels (marked in purple) measured in the human populations included in this paper are shown in the context of their neighboring SNPs and genes. Circles represent annotated SNPs. Colors depict the squared correlation (r^2) of each SNP with the most associated SNP (marked in purple). Gray circles represent SNP for which r^2 information was missing. (A) Association of *rs459465* in TwinsUK sample (adjusted *P*-value = 0.001). (B) association of *rs6022999* with EE assessed using questionnaire in ALSPAC sample (adjusted *P*-value = 0.011). (C) association of *rs6092090* with EE assessed using accelerometer in ALSPAC sample (adjusted *P*-value = 0.012). Plots were produced using the LocusZoom program [63]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Overlap of EE classification assessed by two methods of physical activity measurement.

	Accelerometer – nonEE	Accelerometer – EE
Questionnaire – nonEE	766	57
Questionnaire – EE	130	16

The overlap between the classification into EE group between the subjective (questionnaire based) and objective (based on accelerometer data) is presented. Chi-square test confirmed that there is no significant overlap between the two assessment methods ($\chi^2(1) = 2.90$, $P > 0.05$).

of the SNPs in LD with the two reported SNPs appeared to be associated or trend-associated with the phenotype (Fig. 2 panel B and C). None of the SNPs in LD with the two reported SNPs appeared to be associated or trend-associated with the phenotype.

For the analysis of EE defined on the basis of the accelerometer measurement, there was no significant genetic association.

3.4. Accordance of physical activity measurement between questionnaire and accelerometer

We assessed is the extent of the overlap for EE classification based on the self-reported questionnaire and objectively measured with accelerometer. In the total sample 203 participants were classified into EE group according to one of the methods of EE assessment. However, only 16 participants were classified into EE group according to both physical activity assessment methods (Table 2). Chi-square test confirmed that there is no significant overlap between the two assessment methods ($\chi^2(1) = 2.90$, $P > 0.05$).

3.5. Candidate gene analysis

In order to point possible candidate genes which could be associated with the levels of PA, we explored the candidate locus indicated in the current study to be associated with PA in mice and humans.

3.5.1. Gene ontology analysis

We conducted a gene ontology (GO) analysis using a list of genes present in the genetic region pointed out by QTL analysis. Using DAVID Gene Ontology database [46,47], we assessed if any of the genes found in the QTL region on chromosome 2 was previously associated with locomotor activity or energy expenditure. None of the transcripts was previously associated with these phenotypes according to GO classification. However, it has previously been shown that homozygous *Mc3r* KO mice are characterized by obesity and reduced locomotor activity on the running wheel [44] suggesting that *Mc3r* could potentially be an interesting candidate gene in the assessed region.

3.5.2. Regulatory element analysis

In order to broaden the search of potential candidate genes from the region, we assessed if there are any known trans-regulatory elements in the region tested. To estimate if there are any eQTLs located within the region on chromosome 20 we used the GTEx (Genotype-Tissue Expression) eQTL Browser (http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi#) however, no eQTL association was found for any of the SNPs within the region.

We went back to mouse data and assessed what genes have trans regulatory elements within the region on chromosome 2 using the resource of GeneNetwork – WebQTL database (http://www.genenetwork.org/webqtl/main.py). We used Hippocampus Consortium M430v2 (Jun06) PDNN Database, which contains data for mRNA expression levels assessed in the adult hippocampus of 99 genetically diverse strains of mice including 67 BXD recombinant inbred strains, 13 CXB recombinant inbred strains, a diverse set of common inbred strains (including A and B6 lines), and two reciprocal F_1 hybrids. WebQTL searched the Hippocampus Consortium M430v2 (Jun06) PDNN Database for all records with a trans-QTL having an LRS between 9.2 and 1000 using a 20 Mb exclusion buffer and with LRS between 9.2 and 1000 on mouse chromosome 2 within the range of 169–173 Mbp. The search pointed to 15 genes having regulatory elements within the tested region of chromosome 2. One of those genes, *Grm8* (glutamate receptor, metabotropic 8) is described further in Section 4.

4. Discussion

By using a panel of mouse CS-strains we identified a significant QTL for RWA located on chromosome 2. This genetic region was considered a candidate region for voluntary exercise in humans. To test this hypothesis, we conducted a gene association analysis of a human genetic region, syntenic to this mouse candidate gene region, in two independent human populations. Firstly, using a population of adult women (average age 44.16 years) we could confirm that there is a genetic association of the region with high levels of voluntary physical activity in humans. Secondly, taking under consideration the methodological challenges embedded in this translational approach, we validated this finding in an independent human sample of adolescent girls (assessed at the age of 17).

We further explored the candidate region for potential protein-coding candidate genes which could be associated with the levels of PA in mice or human. We based our exploratory analysis on known functional data and on regulatory elements analysis. At this stage, it is not possible to determine whether any of the proposed candidate genes is the causal factor for the observed phenotypes in mice and humans. The proposed genes should be treated as a hypothesis and tested using functional assays. Furthermore, it is plausible that other genetic elements, such as miRNA, may be in fact responsible for the observed decrease in RWA. Nevertheless, here we would like to propose three genes which could be the primary candidates for the future research efforts. First, KO mice of melanocortin 3 receptor (*Mc3r*, KO mice MGI:5302394) gene express reduced voluntary RWA levels [44]. Second, the protein product of the *CYP24A1* (cytochrome P450, family 24, subfamily A, polypeptide 1; HGNC:2602) gene is responsible for degradation of vitamin D into a physiologically inactive form. Vitamin D was shown to be essential for proper muscle functioning [48,49] and polymorphisms in the vitamin D receptor are associated in humans with changed muscle strength in both genders [50]; these changes are likely to influence the levels of physical activity. However, neither *Mc3r* nor *Cyp24a1* contain a known non-synonymous coding SNP between the progenitor strains, A and B6. Last but not least, out of the fifteen genes that have trans-regulatory elements within the mouse QTL region *GRM8* (glutamate receptor, metabotropic 8; HGNC:4600) could be considered a potential candidate. *Grm8* KO mice are characterized by decreased locomotor activity in the home-cage during the first 3 days of testing; however, this phenotype is accompanied by increased body weight, slight insulin intolerance as well as reduced speed of swimming in the Morris water maze [51]. Furthermore, the home-cage activity and the RWA should be treated as distinct phenotypes [10] and therefore, the *Grm8* deficient mice should be tested for their RWA.

The candidate genes mentioned above may affect various behavioral and/or physiological domains due to their potential pleiotropic effects. For example, in addition to reduction in physical activity levels, Mc3r gene KO mice are also know to show reduced

feeding efficiency. This observation of pleiotropic effects of Mc3r gene (effects of Mc3r on physical activity and feeding efficiency) can be explained by independent effects of the gene on these separate domains. Alternatively, one could easily conceive that the effects of Mc3r are interrelated; namely, that the observed reduced physical activity levels are a consequence of decreased feeding efficiency. Finally, it is possible that Mc3r KO influences a yet to be discovered behavioral or physiological domain ('lower-order phenotype') which in turn influences both, the levels of physical activity and feeding efficiency. This type of pleiotropic effects of genes and complex hierarchical connections between behavioral domains are characteristic for studies regarding neuropsychiatric clinical disorders. In the context of the current study, eating disorders might be used as an adequate example. A majority of clinicians (85%) regard high levels of physical activity to be an essential factor in the development and maintenance of the eating disorders [52]. Furthermore, there is a positive correlation between the levels of physical activity and signs of pathology such as anxiety levels and levels of food restriction [53]. The behavioral and molecular pathways which connect these higher-order phenotypes (anxiety, physical activity and food restriction) require further examination. Finally, the exact significance for eating disorders of proposed candidate genes remains to be elucidated in future studies.

The comparative interspecies behavioral genetics approach is complicated by question regarding the translational value of animal models of human behavior. Although the translational value of voluntary RWA as a model of VE is well documented in literature [10,12,54,55], there are also arguments against this hypothesis [10,13,56]. Sherwin [13], for example, suggested that RWA may be solely a behavioral artifact of poor laboratory housing conditions. This raises a question whether RWA can be used to model human VE [10]. If the translational value of the RWA as a model of VE were undermined, it would make the approach of interspecies behavioral genetics pointless. These arguments should be kept in mind when interpreting current results.

The translational approach is further complicated by difficulty in creating a precise definition and operationalization of human phenotypes. However, numerous validated methodologies exist for assessing voluntary exercise in humans. Accelerometer data as well as questionnaire data are established and most commonly used methods of measuring voluntary exercise in humans [10,11]. Although they both have limitations, they may be chosen for answering specific research questions after informed analysis of feasibility and reliability of these measurement methods [10,57]. In the current analysis, there was a clear discrepancy between categorization to EE group based on the objective (accelerometer) and subjective (questionnaire) assessment method. This stays in agreement with previous findings which showed that the heritability estimates of physical activity are higher when physical activity is assessed using accelerometers [6,58–61]. Furthermore, various physical activity assessment methods lead to discrepant results regarding genetic associations [6,62]. These observations suggest that various physical activity assessment methods may be, in fact, measuring different constructs [6]. This should be considered a limitation of the current study.

There are several other limitations of the current study which should be taken under consideration. First, the logistic regression model for the genetic association in ALSPAC sample was corrected for BMI; however, we did not correct the analysis for other potentially relevant variables such as socio-economic status, urban/rural living, and education. Second, in case of the genetic association analysis in the ALSPAC sample, none of the SNPs in linkage disequilibrium with the two reported SNPs appeared to be trend-associated with the EE. This suggests that the results obtained in this analysis could be obtained by chance.

5. Conclusions

Taken together, this study identified a QTL for voluntary RWA in mice. As this phenotype is considered to be a model of voluntary exercise levels in humans, we tested association of voluntary exercise in humans with a genetic human region syntenic with this candidate mouse locus. The association of genetic variants within 20q13.2 region with voluntary exercise was demonstrated in two independent human cohorts. The analysis (based on bioinformatics and a search for trans-regulatory elements) suggested some possible candidate genes which could contribute to the voluntary exercise levels. However, further studies are needed to test their functional significance for this complex phenotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.05.006.

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