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A neurobiological pathway to smoking in adolescence: *TTC12-ANKK1-DRD2* variants and reward response

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Abstract
The *TTC12-ANKK1-DRD2* gene-cluster has been implicated in adult smoking. Here, we investigated the contribution of individual genes in the *TTC12-ANKK1-DRD2* cluster in smoking and their association with smoking-associated reward processing in adolescence. A meta-analysis of *TTC12-ANKK1-DRD2* variants and self-reported smoking behaviours was performed in four European adolescent cohorts (N = 14,084). The minor G-allele of rs2236709, mapping *TTC12*, was associated with self-reported smoking ($p = 5.0 \times 10^{-4}$) and higher plasma cotinine levels ($p = 7.0 \times 10^{-5}$). This risk allele was linked to an increased ventral-striatal blood-oxygen level-dependent (BOLD) response during reward anticipation (n = 1,263) and with higher *DRD2* gene expression in the striatum ($p = 0.013$), but not with *TTC12* or *ANKK* gene expression. These data suggest a role for the *TTC12-ANKK1-DRD2* gene-cluster in adolescent smoking behaviours, provide evidence for the involvement of *DRD2* in the early stages of addiction and support the notion that genetically-driven inter-individual differences in dopaminergic transmission mediate reward sensitivity and risk to smoking.
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Introduction

Smoking is one of the leading causes of premature death (World Health Organization, 2015). The majority of adult smokers (80–90%) initiate smoking during adolescence (Wittchen et al., 2008) and genetic factors were found to explain 44% of the individual differences (Vink et al., 2005). Adolescence is thus associated with an increased risk of developing long-lasting dependencies to nicotine and other substances (Van De Ven et al., 2010), rendering this developmental period critical for the investigation of genetic risk factors and the resulting neurobehavioral mechanisms implicated in tobacco smoking. However, our knowledge on how genes influence brain mechanisms in smoking is limited, and existing findings are often based on sample sizes not large enough to yield conclusive results (Munafo and Flint, 2011). Moreover, the investigation of smoking in adolescents is often hampered by reporting biases resulting from self-report measures (Kandel et al., 2006), thus requiring complementation by biologically-verified markers of nicotine use such as cotinine (Keskitalo et al., 2009).

Smoking in adolescence, in particular smoking initiation, is influenced by behaviours such as risk taking and impulsiveness, which reflect dissociation between the development of the subcortical reward system and a comparatively delayed maturation of cortical inhibitory functions characteristic for this developmental period (Lydon et al., 2014). In adult smokers this maturation is complete, together with the direct consequence of chronic tobacco exposure, resulting in a neurobehavioral context distinct from that in adolescents. This suggests that genetic factors and the biological processes mediating smoking behaviour may be different in adolescents and adults. For example in adults, it has previously shown that a gene-cluster containing the dopamine receptor 2 (*DRD2*), the ankyrin repeat and kinase domain containing 1 (*ANKK1*) and the tetratricopeptide repeat domain 12 (*TTC12*) genes, is associated with tobacco smoking (Ducci et al., 2011; Gelernter et al., 2006). Similar results were found in adolescents with associations stronger in adolescence than in mid-adulthood (Ducci et al., 2011). While this observation supports the notion of shifting biological processes underlying tobacco smoking across the life span, the mechanisms by which variations in this gene-cluster exert their biological effect on smoking behaviour and smoking initiation in adolescents have not yet been elucidated.

TTC12, *ANKK1* and *DRD2* are located in close proximity on chromosome 11 in a region of high linkage disequilibrium (LD). In addition to nicotine, the gene-cluster has been implicated in alcohol and opiate addiction (Nelson et al., 2013; Xu et al., 2004; Yang et al., 2007), suggesting that its influence on smoking may be exerted through a mechanism which is not substance-specific. *TTC12* encodes for the tetratricopeptide repeat domain 12 protein, which is implicated in dopaminergic transmission and neurodevelopment (Castelo-Branco and Arenas, 2006). *ANKK1* is hypothesized to encode a signalling protein which mediates the expression of *DRD2* (Huang et al., 2009). *DRD2* in turn has a central role in regulating the dopamine reward system that mediates the reinforcing effects of all known addictive substances including nicotine mainly through striatal dopaminergic transmission (Sweitzer et al., 2012). While other dopamine-related genes have been associated to smoking behaviours (Herman et al., 2014), we were interested in further investigating the role of this genetic region in early smoking initiation (Mayhew et al., 2000) and in characterising the molecular and neurological mechanisms underlying this association.

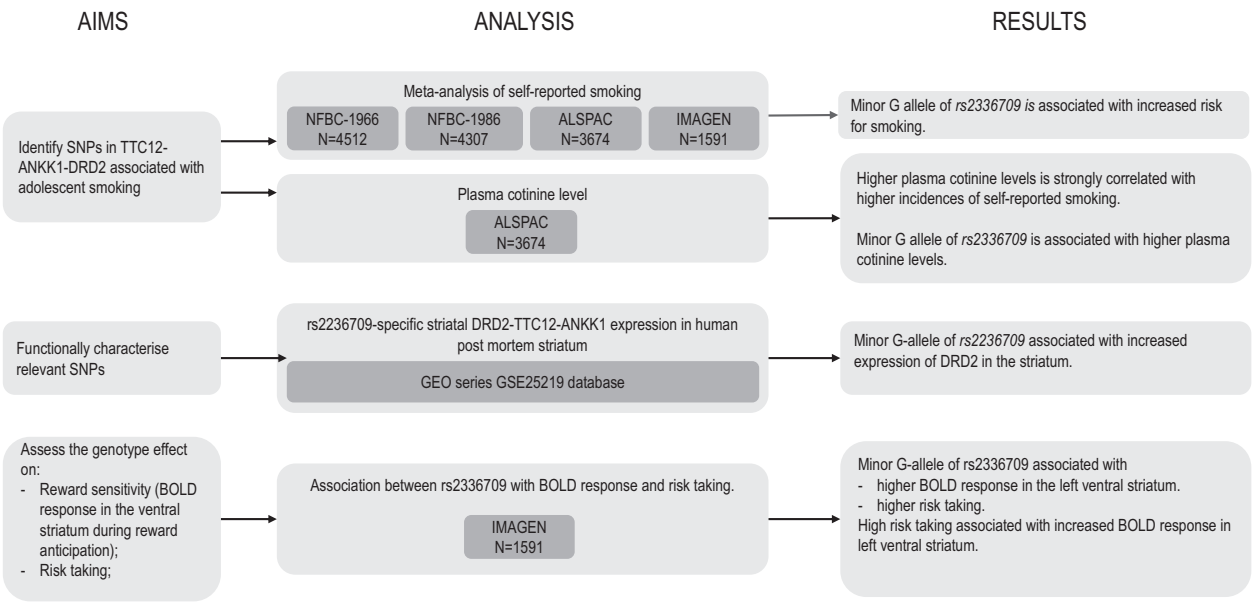

Fig. 1 Study aims, statistical analysis strategy, and results.

Specifically, we aimed (i) to identify single nucleotide polymorphisms (SNPs) in the *TTC12-ANKK1-DRD2* gene-cluster, associated with self-reported cigarette smoking in adolescents through meta-analysis of smoking data from 14,084 youths and blood cotinine levels, an objective measure of nicotine exposure, in a subset of 2,540 youths, (ii) to functionally characterize relevant SNPs by measuring allele-specific gene expression in human post-mortem striatal brain tissue and (iii) to assess the genotype effect on reward sensitivity by analysing its relation with the blood-oxygen level-dependent (BOLD) response in the ventral striatum during reward anticipation, risk taking and tobacco smoking in a subset of adolescents from the IMAGEN sample (Fig. 1).

Experimental procedures

Participants

We included 4512 adolescents from the Northern Finland Birth Cohort NFBC1966 (Sabatti et al., 2009), 4307 from NFBC1986 (Vaarasmaki et al., 2009), 3674 from the Avon Longitudinal Study of Parents and Children (ALSPAC) (Golding, 1990), and 1591 (of which 1263 had data on functional Magnetic Resonance Imaging (fMRI) data and 1085 on risk taking) from IMAGEN (Schumann et al., 2010). Demographic characteristics and phenotypic distribution of the samples are reported in Table 1.

Smoking-related phenotypes

Lifetime smoking, measuring the number and occasions of cigarette smoking adapted for adolescents who have lesser degree of exposure to smoke than adults, was self-reported in each cohort (see Supplementary-Material for details in each cohort; Ducci et al., 2011), and recoded into four categories: *Never-Tried* (never smoked), *Ever-Tried* (smoked at least once), *Smokers* (smoked more than once in the last 30 days), as previously used (Ducci et al., 2011) and *Weekly-Smokers* (smoked at least once a week in the last month). We used *Never-Tried* as our reference group to contrast with *Ever-Tried*, and subsequently validated our results by further contrasting with adolescents who smoke occasionally (*Smokers*) and regularly (*Weekly-Smokers*).

Genetic data

We chose 33 SNPs based on data availability in the cohort that was genotyped first, i.e. NFBC1966. Not all of these 33 SNPs were available in each sample. (SNPs and LD structures for each sample can be found in Supplementary-Fig. 1, MAF of the separated cohorts can be found in Supplementary Table 1).

Cotinine level

Plasma cotinine and genotypic data were available in $n = 2540$ ALSPAC participants (see Supplementary-Material).

Brain genotype and gene expression

We extracted gene expression levels of *TTC12*, *ANKK1* and *DRD2* in the striatum from the GEO series GSE25219 database (Kang et al., 2011). Probe clusters assessing expression across the entire transcript of *TTC12*, *ANKK1* and *DRD2* were extracted (Transcript Cluster IDs 3349453, 3349535 and 3391653, respectively). We independently replicated the association between *DRD2* expression levels

Table 1 Demographic characteristics and phenotypic distribution of the study samples.

	NFBC1966	NFBC1986	ALSPAC	IMAGEN		
				Whole sample	fMRI sample	Risk-taking sample
Total (*N*)	4512	4307	3674	1591	1263	1085
Male (in %)	47.3	47.6	51	49.6	48.9	48.9
Age in years	14	16	15	14	14	14
Country	Finland	Finland	U.K	Germany, U.K., France, Ireland	Germany, U.K., France, Ireland	Germany, U.K., France, Ireland
Year of assessment	1980	2002	2006	2008	2008	2008
Never-Tried						
n	1436	1539	1935	1139	914	771
%	31.83	35.73	52.67	71.59	72.40	71.10
Ever-Tried						
n	3076	2768	1739	452	349	314
%	68.17	64.26	47.33	28.41	27.60	28.90
Smokers						
n	757	982	891	189	146	133
%	16.78	22.8	24.25	11.88	11.40	12.30
Weekly-smokers						
n	309	844	371	100	71	65
%	6.85	19.60	10.10	6.29	5.60	6.00

Notes: The total sample size *N* was decomposed into participants, who never tried smoking (*Never-Tried*) as contrasted to those who have tried smoking at least once (*Ever-Tried*); this comparison is mutually exclusive. Out of those *Ever-Tried* participants, smokers and weekly smokers were taken; percentages refer to the total sample size (*N*).

(Transcript exon probe ID 3391671) and rs2236709 genotypes in 93 post-mortem cortical brain tissue samples of European ethnicity, SNPExpress (Heinzen et al., 2008).

Functional MRI

Brain activation during reward anticipation was measured using a modified monetary incentive delay (MID) fMRI task (Knutson et al., 2000) in a sample of 1263 individuals from the IMAGEN sample. Gender, recruitment site and handedness were included as covariates. Based on previous findings (Yacubian et al., 2006), we focused on the ventral striatum (bilateral) as regions of interest (ROIs) (family-wise-error (FWE)-corrected, $p < 0.05$) as 9-mm spheres from the contrast 'anticipation of large reward > anticipation of no reward' (± 15, 9, -9 in Montreal Neurological Institute (MNI) space) (see Supplementary-Materials for more details).

Risk taking

We used the Cambridge Gambling Task (CGT) from the Cambridge Cognition Neuropsychological Test Automated Battery (CANTAB; Cambridge Cognition http://www.cambridgecognition.com/).

Statistical analyses

Genetic-association analyses and meta-analysis

Genetic data analysis used Plink v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) if not otherwise indicated. Single marker association analyses were conducted using logistic or linear regression (for binary or quantitative traits, respectively) (see also Supplementary-Materials). Each genotype was coded as the number of copies of the minor allele in all cohorts. Analyses controlled for gender, (all samples) age and site (IMAGEN).

Meta-analysis was performed using the meta-analysis procedure from Plink. Significant results after Bonferroni-correction were investigated further (see also Table 2).

Association of genotype and BOLD responses in the ventral striatum, brain activation and risk taking in the IMAGEN cohort

We carried forward the results of the confirmatory analysis to investigate associations with brain activation and behavioural phenotypes underlying reward processing. ANOVAs comparing extracted mean ventral striatum BOLD responses across genotypes for those SNPs showing associations to self-reported smoking and cotinine were carried out in SPSS version 20.0 (IBM Corp., Armonk, NY). We explored associations of the smoking risk variants within *TTC12-ANKK1-DRD2* gene-cluster with risk taking. We expected to find positive associations between genetic risk for smoking and risk taking and tested for this association one-tailed.

Results

A summary of the main findings is illustrated in Fig. 1.

Table 2 Association between SNPs spanning the *TTC12-ANKK1-DRD2* gene-cluster and smoking for *Ever-Tried* vs *Never-Tried*.

BP	Gene	SNP	Location	MAF	Ref allele/	N		*P*		*OR*		*Q*	I^2
					Other allele	Samples	Individuals	(FEM)	(REM)	(FEM)	(REM)		
112685412	*TTC12*	rs4517559	Flanking 5′UTR	0.3736	C/T	4	13553	0.002		1.08		0.27	23.84
112691986	*TTC12*	rs2236709	Intron	0.2588	G/A	4	14081	0.0005*		1.1		0.66	0
112693407	*TTC12*	rs7927508	Intron	0.3667	G/A	3	9762	0.009		1.08		0.15	46.93
112694133	*TTC12*	rs2156486	Intron	0.1814	G/T	4	13555	0.008		0.92		0.83	0
112699378	*TTC12*	rs723077	Coding, Met73Leu	0.4894	C/A	3	9771	0.06		0.94		0.63	0
112704356	*TTC12*	rs10502172	Intron	0.4596	T/C	4	13675	0.004		0.93		0.16	41.71
112705919	*TTC12*	rs2303380	Intron splice site	0.3819	G/A	3	9771	0.49		1.02		0.18	41.76
112716539	*TTC12*	rs2288159	Intron	0.1561	T/G	3	9768	0.13		1.06		0.63	0
112721133	*TTC12*	rs4987094	Intron	0.1004	A/G	3	9777	0.12		1.07		0.67	0
112735810	*TTC12*	rs2276070	Intron	0.1541	T/C	3	9774	0.13		1.06		0.65	0
112739889	*TTC12*	rs719802	Intron	0.3953	T/C	3	9772	0.36		1.03		0.33	8.76
112739985	*TTC12*	rs719804	Intron	0.249	G/A	3	9759	0.06		1.07		0.76	0
112749387	*TTC12*	rs2282511	Flanking 3′UTR	0.3455	A/C	3	11764		0.11		1.08	0.09	57.82
112754346	*TTC12*	rs754672	Flanking 3′UTR	0.4894	T/C	4	13552	0.002		0.92		0.48	0
112761718	*ANKK1*	rs877138	Flanking 5′UTR	0.3431	G/A	3	9777	0.25		1.04		0.37	0
112768580	*ANKK1*	rs4590907	Intron	0.1436	G/T	4	13577	0.23		1.04		0.76	0
112772031	*ANKK1*	rs7118900	Coding, Ala239Thr	0.1896	A/G	3	9767	0.24		0.95		0.88	0
112775225	*ANKK1*	rs4938016	Coding, Gly442Arg	0.4452	G/C	3	11966	0.002		1.09		0.5	0
112775370	*ANKK1*	rs2734849	Coding, His490Arg	0.4886	G/A	4	13580	0.01		0.94		0.3	18.9
112776038	*ANKK1*	rs1800497	Coding, Glu713Lys, TaqIA	0.2026	A/G	4	13549	0.47		0.98		0.71	0
112788669	*DRD2*	rs6277	Coding, Pro219Pro	0.12201	A/G	3	11981	0.004		0.92		0.57	0
112801119	*DRD2*	rs1076563	Intron	0.4127	C/A	3	9773	0.07		0.95		0.78	0
112803549	*DRD2*	rs2471857	Intron	0.1577	T/C	3	9772	0.38		0.96		0.7	0
112815891	*DRD2*	rs7125415	Intron	0.09914	T/C	3	9775	0.09		1.08		0.43	0
112818599	*DRD2*	rs4648318	Intron	0.2538	C/T	3	9768	0.02		1.08		0.53	0
112824662	*DRD2*	rs4274224	Intron	0.4823	G/A	3	9771	0.48		1.02		0.42	0
112829684	*DRD2*	rs4581480	Intron	0.09919	C/T	3	9768	0.42		1.04		0.28	20.71
112834984	*DRD2*	rs7131056	Intron	0.4205	C/A	3	9762	0.14		1.05		0.37	0
112846601	*DRD2*	rs4938019	Intron	0.1447	C/T	4	13588	0.29		1.04		0.41	0
112852165	*DRD2*	rs12364283	Flanking 5′UTR	0.07563	G/A	3	9765	0.94		0.1		0.98	0
112857971	*DRD2*	rs10891556	Intergenic	0.1758	T/G	3	9772	0.51		1.03		0.35	5.8
112860946	*DRD2*	rs6589377	Intergenic	0.3776	G/A	4	13581	0.66		1.01		0.34	10.46
112863421	*DRD2*	rs4482060	Intergenic	0.30032	T/A	3	11955	0.39		1.03		0.48	0

Notes: Association between SNPs spanning the *TTC12-ANKK1-DRD2* gene-cluster and self-reported smoking behaviour. *Ever-Tried* ($N = 8722$) are compared to *Never-Tried* ($N = 6049$) (binary logistic regression). Results from NFBC1966, ALSPAC, NFBC1986 and IMAGEN have been combined using meta-analysis. FEM = Fixed effect model, REM = Random effect model, Q = Cochrane's Q statistic, I^2 = heterogeneity index (0–100), N = number of study samples. Significant p values are in bold, of these those that remain significant after Bonferroni correction for multiple testing are indicated by an asterisk. Thick lines indicate boundary between Linkage Disequilibrium blocks.

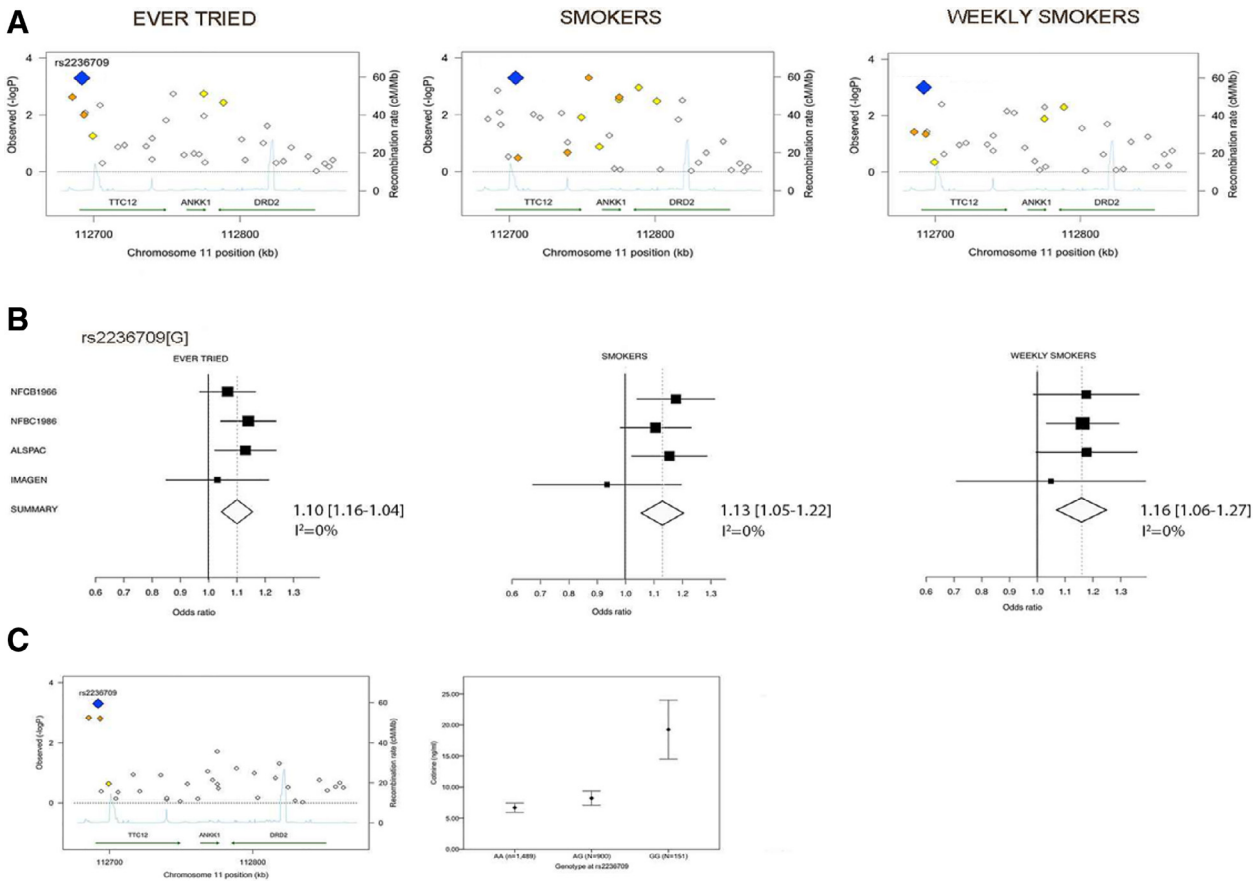

Fig. 2 Association between 33 single nucleotide polymorphisms (SNPs) covering the chromosome 11q23 *TTC12-ANKK1-DRD2* gene-cluster and self-reported smoking behavior in adolescence. Results of the meta-analyses across the NFBC1966, NFBC1986, ALSPAC and IMAGEN cohorts are reported. *p*-values were meta-*p*-values computed under fixed effect models. Given the multiple testing correction for all SNPs a significance threshold of 0.0015 was used. The blue diamond indicates the most significantly associated SNP. This was rs2236709 with $p = 5.0 \times 10^{-4}$ for *Ever-Tried* vs. *Never-Tried*, rs2236709 with $p = 0.001$ for *Smokers* vs. *Never-Tried* and rs2236709 with $p = 0.001$ for *Weekly Smokers* vs. *Never-Tried*. For other SNPs, diamonds are colored in a white-to-red scale corresponding to R^2 values from 0 to 1 with the most significant SNPs. The SNP position refers to National Center for Biotechnology Information build 35. Estimated recombination rates are from HapMap and gene annotations are from UCSC genome browser with build. (B) Forest plots for rs2236709 for the comparisons of the reference group (i.e. *Never-Tried*) with *Ever-Tried, Smokers* and *Weekly-Smokers*. Box areas are proportional to the weight of the individual study. The overall summary odds ratios (OR) computed with a fixed effect model are represented by a diamond whose width indicate the 95% confidence interval (CI). Results of the analyses were as follows: *Ever-Tried* vs. *Never-Tried* OR = 1.10, 95%CI 1.04-1.16, $p = 0.5.0 \times 10^{-4}$; *Smokers* vs. *Never-Tried* OR = 1.13, 95% CI 1.05-1.22, $p = 0.001$ and *Weekly-Smokers* vs. *Never-Tried* OR = 1.16, 95% CI 1.06-1.27, $p = 0.001$ on the left, middle and right forest plots, respectively. (C, left plot) Association between 33 SNPs and cotinine level in the ALSPAC cohort. Blue diamond indicates the most significantly associated SNP (rs2236709), as determined by linear regression analyses. Other diamonds are color-coded in a white-to-orange scale corresponding to increasing R^2 values of the respective SNP with rs2236709. The SNP position refers to National Center for Biotechnology Information build 35. Estimated recombination rates are from HapMap and gene annotations are from UCSC genome browser with build 35 coordinates. (C, right plot) Mean cotinine level (SE) according to rs2236709 genotypes (linear regression analysis: $\beta = 0.11$, $p = 0.5.0 \times 10^{-4}$). $Mean_{AA} = 6.35$, SE = 0.72, $n = 1489$; $Mean_{AG} = 7.94$, SE = 1.18, $n = 900$; and $Mean_{GG} = 19.62$, SE = 4.86, $n = 151$; post-hoc comparisons: AA vs. AG, $p = 0.843$, $R^2 = 0.0005$, AA vs. GG, $p = 3.67E\text{-}5$, $R^2 = 0.02$ and AG vs. GG, $p = 0.001$, $R^2 = 0.013$.

Meta-analysis exploring the association between TTC12-ANKKI-DRD2 and smoking

Comparing adolescents who *Never-Tried* smoking with those who *Ever-Tried* across four cohorts, we identified a significant association with rs2236709 (Risk allele G; Odds ratio (OR) = 1.10, 95% confidence interval (CI): 1.04-1.16, $p = 5.0 \times 10^{-4}$) (Fig. 2 and Table 2). The minor G-allele of rs2236709 was linked to increased risk for smoking amongst subgroups of regular smokers (*Smokers* vs. *Never-Tried*: OR = 1.13, 95% CI: 1.05-1.22, $p = 0.001$; *Weekly-Smokers*

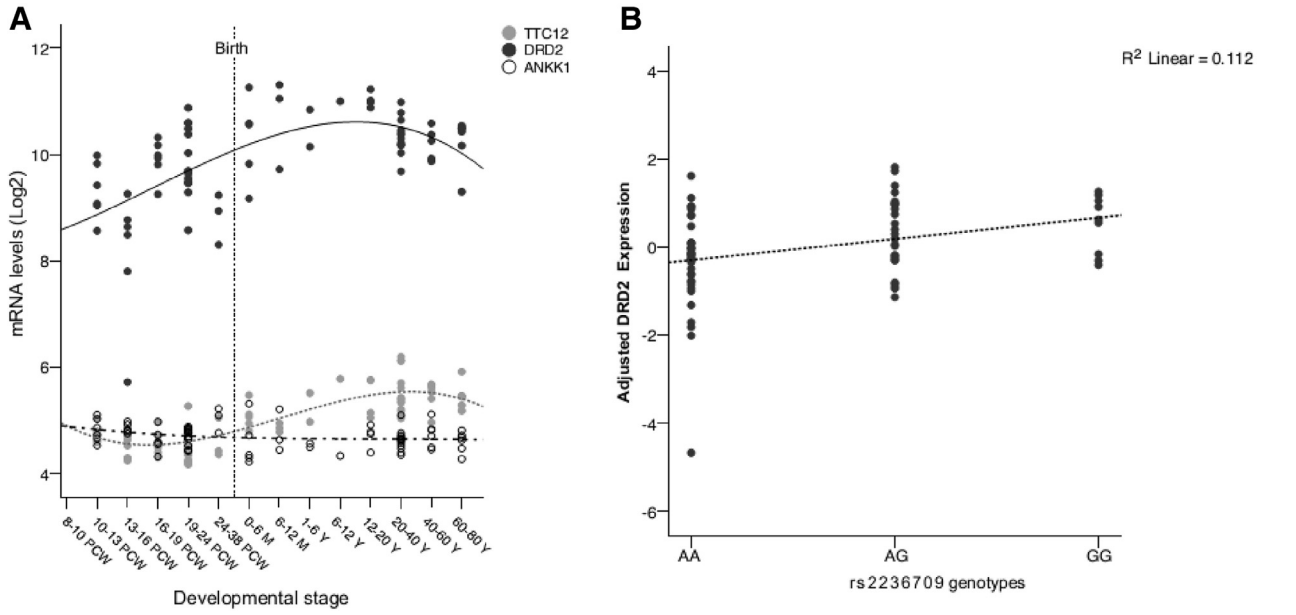

Fig. 3 (A) Expression of *DRD2*, *TTC12* and *ANKK1* in the human striatum across the lifetime. The *x*-axis represents different stages of fetal and postnatal development, as indicated. PCW = post-conception weeks, M = months, Y = years. (B) Striatal expression of *DRD2* corrected for effects of developmental stage and stratified by rs2236709 genotypes. The dotted line visualizes the linear regression against the number of minor G-alleles ($n_{AA} = 22$, $n_{AG} = 16$ and, $n_{GG} = 5$).

vs. *Never-Tried*: OR = 1.16, 95% CI: 1.06-1.27, $p = 0.001$) (Fig. 2B; Supplementary-Material Tables 2 and 3).

Association of cotinine level and self-reported smoking behavior

Higher cotinine levels correlated with a higher number of self-reported cigarettes (Spearman's $\rho = 0.33$, $p = 3.7 \times 10^{-53}$), thus validating self-reported smoking. Mean cotinine levels in ng/ml (SD) for each category of smokers were: *Never-Tried* = 0.73 (2.73), *Ever-Tried* = 15.18 (46.51), *Smokers* = 28.13 (61.64) and *Weekly-Smokers* = 64.97 (82.51). One-way ANOVA revealed significant difference across lifetime smoking categories ($F(3,3319) = 25.09$, $p = 4.80 \times 10^{-16}$, $R^2 = 0.022$). Stratification of mean plasma cotinine levels according to rs2236709 genotypes indicated a significant positive association with the minor G-allele [$F(2,2537) = 9.64$, $p = 6.76 \times 10^{-5}$, $R^2 = 0.003$) (Fig. 2C, Supplementary Table 4), thus confirming the association of rs2236709 with self-reported smoking.

Rs2236709 influences on striatal *DRD2* expression

Rs2236709 is located within *TTC12* and is in LD with the other genes in the locus, *ANKK1* and *DRD2* (see Supplementary-Material Fig. 1). To investigate which of these three genes contributed to the effects of rs2236709 on smoking, we analysed their expression in post-mortem human brain samples. Fig. 3A shows expression of *TTC12*, *ANKK1* and *DRD2* in the striatum across the lifetime. It was indicated that *DRD2* mRNA levels in the striatum increased steadily during the foetal stages and reached a peak in adolescence, while *ANKK1* and *TTC12* expression levels were low overall. We performed association analyses to investigate the effects of rs2236709 on striatal *DRD2*, *TTC12* and *ANKK1* expression and included developmental stage as a covariate. The minor G-allele of rs2236709 was associated with increased expression of *DRD2* in the striatum ($r = 0.39$, $p = 0.013$, df = 37; Fig. 3B). Neither expression of *TTC12* nor that of *ANKK1* was influenced by rs2236709 genotypes ($p = 0.176$ and $p = 0.244$, respectively). There was no association of gender ($p = 0.627$) or RNA integrity ($p = 0.296$) with *DRD2* gene expression.

As there is, to our knowledge, no other dataset contains expression levels of *DRD2* in the striatum and rs2236709 genotype data, we independently replicated the association between *DRD2* expression levels and rs2236709 in cortical brain tissue samples of European ethnicity (Heinzen et al., 2008), and found the association to be significant ($p_{uncorrected} = 0.003$, $p_{corrected} = 0.049$, corrected for the number of *DRD2* probes).

Association of rs2236709 genotype and ventral striatum BOLD response during reward anticipation

As our results suggest that altered dopaminergic transmission in reward-related brain areas (e.g., the ventral striatum) may underlie the association of rs2236709 with smoking, we further measured associations of this variant with BOLD response during reward anticipation in the ventral striatum of 1236 IMAGEN participants. Due to a skewed allele distribution of rs2236709 ($n_{AA} = 706$, $n_{AG} = 467$, $n_{GG} = 90$), we pooled heterozygotes and GG-homozygotes (G-carriers) and compared them to AA-

homozygotes. Our results show a significantly higher BOLD response in the left ventral striatum of G-carriers vs. AA-homozygotes [$F(1,1261) = 5.803$, $p_{uncorrected} = 0.016$, $p_{corrected} = 0.032$, $R^2 = 0.005$, Supplementary-Materials), indicating an association of the risk allele (G) with greater reward sensitivity and increased *DRD2* expression. No significant associations of rs2236709 genotypes with BOLD response in the right ventral striatum ($p = 0.237$) were observed.

Association between risk taking, rs2236709, and ventral striatum BOLD response during reward anticipation

As we expected a positive association between genetic risk for smoking and risk taking, this association was tested one-tailed. Risk taking positively associated with risk allele (G) of rs2236709 ($r = 0.056$, $p = 0.033$), and ventral striatal BOLD response (left: $r = 0.033$, $p = 0.112$; right: $r = 0.049$, $p = 0.034$). Risk taking also significantly associated with the number of occasions of lifetime cigarette smoking ($r = 0.056$, $p = 0.034$).

Discussion

The current study carried out a neurobehavioral characterization of the *TTC12-ANKK1-DRD2* gene-cluster and smoking in adolescence using four large datasets from different European countries. Our result demonstrated that this gene-locus exerts its effect on smoking behaviors such as smoking initiation and frequency already in the very early stages of nicotine abuse. To attain a mechanistic understanding of the associations of the minor G-allele of rs2236709 with both increased self-reported nicotine intake and higher cotinine levels in adolescents, we found that the genetic risk factor rs2236709 regulates DRD2 gene expression, and is also associated with activation of ventral striatum during reward anticipation and risk taking, a behaviour associated with drug initiation.

DRD2 is a key molecular determinant of reward sensitivity (Sweitzer et al., 2012), which reaches its peak expression in the striatum during adolescence. High *DRD2* expression increases sensitivity to rewarding stimuli, thus increases sensitivity to the effects of substances (DiNieri et al., 2011) as well as risk taking (Cocker et al., 2012). This combination increases vulnerability for addictive behaviors (Leyton and Vezina, 2014). Thus, enhanced *DRD2* expression in adolescents is likely to be one component of risk for substance abuse and smoking in particular. Whereas *DRD2* receptor availability is reduced during the compulsive stages of substance addiction (Johnson and Kenny, 2010), early substance use has been linked to increased *DRD2* expression levels in the ventral striatum (Koob and Volkow, 2009), suggesting a differential regulation of *DRD2* in adolescents experimenting with drugs compared to established nicotine dependence in adults.

Our results suggest that carriers of rs2236709 G-risk allele are at a particularly high risk of nicotine abuse, presumably due to an allele-specific increase in *DRD2* expression, in carriers of the G-risk allele found in human post-mortem brain tissue retrieved from the striatum. While our cohorts have different ancestry the LD structure of the rs2236709 locus is similar, suggesting that the findings in the post-mortem human brains can be extended to all our cohorts. Concurrently, increased ventro-striatal response during reward anticipation was observed in carriers of the G-risk allele in functional neuroimaging analyses of 14 year-old IMAGEN participants. Together these findings suggest an enhanced (ventro-)striatal dopaminergic activity underlying the observed association. Our cognitive findings using the IMAGEN sample are in keeping with this interpretation, namely that carriers of the risk allele showed higher risk taking behavior, which in turn was associated with increased ventral striatal activation and with increased nicotine use.

We did not detect significant association between smoking frequency and ventral striatal activity in this sample of 14 year-old adolescents in which 72% had never smoked. This might be explained by insufficient exposure to cigarettes due to young age and/or reduced availability of cigarettes compared to older cohorts, such as NFBC1966 where only 32% adolescents had never smoked at that age.

Rs2236709 is localized in the second intron of the *TTC12* gene locus and tags a haplotype that ranges from the promoter to the third exon of the *TTC12* gene. While it is correlated with *DRD2* expression in the striatum, it is not associated with the expression of *TTC12* itself. However, it is well known that even *Cis*-acting gene expression can be regulated by genetic variations that are not immediately adjacent to the gene they are regulating (Kirsten et al., 2015).

This study has some limitations: (i) different cohorts applied different measures to assess smoking behaviour, which limited us from obtaining a continuous measure consistent across studies, but instead combined information into distinct groups. (ii) Smoking habits have been subject to changing societal and legal attitudes over the last decades. Data acquisition among the different cohorts spreads over several decades, which was not accounted for. (iii) Cotinine can also account for second hand smoking, moreover the half-life of cotinine being 16–18 h is much shorter than some of the self-reported smoking behaviours that measure longer tobacco use (Jarvis et al., 1988; Perez-Stable et al., 1995; Vartiainen et al., 2002). (iv) Genetic heterogeneity between studies should be noted as we pooled all samples of European ancestry due to limited data on adolescent smoking. Two cohorts (NFBC1966, NFBC1986) were of Finnish ancestry, and genetically distinct from the other two European cohorts (IMAGEN, ALSPAC), which might limit generalization of our findings. Notably, despite some differences of the minor allele frequency (MAF), the direction of the smoking-risk SNP effects is consistent across cohorts and I^2 of rs2236709 indicated no heterogeneity across cohorts.

Our work proposes a neurobiological pathway to nicotine abuse in a developmental period characterized by both vulnerability to the effects of nicotine, and great therapeutic potential to prevent or overcome its abuse (Toumbourou et al., 2007). Noting the recent success of interventions targeting behavioral risk profiles (Conrod et al., 2013), our neurobehavioral characterization provided a basis for developing interventions targeting biological mechanisms underlying nicotine abuse.

Conflict of interest

Dr. Banaschewski has served as an advisor or consultant to Bristol-Myers Squibb, Desitin Arzneimittel, Eli Lilly, Medice, Novartis, Pfizer, Shire, UCB, and Vifor Pharma; he has received conference attendance support, conference support, or speaking fees from Eli Lilly, Janssen McNeil, Medice, Novartis, Shire, and UCB; and he is involved in clinical trials conducted by Eli Lilly, Novartis, and Shire; the present work is unrelated to these relationships. Dr. Gallinat has received research funding from the German Federal Ministry of Education and Research, AstraZeneca, Eli Lilly, Janssen-Cilag, and Bristol-Myers Squibb; he has received speaking fees from AstraZeneca, Janssen-Cilag, and Bristol-Myers Squibb; the present work is unrelated to these relationships. The other authors report no financial interests or potential conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2018.07.101.

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