Neurotox Res (2016) 29:345–350
DOI 10.1007/s12640-015-9545-9

SHORT REPORT/RAPID COMMUNICATION

The Addiction-Related Gene *Ankk1* is Oppositely Regulated by D1R- and D2R-Like Dopamine Receptors

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Received: 29 April 2015 / Revised: 2 July 2015 / Accepted: 8 July 2015 / Published online: 21 July 2015
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Abstract The ankyrin repeat and kinase domain containing 1 (*ANKK1*) *Taq*IA polymorphism has been extensively studied as a marker of the gene for dopamine receptor D2 (*DRD2*) in addictions and other dopamine-associated traits. *In vitro* mRNA and protein studies have shown a potential connection between *ANKK1* and the dopaminergic system functioning. Here, we have investigated whether *Ankk1* expression in the brain is regulated by treatment with dopaminergic agonists. We used quantitative RT-PCR of total brain and Western blots of specific brain areas to study *Ankk1* in murine brain after dopaminergic treatments. We found that *Ankk1* mRNA was upregulated after activation of D1R-like dopamine receptors with SKF38393 (2.660 ± 1.035-fold; t: 4.066, df: 11, $P = 0.002$) and apomorphine (2.043 ± 0.595-fold; t: 3.782, df: 8, $P = 0.005$). The D2R-like agonist quinelorane has no effect upon *Ankk1* mRNA (1.004 ± 0.580-fold; t: 0.015, df: 10, $P = 0.9885$). In contrast, mice treatment with the D2R-like agonists 7-OH-DPAT and aripiprazole caused a significant *Ankk1* mRNA downregulation (0.606 ± 0.057-fold; t: 2.786, df: 10, $P = 0.02$ and 0.588 ± 0.130-fold; t: 2.394, df: 11, $P = 0.036$, respectively). With respect the Ankk1 proteins profile, no effects were found after SKF38393 (t: 0.54, df: 2, $P = 0.643$) and Quinelorane (t: 0.286, df: 8, $P = 0.782$) treatments. In contrast, the D2R-like agonist 7-OH-DPAT (±) caused a significant increment of Ankk1 in the striatum (t: 2.718, df: 7; $P = 0.03$) when compared to the prefrontal cortex. The activation of D1R-like and D2-R-like leads to opposite transcriptional regulation of *Ankk1* by specific pathways.

Keywords *Ankk1/Drd2* · *Drd1* · *Drd3* · *Taq*IA · Addiction

The *Taq*IA single nucleotide polymorphism (SNP) (rs1800497; locus 11q22–q23) is the most studied genetic variant in addictions (Ponce et al. 2009). Blum et al. first reported the association between alcoholism and the *Taq*IA A1 allele and the $A1^+$ genotype (hetero- or homozygous for A1) (Blum et al. 1990). Two meta-analyses of Caucasian alcoholics and controls nonetheless support a link between the A1 allele and alcoholism (Munafo et al. 2007; Smith et al. 2008). Currently, the A1 allele is recognized as a risk factor for alcoholism (Agrawal et al. 2012). The *Taq*IA SNP has also been related to a variety of addictions and impulsive disorders (Ponce et al. 2009), antisocial traits in alcoholics (Hoenicka et al. 2007; Ponce et al. 2003), and a number of dopaminergic-related endophenotypes (Ponce et al. 2009).

*Taq*IA is located in the coding region of the ankyrin repeat and kinase domain containing 1 gene (*ANKK1*), which is near the *DRD2* gene of dopamine receptor D2 (D2R) (Neville et al. 2004). This SNP produces a Glu713-to-Lys (E713K) substitution in the ANKK1 aminoacid sequence (Neville et al. 2004). However, *Taq*IA has been extensively studied as a marker of the nearby *DRD2* gene.

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Moreover, the close relationship between the *Taq*IA and *DRD2* functional polymorphisms (Ponce et al. 2009) has masked the possibility that *ANKK1* might also be associated with dopaminergic transmission. In vitro mRNA and protein studies have shown a potential connection between *ANKK1* and the dopaminergic system functioning (Garrido et al. 2011; Hoenicka et al. 2010). In a sample of alcoholic patients, the epistatic effect of the *Taq*IA *ANKK1* and C957T *DRD2* SNPs on expression of antisocial traits suggests that these genes act in a coordinated manner (Ponce et al. 2008). However, it remains unknown the molecular mechanisms by which *ANKK1* could be associated with the dopaminergic system and how *ANKK1* polymorphic alleles would impact addictions vulnerability. Dopamine acts through five receptor subtypes expressed differently in the brain. These receptors belong to the G-protein-coupled superfamily, which has been grouped in two major subclasses (Jackson and Westlind-Danielsson 1994): the D1R-like subclass, which includes dopamine receptor D1 (D1R) and D5 (D5R) and stimulates adenylyl cyclase (AC) via $G_{s/olf}$ signaling proteins that result in increased cyclic AMP (cAMP) and activation of protein kinase A (PKA); and the D2R-like subclass, which includes dopamine D2 (D2R), D3 (D3R), and D4 (D4R) receptors that interact with $G_{\alpha i/o}$ proteins to inhibit production of cAMP (Missale et al. 1998). Other dopamine-related pathways have been reported. On the D1R-like side, stimulation of D5R activates phospholipid and calcium signaling via phospholipase C (PLC) (So et al. 2009). The phospholipid signal transduction system is also initiated when dopamine binds to the D1R-like/D2R hetero-oligomers, leading to activation of PLC and increased calcium levels (Rashid et al. 2007; So et al. 2009).

Other D2-like receptor-coupled signaling pathways have been reported, including the regulation of $G_{\beta/\gamma}$ activity (Hernandez-Lopez et al. 2000), the complex β-arrestin 2/protein phosphatase 2A-Akt pathway which stimulates the glycogen synthase kinase 3 (GSK-3) (Neve et al. 2004) and the activation of PAR4 (Bibb 2005). In addition, in the presence of the D3R preferring agonist 7-OH-DPAT, D3R shows unique properties involving activation of phospholipase D (PLD) through a complex formed with the monomeric G-protein Rho (Everett and Senogles 2010).

Given that alterations in dopamine receptors (DRs) signaling are widely known to contribute to addictions, here we hypothesized that stimulation of different DR could regulate *ANKK1* expression levels.

We carried out dopaminergic treatments, which activate specific signaling pathways, to study their effects upon *Ankk1* expression. Adult C57BL/6 male mice 3 months-old weighing approximately 25–30 g, (Harlan Laboratories, Barcelona, Spain) were handled according to Directive 86/609/EU on the protection of animals used for scientific purposes. The present study was also approved by the Institutional Animal Use and Care Committee of the "Instituto de Investigación Hospital 12 de Octubre".

The study reports data from a total of $n = 68$ mice, with $n = 6$–8 animals per group in each molecular assay. All dopaminergic drugs used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dissociation constant values (Ki) were obtained from the TOCRIS bioscience webpage (http://www.tocris.com). Mice were treated with the following: D1R-like agonist 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine (±) SKF38393 (D1R Ki = 1 μM and D5R Ki = 0.5 μM), D2R-like agonists quinelorane (D2R Ki = 5.7 μM and D3R Ki = 3.4 μM), and 7-OH-DPAT (7-Hydroxy-2-(di-n-propylamino) tetralin hydrobromide (D2R Ki = 10 μM and D3R Ki = 1 μM); the DRs agonist (R)-(−) apomorphine (Ki = 6.43, 7.08, 7.59, and 7.83 μM for D1R, D2R, D3R, and D5R, respectively) and the DR partial agonist aripiprazole (7-[4-[4-(2,3-dichlorophenyl)-1-piperazinyl]butoxy]-3,4-dihydrocarbostyril; Ki = 0.34 μM and 0.8 μM for D2R and D3R, respectively). The DA receptor agonists dissolved in sterile 0.9 % saline solution were administered to different groups ($n = 6$ each) of C57BL/6 mice. Mice received intraperitoneal (±) SKF-38393 (10 mg/kg), quinelorane (0.5 mg/kg), 7-OH-DPAT (0.75 mg/kg), (R)-(−) apomorphine (20 mg/kg), aripiprazole (125 mg/kg), or saline. After one hour, animals were euthanized using cervical dislocation for brain extraction. One hemisphere was treated with RNAlater buffer (Ambion®; Thermo Fisher Scientific, Waltham, MA, USA) for further expression profiles by quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR). The striatum and the prefrontal cortex were dissected from the other hemisphere, immediately frozen in liquid nitrogen, and stored at −80 °C for immunoblots.

For RT-PCR experiments, total RNA was isolated from mouse brains with Trizol reagent (Invitrogen) and purified (RNeasy Protect Mini Kit, Qiagen, Valencia, CA, USA). PolyT primers were used for the RT reaction using the Maxima® First Strand cDNA Synthesis Kit (Fermentas, Life Technologies). All quantitative real-time PCR reactions were carried out in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Gene expression was analyzed using FastStart Universal SYBR Green Master Rox (Roche Diagnostics, Indianapolis, Indiana, USA). Amplification was performed at 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s (denaturation), 60 °C for 1 min (annealing) and 72 °C for 30 s (extension). Samples were quantified by the standard-curve method (Larionov et al. 2005) and normalized to the *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene in the case of quinelorane, SKF38393 and apomorphine treatments. In the case of 7-OH-DPAT and

aripiprazole treatments, that modify *GAPDH* expression, samples were normalized to the *PPIA* [peptidylprolyl Isomerase A (Cyclophilin A)]. Results were analyzed with SDS 2.1 software (Applied Biosystems). The primers used are shown in the Table 1. The normal distribution of data was verified using the Kolmogorov–Smirnov test. Statistical differences were analyzed using the one-way analysis of variance (ANOVA) followed by Student's *t* test (*t*). Statistical analyses were performed using GraphPrism 5 software (GraphPad, La Jolla, CA, USA).

For Western blot studies total protein extracts from the striatum and prefrontal cortex of one hemisphere were obtained after homogenization in lysis buffer (150 mM NaCl, 20 mM Tris HCl, 5 mM EDTA, 10 % glycerol, 1 % NP-40) containing a protease inhibitor cocktail (Complete Mini-Protease Inhibitor Cocktail, Roche Diagnostics). Western blots were performed as previously described (Hoenicka et al. 2010). Membranes were scanned and the band intensity was measured through total area of the peaks using the Image J 1.38v software from the National Institutes of Health (NIH). Primary antibodies were rabbit α-ANKK1 generated against a 14-amino-acid peptide corresponding to the kinase domain of the predicted ANKK1 ORF shared by human, mouse and rat (α-STk antibody); and a 13-amino-acid peptide specific of N-terminal of the predicted mouse Ankk1 ORF (α-STk2 antibody) (Hoenicka et al. 2010). Rabbit sera were collected 7–10 days after the last boost, and antibody titers and specificity were determined by enzyme-linked immunosorbent assay (ELISA) using synthetic peptides-coated plates. Unrelated synthetic peptide and bovine serum albumin were used as negative controls. α-STk and α-STk2 specificity were also assessed by solid-phase preadsorption in tissue with the cognate peptide. α-STk was previously used for immunoblotting and immunostaining (Hoenicka et al. 2010). α-STk2 was previously used for immunostaining of OCT-embedded embryos cut on a cryostat (Hoenicka et al. 2010).

We found in total brain RNA samples a significant interaction between *Ankk1* mRNA expression and dopaminergic treatments ($F = 11.93$; $P < 0.0001$) (Fig. 1a). This interaction effect was subsequently evaluated with pairwise comparison for each specific treatment (Fig. 1a). Mice treatment with the D1R-like agonist (±)

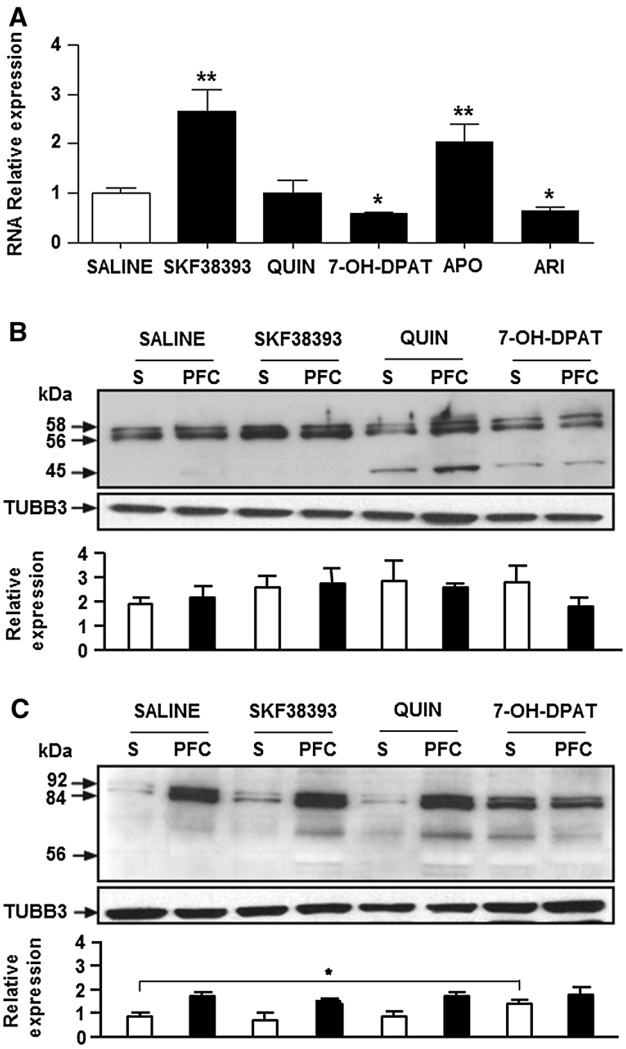

Fig. 1 *Ankk1* mRNA expression level is linked to dopaminergic stimulation in the adult murine brain. **a** Quantitative RT-PCR showing the inverse effect of the D1R-like agonist (±) SKF38393 and apomorphine when compared with the D2R-like agonists 7-OH-DPAT and aripiprazole on *Ankk1* mRNA levels. *QUIN* quinelorane, *ARIP* aripiprazole, *APO* apomorphine. Relative mRNA expression values (±SEM) are shown. $^{*}P < 0.05$; $^{**}P < 0.01$. **b** and **c**, Western blots of striatum (S) and prefrontal cortex (PFC) tissue from mice treated with SKF-38393, QUIN, or 7-OH-DPAT using α-STk **b** and α-STk2 **C** B. α-STk identified bands of ~56–58 and ~45-kDa. C. α-STk2 detected bands of ~56-, ~84-, and ~92-kDa. Relative protein expression of Western blot bands was quantified using Image J 1.37v software

Table 1 RT-PCR oligonucleotides

Gene	Gi (accesion number)	Primer 5′–3′	From	To
Gapdh	52139063	ACGACCCCTTCATTGACC CCAGTGAGCTTCCCGTTCAGC	550	715
Ppia	6679438	GTGCCAGGGTGGTGACTTTAC TTGTGTTTGGTCCAGCATTTG	224	363
Ankk1	146198787	TCCGATTTTGGCCTGTCCAAG AGATGACAATTGCAAAGCTGTAC	145	733

SKF38393 caused a significant upregulation of *Ankk1* (2.660 ± 1.035-fold; *t*: 4.066, d*f*: 11, $P = 0.002$). The D2R-like agonist 7-OH-DPAT caused an inhibitory effect on *Ankk1* mRNA expression (0.606 ± 0.057-fold; *t*: 2.786, d*f*: 10, $P = 0.02$). The decline in *Ankk1* expression was also observed with aripiprazole, a D2R-like partial agonist (0.588 ± 0.130-fold; *t*: 2.394, d*f*: 11, $P = 0.036$). In contrast, the D2R-like agonist quinelorane, had no effect upon *Ankk1* expression in the murine brain (1.004 ± 0.580-fold; *t*: 0.015, d*f*: 10, $P = 0.9885$). Mice were also treated with (R)-(−) apomorphine (APO) a non-selective dopaminergic receptor agonist which acts as a partial agonist of the D1R-like (D1R and D5R) and a full agonist of the D2R-like (D2, D3, and D4). In agreement with previous findings in cultured astrocytes (Hoenicka et al. 2010), we found a significant increase in *Ankk1* expression (2.043 ± 0.595-fold; *t*: 3.782, d*f*: 8, $P = 0.005$) (Fig. 1a).

These data altogether showed that D1R-like and D2R-like activation with specific agonists exerts opposite regulation on *Ankk1* mRNA gene expression levels in adult murine brain. Specifically, the systemic administration of the prototypical D1R-like selective agonist SKF38393 and the non-selective dopaminergic agonist apomorphine causes an *Ankk1* gene upregulation. SKF38393 binds to D1R and D5R that signal via G proteins through PKA and PLC pathways (Sahu et al. 2009), respectively. Apomorphine, that also increased *Ankk1* mRNA expression in vitro (Hoenicka et al. 2010), binds D1R–D2R heterodimers in the striatum that also signal trough PLC pathway (Medvedev et al. 2013). Moreover, we previously found in cells treated with sulpiride, a selective D2R antagonist, a decrease in the *Ankk1* mRNA upregulation after apomorphine treatment (Hoenicka et al. 2010). All these data would suggest that the PLC pathway might be involved in the upregulation of *Ankk1* transcripts in the mice brain. On the other hand, the treatments with D2R-like agonist cause either no effect (Quinelorane) or the decrease of *Ankk1* mRNA gene expression (Aripiprazole and 7-OH-DPAT). These differences in the treatment effect upon *Ankk1* mRNA regulation could be related to different D2R-like agonists profile with respect D2R and D3R: quinelorane shows higher potency at D2R than at D3R sites when activates the β-arrestin 2/Akt/GSK-3 pathway (Mannoury la Cour et al. 2011); aripiprazole is a partial D2R agonist and a strong D3R agonist (Shapiro et al. 2003) and 7-OH-DPAT is a D3R preferring agonist that activates phospholipase D (PLD) pathway(Everett and Senogles 2010). Interesting, the PLD activity is inhibited by the D1R-like signaling (Yang et al. 2005) that upregulates *Ankk1* mRNA. Therefore, our results suggest that *Ankk1* mRNA transcription could be the result of a net effect of competing of D1R-like-related (stimulation) and D2R-like-related (inhibiting) signaling pathways.

After observing significant changes in *Ankk1* mRNA gene expression, we proceeded to study the impact of dopaminergic treatments on Ankk1 proteins profile in specific brain regions by Western blot. We analyzed striatum and prefrontal cortex (PFC) of adult mice brain treated with the SKF38393, 7-OH-DPAT and quinelorane using two antibodies: α-STk, which recognizes identical human and mouse N-terminal peptide (Hoenicka et al. 2010), and α-STk2, which was generated against a second mouse Ankk1 N-terminal peptide (Hoenicka et al. 2010). No differences were observed using α-STk (One-way ANOVA, $F = 0.5779$; P: 0.7678). This antibody detected a ~56-58 kDa band in all samples whilst the ~45-kDa band showed a wide variation among biological/technical replicates (Fig. 1b). α-STk2 detected a different pattern of Ankk1 isoforms in the murine brain (Fig. 1c). The ~56–58 kDa band, which was similar to that detected with α-STk, was expressed at very low levels. In addition, we observed other bands of ~84- and ~92-kDa (Fig. 1c) which may correspond to the predicted longest Ankk1 isoform [RIP kinase and ankyrin repeat domains (Hoenicka et al. 2010)] and Ankk1 protein modified by post-translational mechanisms, respectively. One-way ANOVA showed a significant interaction between the predicted longest Ankk1 isoform and dopaminergic treatments ($F = 5.701$; $P < 0.0005$). The pairwise comparison of striatum and PFC tissues for each specific treatment revealed significant differences only in the striatum samples. Specifically, SKF38393 and quinelorane had no effect upon Ankk1 (*t*: 0.54, d*f*: 2, $P = 0.643$; *t*: 0.286, d*f*: 8, $P = 0.782$, respectively) whilst mice treatment with the D2R-like agonist 7-OH-DPAT caused a significant increment of Ankk1 in the striatum (*t*: 2.718, d*f*: 7, $P = 0.03$) (Fig. 1c). The increment of Ankk1 after 7-OH-DPAT treatment was observed in all biological/technical replicates.

The study of Ankk1 protein profile after dopaminergic treatments in murine brain revealed a lack of direct correlation between mRNA and protein levels after the treatments. D1R stimulation with SKF38393, that significantly increases *Ankk1* mRNA levels, has no effect upon the protein. On the other hand, D2R-like stimulation with 7-OH-DPAT causes an inverse *Ankk1* mRNA/protein correlation in the striatum. There are a number of factors that could affect mRNA/protein correlations in this study. First of all it is possible that *Ankk1* changes probably occur at the mRNA level but not at the protein level, within the 1 h sampling time. When a pharmacological treatment triggers transcriptional changes (upregulation or downregulation), it is more likely that the same treatment triggers concordant responses for translational regulation. Longer and/or chronic treatments could allow the detection of the dopaminergic effect upon Ankk1 protein levels as well. However, we found a significant increment of Ankk1 protein in the striatum but not in the PFC after 7-OH-

DPAT treatment. Therefore, it would be also helpful to analyze the *Ankk1* gene expression in different brain areas after treatment. Important differences in trafficking and intracellular sorting of different DR subtypes may also help to explain our observation, for instance, it has been reported that after specific agonist treatment D1R-like levels are unchanged whilst some D2R-like levels are increased (Bartlett et al. 2005; Kim et al. 2001).

Our results showing opposite regulation of *Ankk1* mRNA expression after D1R- and D2R-like activation would connect the dopamine action, the PLC-calcium signaling, the *Ankk1* gene, and the development of addiction. For instance, the blockade of D1R-like receptors attenuates context-induced renewal of pavlovian-conditioned alcohol-seeking in rats (Sciascia et al. 2014). The D2R-like agonist 7-OH-DPAT, that causes *Ankk1* inhibition, has been associated with an inhibitory effect on the mesocorticolimbic dopaminergic system (Devoto et al. 1995) and with attenuation of the rewarding effects of cocaine and cocaine-seeking behavior (Pilla et al. 1999). A pattern of opposite regulation enhanced by D1R-like and inhibited by D2R-like has also been found, after cocaine administration, in the striatum for the extracellular-signal regulated kinases 1 and 2 (ERK1 and ERK2) (Zhang et al. 2004) as well as in the structural remodeling of dendrites and spines (Zhang et al. 2012).

In conclusion, we found in the adult mouse brain that *Ankk1* is oppositely regulated by D1R- and D2R-like DR. This broader scenario proposes caution when considering the straightforward reasoning linking *Taq*IA-associated phenotypes only with D2R variation regardless the co-expression of different DR subtypes and the ANKK1 protein in brain cells. Our work provides for the first time an evidence of the association between expression of *Ankk1* and the dopaminergic system functioning in the brain.

Acknowledgments We very much appreciate Arantxa Martínez Férriz for her technical assistance. This study was supported by the Fondo de Investigación Sanitaria (FIS), Instituto Salud Carlos III, and Grant No. 011/00737. The CIBERSAM is an initiative of the Instituto de Salud Carlos III. Guillermo Ponce has received research support from Otsuka Pharmaceutical Co, LTD. FIS, and Otsuka Pharmaceutical had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflict of interest.

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