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Remodeling of ion channel expression may contribute to electrophysiological consequences caused by methamphetamine in vitro and in vivo

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ABSTRACT

Methamphetamine (MA) is a psychostimulant. MA may induce numerous cardiotoxic effects, leading to cardiac arrhythmias, heart failure, eventually leading to sudden cardiac death. The deleterious effects of methamphetamine work in tandem to disrupt the coordinated electrical activity of the heart and have been associated with life-threatening cardiac arrhythmias. Remodeling of ion channels is an important mechanism of arrhythmia. Although arrhythmogenic remodeling involves alterations in ion channel expression, it is yet unknown whether MA induced electrical remodeling by affecting gene expression, and whether the changes in protein expression are paralleled by alterations in mRNA expression. Our study focused on the expression of ion channels which were correlated to the electrical remodeling caused by MA. We used RT-PCR and western blot to assess of the transcript and translate levels of ion channel subunits, including I_{to}: kv1.4, kv1.7, kv3.4, kv4.2; I_{K1}: kir2.1, kir2.2, kir2.3, kir2.4; and $I_{Ca\text{-}L}$: $Ca^{2+}\alpha 1$, $Ca^{2+}\beta$, respectively. The reversible effect of these changes after MA withdrawal was also evaluated. MA caused decrease in mRNA and protein levels in all ion channel subunits in vitro and also in vivo, is at this work. The kv3.4 and all 4 subunits of Kir2.0 family showed significant decrease than the other genes. Most of the channel subunit expression started to reverse after MA withdrawal for 4 weeks and significantly reverse in all of the channel subunits after MA withdrawal for 8 weeks. We found that CACNA1C and Kir2.0 family showed lower recoverability than the others after MA withdrawal for 8 weeks. The reduction of the ion channel expression levels may be the molecular mechanism that mediates the electrical remodeling caused by methamphetamine.

1. Introduction

Methamphetamine (MA) is known as amphetamine class of psychoactive drugs. MA is a prevalent substance for abuse, especially in adolescents to pursue a sense of euphoria, resulting in psychologic and physiologic drug dependence. MA targeted towards the central nervous system is associated with numerous cardiotoxic effects. Cardiac arrhythmias and arrhythmic sudden death have been reported widely as indicators of MA toxicity [1,2]. Molecules related to Ca^{2+} homeostasis in cardiomyocytes include sarco(endo)plasmic reticulum Ca^{2+}-ATPase, phospholamban and Na^{+}–Ca^{2+} exchanger. Unchanged expression of sarco(endo)plasmic reticulum Ca^{2+}-ATPase and phospholamban, associated with upregulated expression of Na^{+}–Ca^{2+} exchanger following acute MA exposure indicated that direct cardiomyocyte contractile depression by MA possibly through protein damage and intracellular Ca^{2+} dysregulation [3]. The data indicated that disruption of intracellular Ca^{2+} homeostasis and Ca^{2+} handling proteins may lead to heart dysfunction caused by MA.

As important ionic currents involved in action potential duration, L-type calcium current ($I_{Ca\text{-}L}$), transient outward potassium current (I_{to}) and inwardly rectifying potassium current (I_{K1}) are thought to be associated with the cardiac arrhythmia induced by the psychotropic drugs [4]. Changes in a number of ionic currents have been detected in MA-treated rat ventricular myocytes and the data demonstrated that MA inhibited the $I_{Ca\text{-}L}$, I_{to} and I_{K1} concentration-dependently [5]. The finding of corrected QT interval (QTc) prolongation suggests susceptibility of the methamphetamine user to ventricular arrhythmia, most notably torsades de pointes [6].

Abbreviations: MA, methamphetamine; NRVMs, neonatal rat ventricular myocytes; $I_{Ca\text{-}L}$, L-type calcium current; I_{to}, transient outward potassium current; I_{K1}, inwardly rectifying potassium current; QTc, corrected QT interval.

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These electrophysiological changes may be attributed to the effects of MA on cardiac ion channels. However, the expression changes in cardiac ion channels induced by MA (specifically Ca^{2+} and K^+ channels) have not been demonstrated. Therefore, the purpose of this study was to analyze the expression status of these ion channels in vivo and in vitro by MA exposure. The reversible effect of expression status changes after MA withdrawal was also evaluated. We examined the hypothesis that the electrical remodeling caused by MA is linked to altered expression of cardiac ion channels.

2. Materials and methods

2.1. Cell culture

Neonatal rat ventricular myocytes (NRVMs) were prepared from 0 to 1-day-old neonatal Sprague–Dawley rats (Experimental Animal Center of SMU, China). Rats were sacrificed by immersion in 75% alcohol. Ventricles were removed and washed in Hank's solution, then minced and incubated with 0.25% trypsinase at 4 °C for 12–16 h. Addition of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was used to terminate digestion for 5 min at 37 °C. The supernatant was discarded. 25 ml Hank's solution was supplemented with 25 mg collagenase type II and 125 mg bovine serum albumin as digestive solution. Digestive solution was added and placed in a water bath for 1 min at 37 °C. The supernatant was discarded. Then, fresh digestive solution was added and placed in a water bath on the top of a hot plate stirrer stirring the tissue fragments with a magnetic bar for 15 min at 37 °C and the supernatant was collected. The latter digestion step was repeated 4 times. Cells in the supernatant were isolated by centrifugation for 10 min at 2000 rpm at room temperature. To reduce fibroblast contamination, cells re-suspended in NRVMs culture medium were pre-plated for 1 h. Again, the supernatant was aspirated gently, and cells were plated in six-well plates. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 through the experiments.

2.2. Animal protocols

A total of 80 Sprague–Dawley male rats, weighting from 200 to 220 g, were purchased from The Laboratory Animal Center of Southern Medical University, China. Rats were raised per cage with alternating light–dark cycle (lighting 14 h per day at 25 °C room temperature. Food and water were provided ad libitum. Rats were habituated to the animal facilities for 7 days before use. 40 rats were randomized into MA-injected group, each of which was injected intraperitoneally with MA in 1 ml of saline (0.9%) at the dose of 5 mg per kg body weight twice daily (at 12 h intervals) for 1 week. 8 rats were killed 24 h after the last injection of MA and the hearts were quickly removed. The ventricles were dissected on an ice-cold glass plate, rapidly frozen in liquid N_2. The remaining rats were subdivided into four groups and sacrificed after 1, 2, 4 and 8 weeks. The samples were collected in the same manner. 40 rats were randomized into saline group, 1 ml of 0.9% normal saline injected into each rat twice daily in the same way as with the MA-injected group.

2.3. RNA extraction and real-time PCR

Total RNA was extracted using RNAiso Plus (Takara Biotechnology, Japan) according to the manufacturer's instructions. The concentration and OD260/OD280 value of total RNA were measured by Beckman Coulter DU 520 UV/Vis spectrophotometer (Beckman Coulter, USA). Reverse transcription was carried out starting from 1 μg of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara Biotechnology, Japan) and excluded any potential genomic DNA contamination. The mRNA levels were measured by RT-PCR with an Applied Biosystems 7500 fast real-time PCR system (ABI, USA) using SYBR Premix Ex Taq II (Takara Biotechnology, Japan). Cycling conditions were 2 min at 50 °C, 30 s at 95 °C, 40 cycles of 15 s at 95 °C and 34 s at 60 °C, and Dissociation Stage. RNA content was normalized to 18S ribosomal RNA, and relative changes in gene expression were quantified using the threshold cycle ($2^{-\Delta\Delta Ct}$) method with RQ software (ABI, USA). The primer sequences and amplicon size for the genes are shown in (Table 1).

2.4. Western blot analysis

The total protein was extracted for western blotting. Samples were homogenized in ice-cold RIPA buffer (Santa Cruz Biotechnology) containing protease inhibitors. Protein concentration of the samples was determined by a BCA protein assay kit (Thermo Fisher Scientific). Protein samples were separated by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Non-specific binding was blocked with 5% non-fat skim milk in Tris-buffered saline with Tween 20 at room temperature for 1 h. Membranes were incubated overnight at 4 °C with primary antibodies against CACNA1C (1:200; Millipore), CACNB2 (1:1000; Abcam), KCNA4 (1:200; Alomone), KCNA7 (1:1000; Novus), KCNC4 (1:200; Alomone), KCND2 (1:200; Alomone), KCNJ2 (1:5000; Abcam), KCNJ12 (1:1000; Abcam), KCNJ4 (1:1000; Abcam), KCNJ14 (1:500; Santa cruz). After being washed with TBST, membranes were reacted with horseradish peroxidase conjugated secondary antibodies (1:2000, Amersham) at room temperature for 1 h. The bound secondary antibodies were visualized with Thermo Scientific Pierce ECL Western Blotting Substrate according to the manufacturer's instructions. Signal intensities of bands were analyzed and the relative protein levels were calculated by comparison with the amount of β-actin as a loading control.

2.5. Animal care

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of the Southern Medical University (SMU, Guangzhou, China).

Table 1
Primer sequences and amplicon size of genes.

Gene	Primer sequence	Amplicon size (bp)
CACNA1C	F 5′-TCACCATTGCCTCCGAACACTA-3′ R 5′-CAGGAGCATTTCTGCCGTGA-3′	104
CACNB2	F 5′-GGACCACTGTTTCTTGCTTGT-3′ R 5′-CTGCTGACTTGGCATTAAGA-3′	237
KCNA4	F 5′-CATGACAACTGTGGGCTACGG-3′ R 5′-CGGGCAAAGCAATGGTTAAGA-3′	104
KCNA7	F 5′-AATCCTGGAGGTGCCCTGTG-3′ R 5′-GCCCTATGACGTGCTGGGTAA-3′	92
KCNC4	F 5′-CTCAGGCACACGGGACAGAA-3′ R 5′-TCTGGACACTGCCATCAGCAC-3′	89
KCND2	F 5′-ATGAGCTGACTGCCCAGGTTC-3′ R 5′-AATGACCAGGTGCAAGGTTAAACAA-3′	149
KCNJ2	F 5′-TCTTGGGAATTCTGGTTTGC-3′ R 5′-TGGGAGCCTTGTGGTTCTAC-3′	199
KCNJ12	F 5′-TCGATGTGGGCTTCGACAA-3′ R 5′-GTAGAGGGCACCTCATAGG-3′	269
KCNJ4	F 5′-CAGCTCATCAAGCCCTACA-3′ R 5′-CCTCCGACTCCAGTTCCT-3′	184
KCNJ14	F 5′-AATGGGGTGGAAACAGAAGATG-3′ R 5′-GTCTATACCATTGGCTTCTCAC-3′	378

2.6. Statistical analysis

Statistical analyses of the data were performed using SPSS16.0 software. Statistics were performed using one way ANOVA and Student's *t*-test. The results were summarized as the mean ± SD, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. MA decreases ion channel mRNA and protein expression

Expression of mRNA and protein levels of CACNA1C, CACNB2, KCNA4, KCNA7, KCNC4, KCND2, KCNJ2, KCNJ12, KCNJ4, and KCNJ14 were identified during MA administration and after MA withdrawal using RT-PCR and western blot (Figs. 1–3). We evaluated the reduction rate of gene expression and protein expression with methamphetamine exposure in vitro and in vivo (Table 2). Kir2.0 family (Kir2.1–Kir2.4) showed relatively high in reduction rate than the other genes.

3.2. Reversible change in ion channel expression after MA withdrawal

We evaluated the recovery rate of gene expression and protein expression in vivo after MA withdrawal (Table 3). Most of the ion channel expression showed no change after MA withdrawal for 1 week. The recovery rate of gene expression and protein expression of KCND2 were −43% and −47% after MA withdrawal for 1 week, and it showed −12% and no change for 2 weeks. The expression of KCND2 showed continuous decrease during the first week of MA withdrawal. During the second week of MA withdrawal, about half of the ion channel expression started to recover. Most of the channel subunit expression started to reverse after MA withdrawal for 4 weeks. CACNA1C and Kir2.0 family showed lower recoverability (recovery rate ⩽ 70%) than the others after MA withdrawal for 8 weeks.

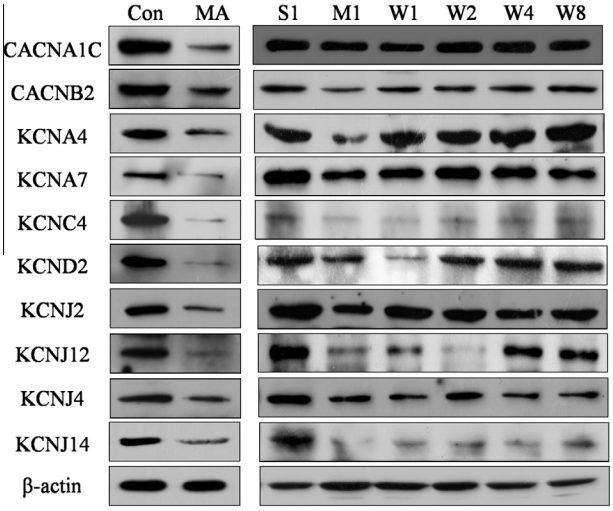

Fig. 2. Expression of protein levels were analyzed by western blot. Representative autoradiographs are shown. Con: control in NRVMs; MA: methamphetamine administration in NRVMs for 24 h. S: normal saline administration in rats; M: methamphetamine administration in rats; W: withdrawal methamphetamine administration in rats; Number: corresponding weeks.

4. Discussion

Arrhythmogenic remodeling involves alterations in ion channel expression [7]. For many ion channel subunits that are remodeled in failing hearts, changes in protein expression are paralleled by alterations in mRNA expression [8]. Our study focused on the expression of ion channels which were correlated to the electrical remodeling caused by MA in vitro and in vivo. MA had inhibitory effects on the I_{to}, I_{K1}, and $I_{Ca\text{-}L}$, in ventricular myocytes, which might be one of the possible electrophysiological mechanisms of cardiac arrhythmias caused by MA. The I_{to}, I_{K1} and $I_{Ca\text{-}L}$ were blocked by MA concentration-dependently, and the IC_{50} value of approximately

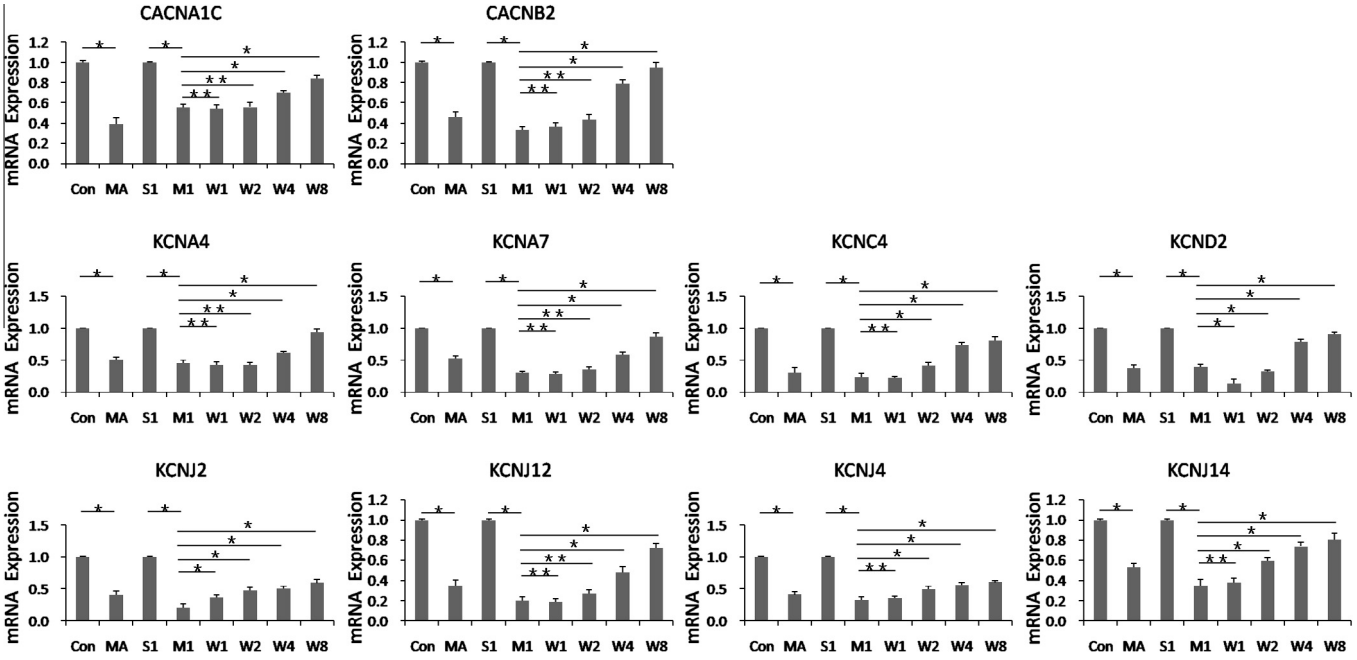

Fig. 1. Expression of mRNA levels of CACNA1C, CACNB2, KCNA4, KCNA7, KCNC4, KCND2, KCNJ2, KCNJ12, KCNJ4, and KCNJ14 during MA administration and after MA withdrawal. Con: control in NRVMs; MA: methamphetamine administration in NRVMs for 24 h. S: normal saline administration in rats; M: methamphetamine administration in rats; W: withdrawal methamphetamine administration in rats; Number: corresponding weeks. The data are represented as mean ± SD ($n = 6$, $^{*}P < 0.01$, $^{**}P > 0.05$).

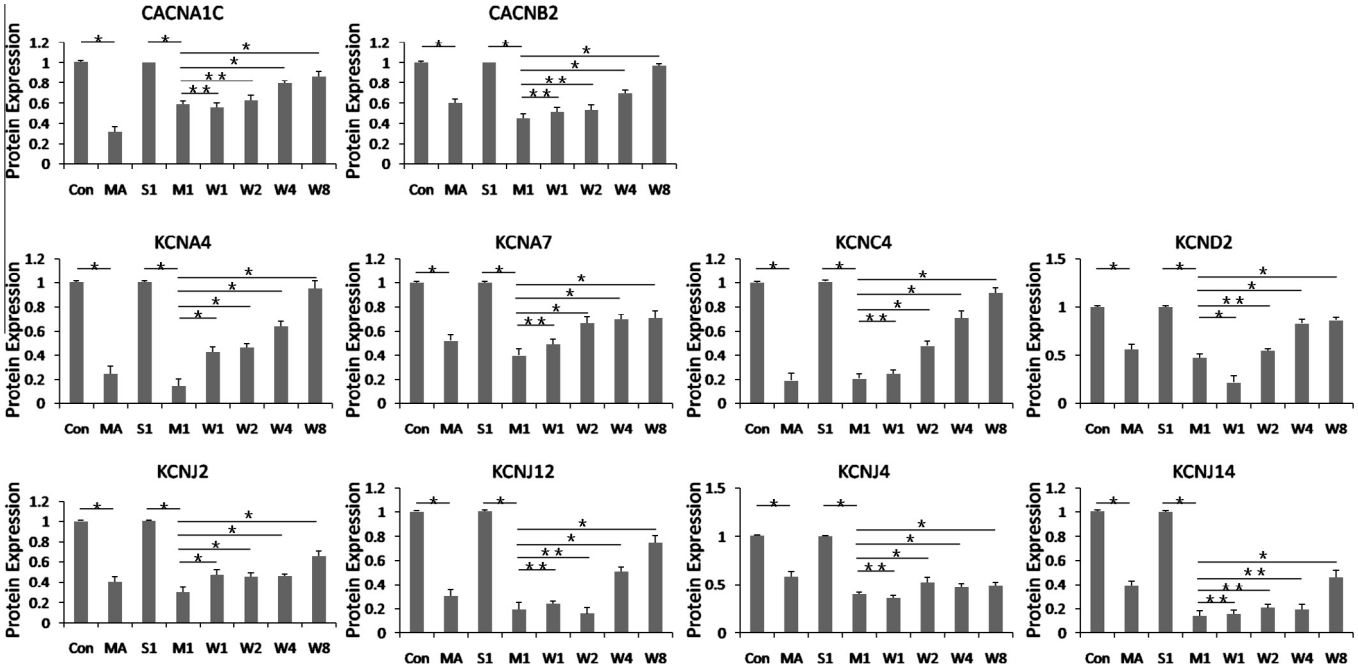

Fig. 3. Expression of protein levels of CACNA1C, CACNB2, KCNA4, KCNA7, KCNC4, KCND2, KCNJ2, KCNJ12, KCNJ4, and KCNJ14 during MA administration and after MA withdrawal. Con: control in NRVMs; MA: methamphetamine administration in NRVMs for 24 h. S: normal saline administration in rats; M: methamphetamine administration in rats; W: withdrawal methamphetamine administration in rats; Number: corresponding weeks. The data are represented as mean ± SD ($n = 6$, $^{*}P < 0.01$, $^{**}P > 0.05$).

Table 2
Reduction rate of gene expression and protein expression with methamphetamine exposure in vitro and in vivo.

Channel	Gene	In vitro		In vivo	
		mRNA (%)	Protein (%)	mRNA (%)	Protein (%)
LTCC	CACNA1C	61	69	44	41
LTCC	CACNB2	54	40	67	55
Kv1.4	KCNA4	49	76	55	86
Kv1.7	KCNA7	47	48	69	60
Kv3.4	KCNC4	69	81	77	80
Kv4.2	KCND2	63	44	60	53
Kir2.1	KCNJ2	59	60	79	70
Kir2.2	KCNJ12	65	70	80	81
Kir2.3	KCNJ4	59	42	67	60
Kir2.4	KCNJ14	47	61	65	86

Reduction rate in vitro = (Con − MA)/Con; Reduction rate in vivo = (S − M)/S.

0.5 mM for MA has been reported previously [5]. The obvious inhibitory effects could be observed in MA of 0.5 mM for the electrical remodeling. Therefore, we chose the concentration of 0.5 mM MA for our subsequent studies on ion expression in vitro. In vivo, we focus on whether there was reversible change of ion channel expressions after withdrawal MA.

Potassium channels are involved in the generation of the resting potential and repolarization of the action potential. Major potassium channels (such as I_{to}, I_{K1}) contribute to repolarization in ventricular myocardium [9,10]. I_{to} is characteristic of neurons and cardiac muscle, and its voltage-dependent activation and inactivation kinetics is much faster than that of other cardiac K^+ currents. Kv4.2 expression correlates with fast I_{to} ($I_{to,f}$), which shows fast inactivation and recovery from steady-state inactivation. Kv1.4 and Kv1.7 are thought to form the channels that mediate slow I_{to} ($I_{to,s}$), which displays a longer time course, especially of recovery from steady-state in-activation [11].

Transcriptional down-regulation underlies the molecular basis of changes in K^+ currents. Down-regulation of Kv4.3 and Kv1.4 subunits underlies I_{to} reduction in failing hearts induced by ventricular tachycardia [12,13]. Similar discrepancies exist in the study of I_{K1} unit Kir2.1 [14]. Inwardly rectifying K^+ (Kir) channels that contribute to maintaining the resting membrane potential are encoded by

Table 3
Recovery rate of gene expression and protein expression in vivo after methamphetamine withdrawal.

Channel	Gene	W1		W2		W4		W8	
		mRNA	Protein	mRNA	Protein	mRNA (%)	Protein (%)	mRNA (%)	Protein (%)
LTCC	CACNA1C	No change	No change	No change	No change	33	50	64	66
LTCC	CACNB2	No change	No change	No change	No change	68	44	93	94
Kv1.4	KCNA4	No change	33%	No change	37%	30	57	89	94
Kv1.7	KCNA7	No change	No change	No change	45%	41	50	81	52
Kv3.4	KCNC4	No change	No change	24%	34%	66	62	75	89
Kv4.2	KCND2	−43%	−47%	−12%	No change	65	67	85	74
Kir2.1	KCNJ2	20%	25%	34%	22%	38	23	49	50
Kir2.2	KCNJ12	No change	No change	No change	No change	35	39	66	68
Kir2.3	KCNJ4	No change	No change	26%	21%	36	12	42	16
Kir2.4	KCNJ14	No change	No change	38%	No change	60	No change	70	37

Recovery rate = (W − M)/(S − M). W: withdrawal methamphetamine administration in rats; Number: corresponding weeks.

the Kir2.0 family (Kir2.1–Kir2.4). Three of these subunits (Kir2.1–Kir2.3) are expressed in cardiac cells [15–17]. Herein we show that all four subunits are expressed in vitro and in vivo at both the transcript level and translate level.

The main pore-forming α1c (Cav1.2) subunit determines the main biophysical and pharmacologic properties of the Ca^{2+} channel. *CACNA1C*, a gene encoding Cav1.2 protein, associated with a multiorgan dysfunction causing long QT intervals, arrhythmias and autism known as Timothy syndrome [18]. The auxiliary subunits β2 (*CACNB2*), the dominant isoform known to play an essential role in the voltage dependence of LTCC, has been also studied.

We hypothesized that change in the expression of ion channels may contribute to electrophysiological consequences induced by MA. Herein we observe the link between electrical remodeling and ion channel expression remodeling induced by MA. Our observations that MA altered potassium and calcium channel subunit expression in vivo and in vitro suggest the correlation with the change in I_{to}, I_{K1} and $I_{Ca\text{-}L}$ currents, is at work. We used RT-PCR and western blot to assess of the transcript and translate levels of ion channel subunits, including I_{to}: kv1.4, kv1.7, kv3.4, kv4.2; I_{K1}: kir2.1, kir2.2, kir2.3, kir2.4; and $I_{Ca\text{-}L}$: Ca^{2+}α1, Ca^{2+}β.

Our results show that there was a corresponding change in the transcript and translate levels. In vitro and in vivo, MA caused decrease in mRNA and protein levels in these ion channel subunits (Table 2). And, more remarkable, kv3.4 and all 4 subunits of Kir2.0 family showed significant decrease. MA had inhibitory effects on the I_{to}, I_{K1}, and $I_{Ca\text{-}L}$ and had inhibitory effects on the corresponding ion channel expression, which provide a possible link between electrical remodeling and ion channel expression remodeling induced by MA. Thus, the accumulated data support the view that arrhythmogenic remodeling is one of the possible mechanisms evoked MA-induced arrhythmias.

Interestingly, we observed the reversible changes in ion channel expression after withdrawal MA (Table 3). Very slightly reversible regulation of expression could be observed after MA withdrawal for 2 weeks in some of the ion channel subunits. Most of the ion channel subunit expression showed no change. Evaluation of cardiac lesion changes attributable to MA withdrawal may evidence a number of events and the reversible character of pathological findings were well documented [19,20]. These studies indicated that at the first and second week of withdrawal of MA, the severity of pathological changes slightly reversed. Some of cardiac effects of MA use remained relatively irreversible in this period. The accumulated data suggest that the state of cardiac lesions still remained, even if MA administration had ceased for 2 weeks. Most of the ion channel subunit expression remained relatively irreversible within 2 weeks after MA withdrawal. To a certain extent that the functions of ion channels were affected, and the effects could induced electrical remodeling. Thus, it is high time that the risk of arrhythmias was possible to appear.

Most of the ion channel subunit expression started to reverse after MA withdrawal for 4 weeks. Recovery rates of fourth week of MA withdrawal were higher than second week of MA withdrawal significantly. The pathological recovery was also pronounced at the fourth week of MA withdrawal, indicating the gradual reversal of cardiac lesions [19,20].

Reversible expression could be observed significantly in all of the ion channel subunits after MA withdrawal for 8 weeks. Recovery rates of eighth week of MA withdrawal were higher than fourth week of MA withdrawal. Recovery could help to improve the functions of ion channels, thus, to ease the effects on electrical remodeling. These data suggest that the risk of arrhythmogenesis may be reduced, in some way. A persistent pathological recovery was seen from a grade of marked change towards mild to normal. Most of the pathological changes returned or were on the way to returning to a normal state [19,20]. Nonetheless, there was a disparity between the ion channel subunit expression of eighth week of MA withdrawal and the control. Our data showed that the expression of ion channel subunits were still unable to return to normal state, further suggest that the ion channel expression remodeling still exist for a period of time even after MA withdrawal for two months, therefore, the risk of arrhythmias may present for a period of time.

We found that CACNA1C and Kir2.0 family showed lower recoverability (recovery rate $\leqslant$ 70%) than the others after MA withdrawal for 8 weeks. Lower recoverability of CACNA1C and Kir2.0 family suggest pronounced alterations of $I_{Ca\text{-}L}$ and I_{K1} electrical and ion signaling and increased risk of arrhythmogenesis. The physiological function of I_{K1} was adjusting the resting potential and joining in the repolarization of action potential. I_{K1} inhibition could elevate membrane potential and prolong the action potential duration. $I_{Ca\text{-}L}$ was another important ionic current in the repolarization of ventricular myocytes. The early after depolarizations and delayed after depolarizations caused by the enhancement of $I_{Ca\text{-}L}$ in the repolarization were important incentives of the reentrant arrhythmia. Our findings suggest that remodeling of CACNA1C and Kir2.0 family expression may be a possible factor inducing arrhythmogenesis in late convalescence period of MA-induced cardiac lesions.

In conclusion, reduced number of functioning channels may contribute to the reduction in I_{to}, I_{K1} and $I_{Ca\text{-}L}$ and therefore is a reasonable index of the level of channel proteins. Alternatively, reduction in transcription or translation of I_{to}, I_{K1} and $I_{Ca\text{-}L}$ genes could reduce channel number. At present, these mechanisms remain speculative, given the uncertain relation of the reversible change to the electrical remodeling caused by MA. The mechanism of ion channel expression remodeling is still under investigation. Meanwhile, awaiting future studies in response to the mechanisms of cardiac toxicity induced by MA exposure.

Conflict of interest

The authors declare no conflict of interest.

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