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Differentially Expressed Genes in *Period 2*-Overexpressing Mice Striatum May Underlie Their Lower Sensitivity to Methamphetamine Addiction-Like Behavior

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Abstract

Previous reports have demonstrated that genetic mechanisms greatly mediate responses to drugs of abuse, including methamphetamine (METH). The circadian gene Period 2 (*Per2*) has been previously associated with differential responses towards METH in mice. While the behavioral consequences of eliminating *Per2* have been illustrated previously, *Per2* overexpression has not yet been comprehensively described; although, *Per2*-overexpressing (*Per2* OE) mice previously showed reduced sensitivity towards METH-induced addiction-like behaviors. To further elucidate this distinct behavior of *Per2* OE mice to METH, we identified possible candidate biomarkers by determining striatal differentially expressed genes (DEGs) in both drug-naïve and METH-treated *Per2* OE mice relative to wild-type (WT), through RNA sequencing. Of the several DEGs in drug naïve *Per2* OE mice, we identified six genes that were altered after repeated METH treatment in WT mice, but not in *Per2* OE mice. These results, validated by quantitative real-time polymerase chain reaction, could suggest that the identified DEGs might underlie the previously reported weaker METH-induced responses of *Per2* OE mice compared to WT. Gene network analysis also revealed that *Asic3*, *Hba-a1*, and *Rnf17* are possibly associated with *Per2* through physical interactions and predicted correlations, and might potentially participate in addiction. Inhibiting the functional protein of *Asic3* prior to METH administration resulted in the partial reduction of METH-induced conditioned place preference in WT mice, supporting a possible involvement of *Asic3* in METH-induced reward. Although encouraging further investigations, our findings suggest that these DEGs, including *Asic3*, may play significant roles in the lower sensitivity of *Per2* OE mice to METH.

Key Words: Methamphetamine, *Per2* overexpression, Gene expression, Substance abuse, Drug addiction, Conditioned place preference

INTRODUCTION

For several years, the psychostimulant methamphetamine (METH) has been one of the most commonly abused substances in the world. According to a 2017 study by the National Survey on Drug Use and Health (NSDUH), a staggering 14.7 million individuals (5.4% of the population) have used METH at least once in their lives (National Institute on Drug Abuse, 2018). Despite various drawbacks, the number of cases of METH abuse has increased over the years because of its potent short-term rewarding effects (Passaro *et al*., 2015), placing a burden on the global efforts for its regulation (Sliman *et al*., 2016). One of the challenges of METH abuse is the treatment and recovery of addicted users, which at present is still problematic for current healthcare systems worldwide (Ballester *et al*., 2016). Therefore, in-depth comprehension of the possible biological underpinnings for the development of METH addiction may be crucial for both its treatment and pre-

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vention. As with any other neurological compulsive disorder, drug addiction involves the display of behavioral alterations, including substance-seeking and uncontrollable drug intake, which are usually accompanied by changes in gene and/or protein expressions and modified neuronal plasticity (Nestler, 2000; Ducci and Goldman, 2012; Yazdani *et al*., 2015). Such modifications may lead to the manifestation and maintenance of rewarding effects after chronic exposure to abused substances, providing candidate neurobiological mechanisms for the development of addiction. Hence, endogenous biological variations in the expression of key genes contributing to addictive behavior would probably alter individual responses to addictive drugs.

The Period 2 (*Per2*) gene, aside from participating in circadian rhythm, was previously found to be potentially involved in drug addiction (Shumay *et al*., 2012; Zhao *et al*., 2014; Kim *et al*., 2019). *Per2* mutant mice exhibited greater susceptibility to the addiction-like behaviors induced by drugs of abuse, e.g., increased alcohol consumption (Spanagel *et al*., 2005). While addiction-associated behaviors are commonly attributed to changes in the mesolimbic dopamine system, *Per2* was also found to potentially influence dopaminergic proteins (McClung, 2007; Hood *et al*., 2010; Kim *et al*., 2019). Previous studies have pointed out that *Per2* expression levels affect dopamine concentration through monoamine oxidase A activity regulation (Hampp *et al*., 2008). Since striatal dopamine concentration could contribute to the development of addiction-like phenotypes, differential *Per2* expression may also have the potential to influence responses to different drugs of abuse. We previously attempted to associate *Per2* deficiency with the predisposition to display increased METH addiction (Kim *et al*., 2019, 2021), although reports on *Per2* overexpression in addiction-like phenotypes have been limited. However, in our previous study, we showed that *Per2*-overexpressing (*Per2* OE) mice possessed lower METH-induced conditioned place preference (CPP) and were less sensitized to repeated METH treatment. *Per2* OE mice also displayed weaker METH withdrawal symptoms and had generally contrasting striatal dopamine-related gene expressions and dopamine levels relative to *Per2* knockout mice. This indicates a probable role for varying *Per2* expression levels not only in the cerebral mechanism of METH addiction, but also in the mediation of genetic factors in the development of such disorders in general.

In this study, we aimed to determine transcriptional differences between *Per2* OE and wild type (WT) mice that could serve as potential genetic foundations and possible biomarkers for the weaker responses of *Per2* OE mice to METH-induced addiction-like behavior. To elucidate further, we used RNA sequencing (RNA-seq) to identify differentially expressed genes (DEGs) in mice striatum, the brain region generally implicated in METH-induced behaviors and addiction (Chavoshi *et al*., 2020). We then used quantitative real-time polymerase chain reaction (qRT-PCR) to confirm RNA-seq results. In parallel, we treated *Per2* OE and WT mice with METH once a day for 7 days to determine the transcriptional response of the identified genes to repeated METH treatment. Additionally, we investigated the potential involvement of *Asic3* in METH-induced reward by inhibiting the functional protein of *Asic3* in the CPP test.

MATERIALS AND METHODS

Animals

Male *Per2* OE and C57BL/6J mice, 8 to 12 weeks old, were used in this study. *Per2* OE mice were produced according to previously described methods (Kim *et al*., 2019). *Per2* OE mice were cross-bred with C57BL/6J mice to produce *Per2* OE pups and genotyped through gel electrophoresis using DNA extracted from 3-week-old mice tails. Primers used for polymerase chain reaction (PCR) genotyping are as follows: *Per2* forward (5'-CACGTCAAG TGACCTGCTCAA-3') and *Per2* reverse (5'-GCAGATGAACTTCAGGGTCAG-3'). C57BL/6J mice served as the WT group since *Per2* OE mice possessed a C57BL/6J background and were acquired from Hanlim Laboratory Animals Co (Hwaseong, Korea). All mice were housed (3-6 per cage) in a condition-controlled animal room (12 h/12 h light/dark cycle, 7:00-19:00, and 22 ± 2°C) with food and water *ad libitum*. Sufficient efforts were done to minimize the stress experienced by all animals during experiments. Animal treatment and maintenance were performed in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23 revised 1985), and the Animal Care and Use Guidelines of Sahmyook University, Seoul, Korea (SYUIACUC2020-007).

Drugs

METH hydrochloride was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and APETx2 was from Tocris Bioscience (Bristol, UK). Both were dissolved in physiological saline (SAL) and delivered intraperitoneally at the doses of 0.5 mg/kg (METH) or 0.02 mg/kg (APETx2). The doses are based on previous studies demonstrating the addiction-like behavior-inducing effects of METH (Kim *et al*., 2019, 2021) and the moderate ASIC3-inhibiting effect of APETx2 (Andreev *et al*., 2018). Treatments for molecular experiments were performed at zeitgeber (ZT) 4-5, where ZT 0 and 12 are lights on and off, respectively, based on previous studies (Abarca *et al*., 2002; Hood *et al*., 2010).

RNA sequencing

Drug-naïve mice and METH-treated (7 days) mice (3 per group) were sacrificed at ZT 4-5. Their brains were extracted, the striatum was isolated using mouse brain matrix, and samples were stored at –80°C before further processing. RNA-seq libraries were constructed using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) and subjected to 100 nt paired-end sequencing using Illumina NovaSeq 6000 (Illumina). Read quality and possible contamination were examined Agilent Technologies 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The raw reads were aligned to *Mus musculus* genome using HISAT v2.1.0 (https://github.com/DaehwanKimLab/hisat-genotype), which is publicly available in a GitHub repository. These indexes were created using the same Burrows–Wheeler transform (BWT)/graph FM index (GFM) as Bowtie2 (Johns Hopkins University, MD, USA). The transcript assembly of recognized transcripts was then processed using StringTie v1.3.4d (Johns Hopkins University). The expression abundance of the transcript and gene were calculated as read count or FPKM value (fragments per kilobase of exon per million fragments mapped) per sample. The expression profiles were used for additional analyses, such as those of DEGs. In differently conditioned groups, DEGs or transcripts were filtered using statistical hypothesis testing.

Tissue collection and RNA preparation

Per2 OE and WT mice treated with SAL or METH for 7 days (5-6 mice per group) were euthanized and decapitated at ZT 4-5 for brain extraction 30 min after the last treatment. The striatum was isolated using a mouse brain matrix on ice and was quickly frozen at –80°C before further processing. Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was further processed using Hybrid-R™ Kit (Geneall Biotechnology, Seoul, Korea). RNA concentrations were measured with Colibri Microvolume Spectrometer (Titertek-Berthold, Pforzheim, Germany).

Confirmatory qRT-PCR analyses of selected DEGs

qRT-PCR was performed to validate the RNA-seq data of the selected DEGs. Briefly, 1 μg of total RNA was reverse-transcribed into cDNA using AccuPower® CycleScript RT PreMix (Bioneer, Seoul, Korea) following the manufacturer's protocol, and aliquots were stored at –20°C. The cDNA was amplified using custom sequence-specific primers (Cosmogenetech, Seoul, Korea) and detected using SYBR® Green (Solgent, Daejeon, Korea). The primer sequences of the selected DEGs are shown in Supplementary Table 1. The input concentration for cDNA synthesis was 2.5 μg/μL. The synthesis conditions were as follows: 94°C for 1 min (denaturing step), followed by annealing at primer-specific temperature for 1 min, and then 72°C for 45 s. Triplicate samples were done during qRT-PCR analyses. Values were normalized relative to *Gapdh*. The results were shown as relative expression levels calculated through the $2^{-\Delta\Delta CT}$ method (VanGuilder *et al*., 2008).

Inhibition of ASIC3 in CPP

Apparatus: The CPP apparatus is comprised of two compartments, each measuring 17.4×12.7×12.7 cm^3, and separated by a removable guillotine door. One compartment had black walls and smooth white flooring, while the other had black walls with white dots and textured white flooring. An illumination of 12 lux was maintained all throughout the experiment. An automated system (Ethovison, Noldus, Netherlands) was used for recording animal movements and stay duration.

Procedure: This method was based on previous studies with some modifications (Kim *et al*., 2019; Custodio *et al*., 2020; Sayson *et al*., 2020). The test consisted of three phases: (A) habituation (days 1-3) and pre-conditioning (day 4; 15 min), (B) conditioning (days 5-12; 30 min), and (C) post-conditioning (day 13; 15 min). Mice were allowed to freely explore the entire apparatus during the habituation phase. Afterwards, an initial trial (pre-conditioning) was used to determine the preferred compartment of each mouse. Their non-preferred side (the compartment with the lower stay duration of a mouse) was designated as the drug-paired compartment. Eight mice were assigned for each treatment group. During the conditioning phase, mice were pretreated with either APETx2 or SAL 30 min prior to each METH or SAL treatment before being placed in the drug-paired compartment. On alternate days, mice received SAL and were confined to the SAL-paired compartment. Finally, during post-conditioning phase, mice were drug-free and allowed to explore both compartments, similar to the pre-conditioning phase. CPP score was calculated as follows: post-conditioning phase (time spent) – pre-conditioning phase (time spent). On the post-conditioning phase, mice brains were extracted right after video recording. Striatal *Asic3* and *Per2* expression levels were subsequently determined through qRT-PCR (5 mice per group).

Statistical analysis

The relative abundances were measured in read counts using StringTie software. We performed a statistical analysis to identify DEGs using the estimates of abundances for each gene in the sample. We excluded genes with more than one "zero" read count value. The filtered data were log2-transformed and subjected to RLE normalization. Statistical significance of the differential expression data was determined by the nbinomWaldTest using DESeq2 and fold change (FC). The null hypothesis was that no difference exists among the groups. The false discovery rate (FDR) was regulated by adjusting the *p* values using the Benjamini-Hochberg algorithm. For the DEG set, hierarchical clustering analysis was performed using complete linkage and Euclidean distance as a similarity measure. Gene enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were also performed based on Gene Ontology (GO, http://geneontology.org/), and KEGG pathway (https://www.genome.jp/kegg/) databases, respectively. We entered the identified DEGs in GeneMANIA (https://genemania.org/) to predict possible gene associations and functions which could support our RNA-seq results. Data are expressed as mean ± SEM and analyzed using two-way or one-way analysis of variance (ANOVA), with Tukey's Post-hoc test. Treatment was considered as the between-subject factor and strain or treatment group was the within-subject factor. Statistical analyses were performed using GraphPad Prism v8 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p<0.05$.

RESULTS

Striatal DEGs in drug naïve *Per2* OE mice vs. WT

RNA-seq analyses indicated that 138 genes were differentially expressed in the striatum of drug-naïve *Per2* OE mice compared to WT mice with |FC|≥2 ($p\leq0.05$) (Fig. 1). Using GO

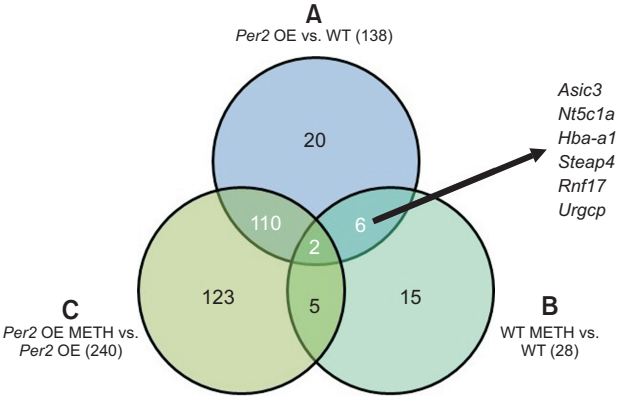

Fig. 1. Common modified genes found from RNA-sequencing. Venn diagram showing the number of (A) differentially expressed genes (DEGs) in the striatum of *Per2* OE mice compared to WT (drug-naïve) mice, (B) altered genes in WT mice after repeated METH treatment, and (C) altered genes in *Per2* OE mice after repeated METH treatment. Arrow indicates the specific genes corresponding to the number.

Table 1. Enrichment analysis of DEGs between *Per2* OE mice and WT mice (drug-naïve)

Biological process		Molecular function		Cellular component	
Top 10 GO Functional Terms	*p*-value	Top 10 GO Functional Terms	*p*-value	Top 10 GO Functional Terms	*p*-value
Regulation of biological quality	<0.001	Ion channel activity	<0.001	Postsynaptic membrane	<0.001
Ion transport		Passive transmembrane transporter activity		Integral component of plasma membrane	
Localization		Channel activity		Synaptic membrane	
Metal ion transport		Ion gated channel activity		Intrinsic component of plasma membrane	
Potassium ion transport		Gated channel activity		Plasma membrane part	
Ion transmembrane transport	<0.01	Voltage-gated cation channel activity		Synapse	
Intracellular signal transduction		Cation channel activity		Postsynapse	
regulation of membrane potential		Potassium ion transmembrane transporter activity		Plasma membrane region	
Second-messenger-mediated signaling		Voltage-gated ion channel activity		Presynaptic membrane	
Inorganic ion transmembrane transport		Binding		Integral component of presynaptic membrane	

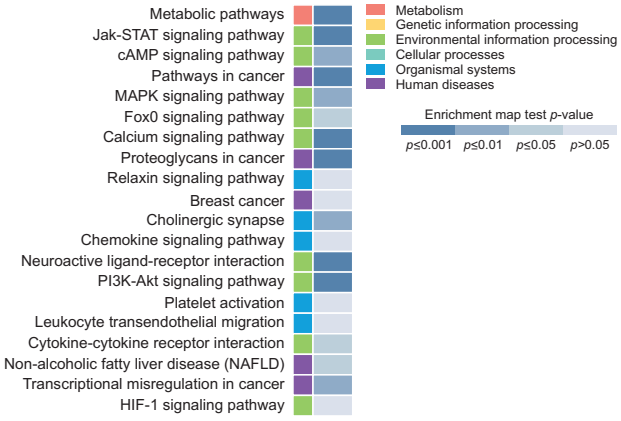

Fig. 2. Heat map of KEGG pathways. Significant pathways for DEGs in drug naïve *Per2* OE mice.

analysis, the top 10 enrichment terms related to these DEGs were identified (Table 1). Enrichments were highly related to the regulation of biological quality, ion transport, localization, intracellular signal transduction, binding, ion channel activity, plasma membrane, and synapse. KEGG analysis (Fig. 2) also revealed that these DEGs were significantly associated with metabolic, Jak-STAT signaling, cancer, calcium signaling, and PI3K-Akt signaling pathways; proteoglycans in cancer; and neuroactive ligand-receptor interaction ($p \leq 0.001$).

DEGs in *Per2* OE mice remained unchanged after repeated METH treatment

As shown in Fig. 1, among 138 DEGs, 118 genes were modified in *Per2* OE and/or WT mice after repeated METH treatment. From these, we determined 6 common DEGs between drug-naïve *Per2* OE/WT and METH-treated WT/WT (Table 2) with |FC|≥2. *Asic3* and *Hba-a1* were significantly downregulated, while *Nt5c1a*, *Steap4*, *Rnf17*, and *Urgcp* were significantly upregulated in *Per2* OE mice compared to WT mice. After repeated METH treatment of both *Per2* OE and WT mice, these genes were modified in WT mice, but not in *Per2* OE mice. *Asic3*, *Nt5c1a*, *Hba-a1*, and *Steap4* were downregulated, whereas *Rnf17* and *Urgcp* were upregulated. We used qRT-PCR to validate the differential expression of these DEGs. *Asic3*: interaction ($F_{1,16}$=8.16, p<0.05); strain ($F_{1,16}$=19.2, p<0.001). *Nt5c1a*: interaction ($F_{1,16}$=11.5, p<0.01); strain ($F_{1,16}$=13.7, p<0.01). *Hba-a1*: interaction ($F_{1,16}$=7.21, p<0.05); strain ($F_{1,16}$=21.4, p<0.001). *Steap4*: treatment ($F_{1,16}$=4.60, p<0.05); strain ($F_{1,16}$=29.2, p<0.001). *Rnf17*: strain ($F_{1,16}$=7.60, p<0.05). *Urgcp*: treatment ($F_{1,16}$=5.66, p<0.05); strain ($F_{1,16}$=4.96, p<0.05). Relative gene expression results corresponded to our RNA-seq data, except for *Nt5c1a*, which exhibited lower expression in *Per2* OE mice than in WT mice, and a lack of significant difference in *Steap4* and *Rnf17* expression after repeated METH administration in WT mice (Fig. 3).

Gene network analysis of identified DEGs

GeneMANIA results (Fig. 4) identified that the six genes were mostly associated with circadian rhythm and ion transport. Only *Hba-a1*, *Asic3*, and *Rnf17* displayed up to two degrees of association with *Per2*.

Table 2. Common differentially expressed genes (A ∩ B-C)

Gene ID	Transcript ID	Gene titles	Gene symbols	Mean fold change (*Per2* OE vs. WT)	Mean fold change (WT METH vs. WT)
171209	NM_001310474, NM_183000	Acid-sensing (proton-gated) ion channel 3	*Asic3*	–2.16	–2.05
230718	NM_001085502	5'-nucleotidase, cytosolic IA	*Nt5c1a*	2.73	–2.04
15122	NM_008218, NM_008218_dup1	Hemoglobin alpha, adult chain 1	*Hba-a1*	–17.52	–14.89
117167	NM_054098	STEAP family member 4	*Steap4*	2.52	–3.21
30054	NM_001033043	Ring finger protein 17	*Rnf17*	3.58	2.41
72046	NM_001077661, NM_178623	Upregulator of cell proliferation	*Urgcp*	2.11	2.09

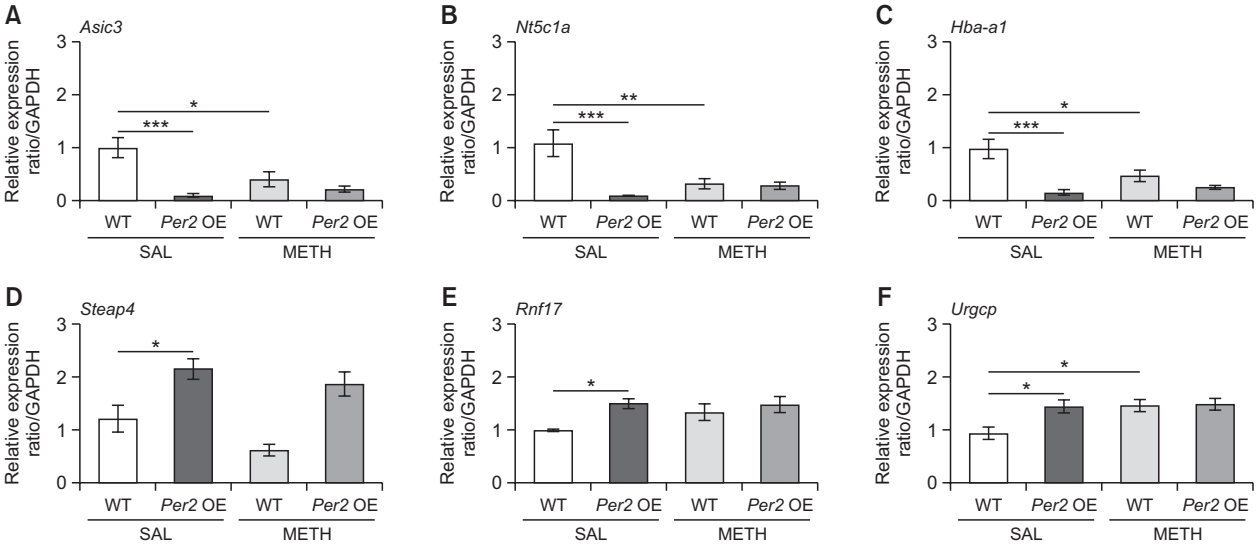

Fig. 3. METH treatment-altered genes in WT but not in *Per2* OE mice. qRT PCR validated the expressions of (A) *Asic3*, (B) *Nt5c1a*, (C) *Hba-a1*, (D) *Steap4*, (E) *Rnf17*, and (F) *Urgcp* in the striatum of *Per2* OE and WT mice before and after repeated METH treatments. n=5. *p<0.05, **p<0.01, ***p<0.001 (Tukey's Post-hoc analysis).

APETx2 pretreatment partially reduced METH-induced CPP in WT mice

Fig. 5A illustrates the place preference of mice pretreated with SAL or APETx2 after METH conditioning. One-way ANOVA showed significant variation among groups ($F_{3,28}$=7.72, p<0.001). Although, Tukey's post-hoc analysis revealed that APETx2 pretreatment during conditioning only partially reduced METH-induced CPP and did not reach statistical significance (p=0.163). Only SAL-pretreated mice showed significantly higher METH-induced CPP compared to SAL-pretreated/treated group (p<0.001). In Fig. 5B, one-way ANOVA revealed significant differences in the expression of striatal *Asic3* among the treatment groups ($F_{3,16}$=5.32, p<0.01). Using Tukey's post-hoc analysis, the results exhibited that WT mice pretreated with APETx2 possessed lower *Asic3* expression than SAL-pretreated mice, with no significant difference in *Asic3* expression between APETx2-pretreated WT mice treated with SAL or METH. One-way ANOVA in Fig. 5C showed no significant differences among the groups regarding *Per2* expression level.

DISCUSSION

Our study determined 138 DEGs in drug-naïve *Per2* OE mice from RNA-seq. We hypothesized that some of these genes might participate in the previously reported behavior of *Per2* OE mice to METH relative to WT mice (Kim *et al*., 2019), given that pertinent genetic differences may potentially result to varied responses to drug treatments (Palmer *et al*., 2005; Hitzemann *et al*., 2019). Our results may suggest that some of the 138 DEGs in drug-naïve *Per2* OE mice might be associated with drug-induced addiction-related behaviors. Among them, 118 genes were altered following repeated METH exposure, thus we could assume the involvement of these genes in METH-induced behavioral changes. Interestingly, six DEGs were only modified in WT mice after repeated METH treatment, and three of them, namely *Asic3*, *Hba-a1*, and *Rnf17*, displayed association with *Per2*. Hence, this may suggest a potential involvement for the three DEGs in the different sensitivity of *Per2* OE mice to METH.

The acid-sensing (proton-gated) ion channel (ASIC) 3 (*Asic3*) gene encodes voltage-independent sodium channels

that respond to changes in extracellular pH levels. A previous study (Kreple *et al*., 2014) has correlated ASICs with drug addiction wherein the elimination of the ASIC1a-subtype was associated with the drug-induced plasticity proliferation. Interestingly, ASICs interact with sigma-1 receptors and influence Ca^{2+} influx and calcium homeostasis, which contributes to the development of psychostimulant abuse (Soriani and Kourrich, 2019). Despite the lack of identified associations between *Asic3* and addiction, several studies have mentioned other subtypes of ASICs to be involved in stimulant addiction, which could provide *Asic3* a possible role in METH addiction.

Moreoever, *Hba-a1* encodes hemoglobin alpha, adult chain 1 and was upregulated in various brain regions of stressed mice (Stankiewicz *et al*., 2015); however, it was also downregulated in rats in a separate chronic stress study (Andrus *et al*., 2012). While this gene was extensively associated with alcohol addiction (Kerns, 2005; Mulligan *et al*., 2011), our study suggests that it may also participate in METH-induced behavior. Interestingly, one clinical study (Tavasolian *et al*., 2015) showed that METH addicts possessed lower levels of hemoglobin than healthy individuals. In contrast, microarray analysis of MDMA-treated mice showed an almost 3-fold increase in *Hba-a1* expression in the striatum (Salzmann *et al*., 2006). Intriguingly, one study also demonstrated a dysregulation of both hepatic *Per2* and *Hba-a1* after experimental chronic jet lag in mice (Wu *et al*., 2012), providing a possible link between the two genes. The reduced *Hba-a1* expression in WT and *Per2* OE mice after repeated METH administration coincides with a previous study showing a downregulated *Hba-a1* expression in mice nucleus accumbens as a putative target for METH-responsive miRNAs (Zhu *et al*., 2016), possibly supporting its role in METH-induced behavior.

The protein encoded by the RING finger protein 17 (*Rnf17*) gene contains zinc-binding motifs that interact with macrobiomolecules and play a role in ubiquitination (Joazeiro and Weissman, 2000). One study showed that a downregulated form of ring finger protein (*Rnf5*) was involved in METH-induced inflammatory bowel disease (Sun *et al*., 2020), which is associated with METH abuse.

Together with these information, qRT-PCR results confirmed the downregulation of *Asic3* and *Hba-a1* and the upregulation of *Rnf17* in the striatum of drug-naïve *Per2* OE mice relative to WT in our RNA-seq results. It was also revealed that these genes remained unaltered even after repeated METH treatment in *Per2* OE mice. These observations may propose two assumptions: (1) repeated METH administration could result in the downregulation of *Asic3* and *Hba-a1* and the upregulation of *Rnf17*, providing them putative roles in METH-induced addiction-like phenotypes, and (2) the inherent downregulation or upregulation of these genes in *Per2* OE mice could probably contribute to their decreased sensitivity to METH-induced effects, since repeated METH administration induced no significant changes in their expressions.

Gene network analysis showed that only *Hba-a1*, *Rnf17*, and *Asic3* were potentially associated with *Per2* through physical interactions and predicted correlations. The lack of potential genetic interactions of *Per2* with *Nt5c1a*, *Steap4*, and *Urgcp*, along with the scarcity of relevant studies pertaining to those genes influencing METH-induced behaviors and addiction, may imply that they could influence other behavioral mechanisms in *Per2* OE mice apart from METH addiction sensitivity. Nevertheless, we selected *Asic3* as an initial gene of

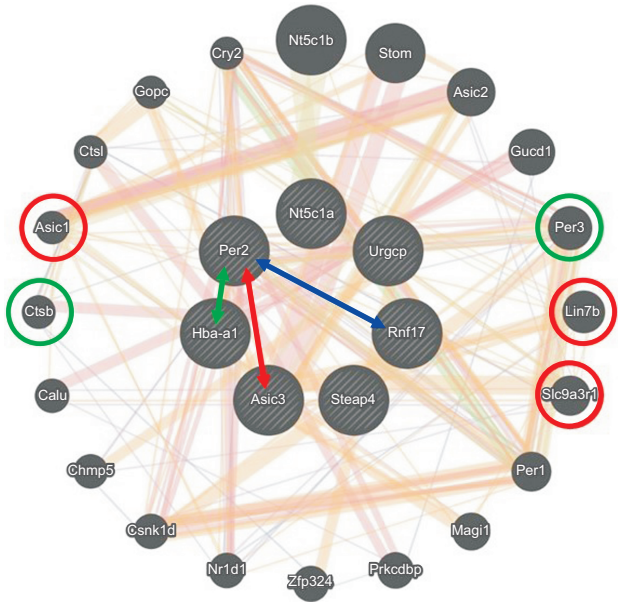

Fig. 4. Gene network analyses. Using GeneMANIA, *Asic3* was shown to be associated with *Per2* through physical interactions and predicted correlations with *Asic1*, *Lin7b*, and *Slc9a3r1*. *Hba-a1* was shown to be associated with *Per2* through physical interactions and predicted correlations with *Ctsb* and *Per3*, while *Rnf17* was associated directly with *Per2*.

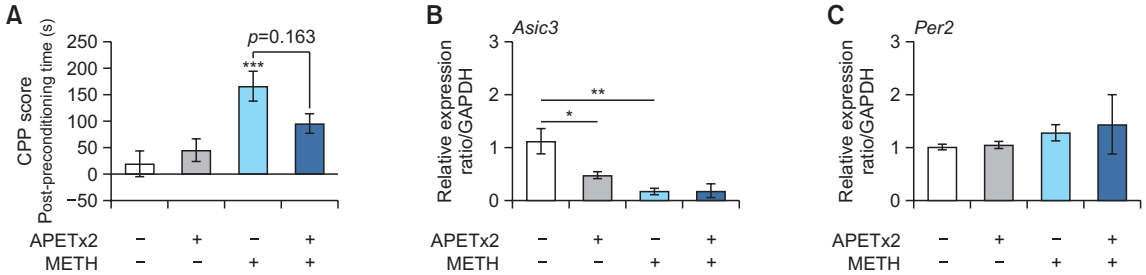

Fig. 5. Possible involvement of *Asic3* in METH-induced reward in WT. WT mice were pre-treated with SAL or APETx2 (ASIC3-selective inhibitor, 0.02 mg/kg, i.p.) 30 min. prior to SAL or METH (0.5 mg/kg, i.p.) treatment. (A) Place preference of WT mice after METH conditioning sessions. n=8. Relative expressions of (B) *Asic3* and (C) *Per2* in WT mice after post-conditioning. n=5. *p<0.05, **p<0.01 and ***p<0.001 (vs. saline pretreated/treated; Turkey's Post-hoc analysis).

interest for further investigation and verified its involvement in METH-induced reward. In Fig. 5A, WT mice pretreated with APETx2, a peptide isolated from sea anemone venom known to inhibit ASIC3 (Andreev *et al*., 2018), before METH conditioning sessions exhibited a decreased trend in CPP score relative to SAL-pretreated mice, suggesting a moderate attenuation of METH-induced rewarding effect. The lack of significant difference may be because ASIC3 inhibition alone may only provide a contributing role in METH-induced reward and involve other undetermined signaling pathways that can possibly lead to significant changes in addiction-like behaviors. Nevertheless, this result could suggest that ASIC3 signaling might still play a role in the mediation of METH-induced behaviors. Fig. 5B also seemed to replicate the expression pattern of *Asic3* in *Per2* OE mice following repeated METH treatment (Fig. 3A), which could potentially support our assumption that an initial downregulation of striatal *Asic3* expression prior to METH exposure may contribute to weaker METH-induced addiction-like behaviors and might be accompanied by an unaltered *Asic3* expression even after METH exposure. The effect of ASIC3 antagonism in METH-induced reward therefore raises the question whether ASIC3 signaling also influences dopaminergic neurotransmission, given that METH addiction development is said to involve extracellular dopamine elevation in presynaptic terminals due to its ability to inhibit vesicular monoamine transporter 2 and reverse the function of dopamine transporters, even though functional ASIC3 receptors are more expressed in the peripheral nervous system (Li and Xu, 2011). However, one study (Wu *et al*., 2019) did demonstrate that *Asic3* knockout mice possessed elevated dopamine turnover rate and pERK activation in the striatum, suggesting a possible relationship between ASIC3 and dopaminergic signaling. Furthermore, these transgenic mice also exhibited decreased dendritic spine density of medium spiny neurons in their striatum. Interestingly, a previous report (Jedynak *et al*., 2007) presented that repeated METH administration was able to lower spine density in the dorsomedial striatum of rats. This may suggest a mutual link between the reduction of *Asic3* expression and repeated METH treatment through possible modifications in spine density of striatal medium spiny neurons. Additionally, deletion of other ASIC subtypes (1a and 2) also diversely modulated chronic cocaine-induced locomotor activity (Jiang *et al*., 2013), while *Asic1a* knockout in mice nucleus accumbens increased cocaine-induced CPP (Kreple *et al*., 2014). Along with our current findings, these previous reports further support the potential of ASICs in influencing psychostimulant-induced behavior and sensitivity, although defining the exact mechanism requires further investigations. We also evaluated the *Per2* expression level in mice post CPP to determine a potential co-expressing interaction between *Asic3* and *Per2*. However, there were no significant differences in *Per2* expression level among the groups, unlike in previous reports showing that METH-exposed mice displayed upregulated *Per2* expressions (Nikaido *et al*., 2001; Yamamoto *et al*., 2005; Piechota *et al*., 2012). This could perhaps suggest that *Per2* may mediate *Asic3* expression, as exhibited by its downregulation in *Per2* OE mice, but may be independent of varying *Asic3* expressions, since it remained unaltered in APETx2-pretreated mice. Given these findings, it is imperative to further investigate the specific mechanistic links between *Asic3* and METH-induced responses, accompanied by further assessing the participation of *Hba-a1* and *Rnf17*.

In summary, we demonstrated that *Per2* overexpression in mice may lead to striatal DEGs that might potentially be involved in the mechanism of METH addiction. We were able to identify DEGs in *Per2* OE mice that were not modified by repeated METH administration, unlike in WT mice. This may suggest that these genes, *Asic3*, *Hba-a1*, and *Rnf17*, could possibly contribute to the lower sensitivity of *Per2* OE mice to METH compared to WT mice. We have also confirmed that ASIC3 antagonism resulted to a partial attenuation of METH-induced CPP in WT mice, suggesting that varied striatal *Asic3* expressions might potentially be one of the contributing factors leading to diverging METH sensitivities. Taken together, the identified DEGs, including *Asic3*, might potentially underlie the weaker sensitivity of *Per2* OE mice to METH addiction.

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