KCNQ3 normalizes hyperactivity of VTA-NAcLat circuit and attenuates methamphetamine addiction in mice

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Abstract

The brain circuit projecting from the ventral tegmental area (VTA) to the lateral shell nucleus accumbens (NAcLat) has a key role in methamphetamine (MA) addiction. As different VTA dopamine (DA) neuronal subpopulations participate in different neuronal circuits, it is still a challenge to isolate these DA subtype cells. Using retrograde tracing, Patch-seq in MA-addicted mice, we isolated the DA neurons of the VTA-NAcLat circuit and profiled its gene expression. We found that in this circuit the KCNQ3 (Kv7.3) gene, encoding K+ channel protein, was among the differentially expressed genes. Injection of the Kv7.3 channels agonist ICA069673 or overexpression of Kv7.3 channels in the VTA-NacLat circuit could reverse MA addiction. Furthermore, enhancement of Kv7.3 channels activity decreased neural oscillation, neuronal excitability, synaptic plasticity and DA release in the VTA-NacLat circuit of MA-addicted mice. Activation of Kv7.3 channels in the VTA may become a potential novel treatment strategy for MA addiction.

Introduction

The abuse of methamphetamine (MA) is a worldwide health concern. Addictive drugs, including MA, stimulate the mesocorticolimbic dopamine (DA) reward system by increasing the activity of DA neurons in the ventral tegmental area (VTA) which then project primarily to the nucleus accumbens (NAc), amygdala, and the medial prefrontal cortex (mPFC). Importantly, these drugs induce long-term changes in DA levels and DA neuronal excitability within the mesocorticolimbic DA system. In particular, there seems to be a rapid release of DA from the VTA in the NAc, which alters NAc neuronal excitability and generates reward mechanisms. There is evidence indicating that different VTA DA neuron subpopulations have distinct projection targets and are also significantly different in anatomy and function. VTA projects to different NAc subdivisions: NAc core and NAc shell (medial shell (NAcMed) and lateral shell (NAcLat)). The two subdivisions of the NAc also have different anatomical characteristics and functions. The NAc shell plays a role in motivational functions and primary reward processing associated with drugs, and the NAc core is involved in the encoding of cue-reward learned associations. Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the NAc shell as compared with the NAc core of the rat. Compared with the NAc core, the NAc shell is more closely related to conditional position preference. VTA neurons projecting to NAcMed and NACLat are two distinct subpopulations with peculiar molecular characteristics and biological functions. Indeed, they display differential gene expression and firing patterns, and also receive different inputs. However, there is scant knowledge on the anatomical features of the VTA-NAcLat circuit and its function in MA addiction. A better understanding of this pivotal circuit in the pathophysiology of MA addiction may lead to the discovery of a novel target for the treatment of MA addiction. It is still a scientific challenge the isolation of the different VTA DA subtype neurons and to analyze their respective molecular characteristics. Patch-seq could solve this problem by combining whole-cell patch-clamp recordings and single-cell RNA-sequencing (RNA-seq) to produce high-
quality sequencing results $^{22,23}$. In this work, by using Patch-seq, we have precisely isolated DA neurons of the VTA-NAcLat circuit in mice addicted to MA, and screened out the differentially expressed genes. Through the results of single-cell RNA-seq, we combined the literature to determine the differential expressed gene KCNQ3 which encodes for Kv7.3 channels.

The KCNQ family includes Kv7.1–Kv7.5 channels, forming homotypic or heterotypic Kv7 channels, which are involved in the formation of M currents $^{24}$. Kv7 channels are voltage-dependent K$^+$ channels with slow activation and non-inactivation properties. All isoforms of Kv7 channels turn on at subthreshold potentials around ~ 60 mV, which stabilizes the neuronal resting potentials $^{25,26}$. There is evidence that Kv7 channels could effectively inhibit neuronal excitability and inhibit repetitive and burst firing of action potentials $^{27,28,29}$. Furthermore, conditional deletion of Kv7.2 and Kv7.3 on hippocampal neurons increases the frequency of excitatory postsynaptic current (EPSC) $^{30}$. Kv7 activation with retigabine treatment also decreases DA release $^{31,32}$. Kv7.2, 7.3, 7.4 and 7.5 subunits are expressed in various neurons in the brain, and Kv7.1 is mainly expressed in cardiomyocytes $^{33}$. Since Kv7.3 has been reported to promote the surface expression of other Kv7 subunits, and some studies have shown that these subunits co-localize in many regions, Kv7.2/3 or Kv7.3/5 heteromers are considered to be Kv7's major subunit channels $^{34,35,36}$. Moreover, it is mainly Kv7.2/3 that forms the M current $^{28,34,37}$. In our study, we found that the expression of Kv7.3 was significantly reduced in the VTA of MA addicted mice and showed that Kv7.3 has a key role in neuronal excitability, synaptic plasticity and DA release in the VTA-NAcLat circuit of MA-administered mice. Therefore, we carried out a series of studies on Kv7.3 channels in an attempt to find a new target for treating MA addiction.

Results

Chemogenetic inhibition of the VTA-NAcLat circuit attenuates MA addictive behavior

Mice injected intraperitoneally with MA during 6 conditioning days displayed significant preference for the MA-paired chamber (white chamber) compared with the saline-paired chamber (black chamber) (Fig. 1a, b). Indeed, CPP scores increased after MA treatment, indicating that mice developed addictive behaviors to MA (Fig. 1c).

To determine the distribution and type of VTA neurons involved in the circuit leading to addiction, mice were injected with red retrobeads into NAcLat to trace the VTA neurons in the circuit (Fig. 1d, e). Consistent with previous research $^{38}$, we observed that VTA neurons projecting to NAcLat were predominantly located in the lateral VTA (lVTA) and only a small amount in the medial VTA (mVTA) (Fig. 1f). Immunofluorescence staining showed that 93.8% of VTA neurons projecting to NAcLat were TH positive and thus DA neurons (Fig. 1g, h). To further confirm the distribution and type of VTA neurons projecting to NAcLat, we injected red retrobeads into NAcLat of the mouse and performed Patch-clamp experiments (Fig. 1i). Consistent with the data previously reported, we found that VTA neurons (red retrobeads labeling) in the circuit were located predominantly in the lVTA and only small amount in the
mVTA (Fig. 1j). We clamped VTA neurons labeled with red retrobeads for whole-cell recording and stimulated with a -300pA current and found that about 90% of the cells showed the typical Sag specific to DA neurons (Fig. 1k, l). In conclusion, VTA neurons of the VTA-NAcLat circuit are mainly distributed in the lVTA, and the vast majority of them are DA neurons.

To investigate whether the VTA-NAcLat circuit exhibits hyperactivity in MA addiction, we injected bilaterally pAAV-EF1a-hM4Di-mCherry-WPRE or the control virus (pAAV-EF1a-mCherry-WPRE) into the VTA and we microinfused bilaterally CNO intra-NAcLat during the CPP paradigm (Fig. 2a, b). We observed a reversal of addictive behavior in mice injected with hM4Di virus, whereas a significant change in the CPP was still present in mice injected with m-Cherry only (Fig. 2c). Confocal images showed the restricted and selective expression of hM4Di-mCherry virus in the VTA of the mice (Fig. 2d).

To further confirm whether the addictive behavior to MA was mediated by the activation of the VTA-NAcLat circuit, we suppressed the activity of the VTA-NAcLat circuit in vivo by using a chemogenetic strategy. We expressed hM4D specifically in the VTA-NAcLat circuit by VTA injection of pAAV-EF1a-DIO-hM4Di-mCherry-WPRE or pAAV-Ef1a-DIO-mCherry-WPRE and NAcLat injection of pAAV-hSyn-EGFP-P2A-NLS-Cre-WPRE (Retro) (Fig. 2e). We silenced the VTA-NAcLat circuit through the administration of CNO intraperitoneally. After CPP behavioral test (Fig. 2f), there was no significant difference in CPP scores between pre- and post-test in mice injected with hM4Di, whereas this difference was significant in mice injected with m-Cherry only (Fig. 2g). Importantly, using confocal microscopy analysis, we showed that the expression of AAVs in the VTA neurons projecting to the NAcLat were selective and restricted (Fig. 2h). Next, we determined the spontaneous discharge of hM4Di-expressing VTA neurons to verify the inhibitory effect of hM4Di on VTA neuron activity. After bath application of CNO (3μM), the frequency of spontaneous discharge in neurons expressing hM4Di did not change, but their amplitude was significantly reduced than in neurons expressing mCherry (Fig. 2i-k). Overall, these data indicated that the activity (particularly the hyperactivity) of the VTA-NAcLat circuit was critically involved in MA addiction, and that inhibition of this circuit could attenuate addictive behavior to MA.

**Single-cell RNA-seq of VTA-NAcLat DA neurons and focused on Kv7.3 Channel**

We next screened out the genes differentially expressed in the VTA-NAcLat circuit between MA addicted and control mice in the attempt to study the possible mechanisms underlying MA addiction. We thus performed Patch-seq in samples from VTA neurons projecting to NAcLat in both MA-addicted and control mice. After the mice injected with red retrobeads into NAcLat were trained in the CPP apparatus, the brains were collected and in VTA-containing slices, patch-clamp technology was used to identify neurons tagged with retrobeads as DA neurons. These neurons were then collected for single-cell RNA-seq (Fig. 3a). Through the analysis of RNA-seq results, we found that in MA mice the mRNA expression levels of 75 genes were down-regulated and 30 genes were up-regulated in comparison with control mice (Fig. 3b). Combined with the literature, we corroborated 11 differential genes from the Patch-seq results by RT-PCR, and we found that MA mice had decreased levels of KCNQ3 and increased levels of PCP4 and FGF20 compared to control mice (Fig. 3c).
Therefore, we have further investigated the ion channel Kv7.3 which can stabilize the neuronal resting potential and is the major isoform of Kv7 channels regulating neuronal activity. First, immunofluorescence staining confirmed the reduced expression of Kv7.3 in the VTA neurons projecting to NAcLat of MA compared with control mice (Fig. 3d, e). Second, we observed that the function of Kv7.3 channels in retrobeads-labeled VTA neurons projecting to NAcLat was altered in MA mice by using patch-clamp. We held the membrane voltage from -20 mV to -60 mV to measure Kv7 channel-mediated M currents (IM). In MA mice, the amplitude of IM was reduced in VTA neurons projecting to NAcLat compared with the control mice indicating reduced function of Kv7 channels (Fig. 3f, g). Since Kv7.2/Kv7.3 heteromers are considered to be the main molecular basis for the IM, the decrease of IM indicates that Kv7.3 function is most likely disrupted in MA mice. Consequently, we speculated that increasing the function of Kv7.3 in the VTA neurons could attenuate addictive behaviors of MA mice.

**Activation Kv7.3 channel reduces CPP scores and neuronal oscillations in MA addicted mice**

Currently, there are no selective agonists for Kv7.3 channels. We therefore chose retigabine which has affinity for Kv7.1-Kv7.5 channels but among them much higher affinity for Kv7.3 channels, and ICA069673 that selectively activates Kv7.2/Kv7.3 heteromers and has little effect on other Kv7 isoforms. To directly test if retigabine and ICA069673 could affect MA addictive behavior, MA-treated mice received an intra-VTA microinfusion of retigabine or ICA069673 prior the CPP test sessions (Fig. 4a, b). We found that both retigabine and ICA069673 reduced CPP scores, reversing the addictive behavior of MA mice (Fig. 4c), indicating that activation of Kv7.3 channels in the VTA may be a valid pharmacological approach to attenuate MA addictive behavior, and consequently, that Kv7.3 channel is a potential novel target for the psychopharmacology of MA addiction. Using ICA069673 to activate Kv7.3 channels, we then examined the potential mechanism of action of this drug and the role of Kv7.3 channels in the VTA-NAcLat circuit and MA addiction.

Immediately after completing the CPP test, we measured local field potential (LFP) of the VTA and NAcLat in three experimental groups to investigate the effect of ICA069673 on neuronal oscillations in these two brain regions of MA-administered mice. We observed that delta and theta oscillations increased in both VTA and NAcLat of MA-administered mice (Fig. 4d). Notably, delta oscillations have been reported to be implicated in the reward process of drugs of abuse, and involved in anticipation and motivation, whereas theta oscillations have been related to active motor behavior and cognitive processes. In addition, in MA-administered mice, alpha, beta and gamma oscillations were increased in the VTA (Fig. 4d). There is evidence that alpha and beta oscillations have been implicated in reward events, and beta and gamma oscillations were involved in episodic memory. Remarkably, oscillation activities in all band frequencies were normalized in the VTA and NAcLat of ICA069673-administered mice (Fig. 4d). LFP traces and spectrograms of VTA and NAcLat are shown in Fig. 4e.

**Activation Kv7.3 channels reduces the levels of Arc and Ca²⁺ in MA addicted mice**
We next investigated whether intra-VTA infusion of ICA069673 could also reduce the activity of neurons in the VTA-NAcLat circuit. We chose the activity-regulated cytoskeleton-associated protein (Arc) as an indicator of neuron activity. Arc is an immediate early gene and could be induced by multiple forms of synaptic and cellular activity. Arc was specifically trafficked and localized to neuronal dendrites. Arc is strongly expressed in many processes involving learning, memory, and reward \(^50, 51, 52\). We first traced VTA neurons in the circuit by injecting retrobeads into NAcLat, and traced the NAcLat neurons in the circuit by injecting pscAAV-Hsyn-Cre-tWPA into the VTA and pAAV-Ef1a-DIO-EGFP-WPRE into the NAcLat. We then imaged mouse brain slices with confocal microscopy to examine the expression of Arc near these neuron bodies. We found that the expression level of Arc in the VTA and NAcLat neurons of MA mice was significantly higher than that of saline-administered (control) mice, and the microinfusion of ICA069673 into the VTA was able to normalize the levels of Arc back to the values of control mice (Fig. 5a-d).

To further confirm that activation of Kv7.3 channels can reduce the neuronal activity of VTA and NAcLat in MA addicted mice, we studied the activity levels of Ca\(^{2+}\) in the VTA and NAcLat. Ca\(^{2+}\)/calmodulin-dependent protein kinase II is involved in long-term potentiation, synaptic plasticity, memory consolidation and synaptic transmission which strongly reflect the activity of neurons. Mice were injected with pAAV-CMV-GCaMP6s-P2A-nls-dtomato into the VTA and the NAcLat to express the Ca\(^{2+}\) indicator GCaMP6s in neurons. Using the confocal microscopy, we found that Ca\(^{2+}\) activity significantly increased in the VTA and the NAcLat of MA-administered mice relative to saline-administered mice. Intra-VTA infusion of ICA069673 into MA-administered mice normalized Ca\(^{2+}\) activity back to the levels of control mice (Fig. 5e-h).

**Activation Kv7.3 channels reduces cellular excitability in VTA and DA release in NAcLat**

To further explore the mechanism by which activation of Kv7.3 channel reduces neuronal activity in the VTA-NAcLat circuit, we used patch-clamp in the slices containing VTA from the three experimental groups of mice injected with red retrobeads into NAcLat after the CPP test (Fig. 6a, b). We recorded action potentials from retrobeads-labeled VTA neurons and observed that the frequency of action potentials in MA mice increased compared to control mice, and the intra-VTA infusion of ICA069673 significantly decreased the frequency of the action potentials in MA-administered mice (Fig. 6c, f). The amplitudes of action potentials did not differ among the three groups of mice (Fig. 6e). We also analyzed the phase plots of the representative action potentials (Fig. 6d) and found that compared with control mice, the permeability of VTA neurons to ions in MA mice was significantly increased, and intra-VTA infusion of ICA069673 decreased the permeability back to control levels. Firing spikes induced by injecting currents of 50-150pA in 50pA steps into the retrobeads-labeled VTA neurons of MA mice were significantly increased compared with control mice, and intra-VTA infusion of ICA069673 decreased the firing spikes induced by injecting currents to control levels (Fig. 6g, h). These data indicated that activating Kv7.3 channels can significantly reduce the cellular excitability of VTA neurons following MA administration.
Since activation Kv7.3 channels reduced the excitability of VTA neurons, we examined whether it may also affect the release of DA from the axon terminals of VTA DA neurons into the NAcLat. (Fig. 6i, j). We observed significantly increased oxidative currents (indicating increased DA release in the NAcLat) in MA-administered mice than in saline-administered mice. Notably, intra-VTA infusion of ICA069673 reduced oxidative currents in MA-administered mice to control levels (Fig. 6k). To calculate the concentration of DA released in the NAcLat, we then performed a calibration curve with standard solutions of DA at different concentrations (Fig. 6l, m). We then observed more than a twofold increase in the DA concentration in the NAcLat of the MA-administered mice relative to saline-administered mice, and intra-VTA infusion of ICA069673 normalized the DA concentration to control levels (Fig. 6n). These data indicated that intra-VTA infusion of ICA069673 in MA-treated mice is able to inhibit the DA release in the NAcLat from the axon terminals of VTA neurons, probably reducing the excitability of NAcLat neurons.

**Effects of Kv7.3 channels activation on synaptic plasticity in the VTA-NAcLat circuit**

To explore whether activation Kv7.3 channels alters synaptic plasticity in the VTA-NAcLat circuit of the MA-administered mice, we injected the AAV-NCSP-YFP-2E5 into the VTA and the mice were then subjected 2 weeks after to the CPP test (Fig. 7a). We examined three subtypes of spines in the VTA neurons: mushroom spines, thin spines and stubby spines (Fig. 7b). We observed that MA increased the total spine density and the three subtypes of spine density compared with control. Intra-VTA infusion of ICA069673 reduced the total spine density and the three subtypes of spine density to control levels (Fig. 7c), indicating that pharmacological activation of Kv7.3 channels can reduce the formation of spines, and thus Kv7.3 channels may have an important role in the formation of new excitatory synapses following MA treatment.

Finally, using electrophysiology, we studied the effects of intra-VTA infusion of ICA069673 on synaptic function in the circuit. Mice were subjected the CPP training two weeks after VTA injection of pscAAV-Hsyn-Cre-tWPA and NAcLat injection of pAAV-Ef1a-DIO-EGFP-WPRE (Fig. 7d, e). We prepared NAcLat-containing slices and employed whole-cell voltage clamping to measure EPSC of EGFP-labeled NAcLat neurons (Fig. 7f). We observed an increase in the mean amplitudes of EPSC in NAcLat neurons of MA-administered mice and intra-VTA infusion of ICA069673 was be able to reverse this change (Fig. 7g, i). No changes in their frequency upon either MA or MA+ICA069673 treatments were observed (Fig. 7h, j).

**Overexpression of Kv7.3 channels in the VTA rescues MA addiction**

To selectively overexpress Kv7.3 channels in the VTA neurons of the VTA-NAcLat circuit, we used the Cre-dependent AAV virus pAAV-CMV-DIO-KCNQ3-WPRE and the pAAV-CMV-DIO-EGFP-WPRE as control (Fig. 8a). We injected the two AAV viruses bilaterally into the VTA of the mice and pAAV-hSyn-mCherry-P2A-NLS-Cre-WPRE (Retro) bilaterally into the NAcLat (Fig. 8b). CPP test was performed in mice 2 weeks after injection of Kv7.3 AAV (Fig. 8c). Overexpression of Kv7.3 channels in VTA neurons of the circuit reduced CPP scores and attenuated addictive behaviors in MA-administered mice (Fig. 8d). Consistent with the previous pharmacological results, overexpression of Kv7.3 channels in mice was efficient to modulate addictive behavior, supporting that Kv7.3 channels have a central role in the reward process. We observed
selective expression of Kv7.3 AAV in VTA neurons of the circuit by immunofluorescence staining (Fig. 8e). Electrophysiological validation was then performed in mice injected with AAVs after the CPP test (Fig. 8f, g). Compared with control AAVs, overexpression of Kv7.3 channels induced increased M currents (Fig. 8h, i) and reduced firing rate (Fig. 8j, m) in VTA neurons of the circuit. In addition, overexpression of Kv7.3 did not alter the amplitude of action potentials in MA-administered mice (Fig. 8l). The phase plots (Fig. 8k) of the representative action potentials indicated that overexpression of Kv7.3 channels decreased the permeability of ions in MA-administered mice. Collectively, these data indicated that enhancing Kv7.3 channels expression in the VTA of the VTA-NAcLat circuit effectively reduces MA addictive behaviors in mice.

Discussion

MA is a potent common stimulant drug that has a high potential for abuse and addiction. Unfortunately, the neurobiological mechanisms underlying MA addiction are only partially known, and there are no current drugs for treating MA addiction. The VTA-NAc circuit is intimately involved in the processing of reward-related stimuli, and in our preclinical study we demonstrated that the VTA-NAcLat circuit plays a central role in MA addiction, and is a key circuit to target for developing novel drugs able to treat MA addictive behaviors. Patch-seq was used to isolate VTA neurons implicated in the VTA-NAcLat circuit allowing to obtain high-quality whole-transcriptome data from single neurons. Among the differentially expressed genes between control and MA addicted mice, we interestingly found decreased expression of the KCNQ3 gene which encodes for Kv7.3 channels. We then confirmed that activation of Kv7.3 channels reversed MA addiction by normalizing the hyperactivity of the VTA-NAcLat circuit. In particular, intra-VTA infusion of the Kv7.3 agonist ICA069673 reduced VTA neurons excitability, synaptic plasticity, and DA release in NAcLat of MA-addicted mice.

In addition, using immunofluorescence and patch-clamp techniques, we found that the VTA neurons in the circuit were mostly DA neurons, thus indicating that the projection terminals from the VTA to the NAcLat which activated NAcLat neurons, mainly released DA. Within the reward system, DA seems to modulate multiple complex and diverse processes including incentive motivation, habit formation, aberrant learning and memory processes and effort-related aspects of motivation. Finally, we have demonstrated that chemogenetic inhibition of this circuit attenuated MA addictive behavior thus indicating that the hyperactivity of this circuit may lead to addictive behaviors, and that Kv7.3 channels in the VTA neurons are a potential novel target for MA addiction since their overexpression reduced addictive behaviors along with the activity of VTA neurons. Previous reports have demonstrated the possible involvement of Kv7.3 channels in disorders including depression, epilepsy and mania.

Interestingly, we also revealed that activation of Kv7.3 channels reduced neuronal oscillations in the VTA of the MA-administered mice. Network oscillations arise from the coordinated interplay of neuronal excitation and inhibition. It is abundantly clear that different subgroups of VTA DA neurons display considerable heterogeneity that depend on differences in their afferent and efferent connections. For example, VTA projections to the NAc are involved in encoding reward prediction, while projections to the
hippocampus contribute to spatial memory formation. At the same time, in the drug-induced CPP paradigm, more complex conditioning processes may result. Consistent with this notion, we found increased neuronal oscillatory activity in multiple frequency bands in the VTA of MA addicted mice. There is evidence that neuronal oscillations in different frequency bands are involved in different processes, such as reward, anticipation, motivation, cognitive process and memory.

Activation of Kv7.3 channels can restore VTA neuronal oscillations to normal levels in MA addicted mice, thus normalizing the activity not only of the VTA itself but also of downstream brain regions innervated/modulated by the VTA. This was demonstrated by multiple findings in the MA-administered mice since activation of Kv7.3 channels could effectively reduce 1) the neuronal excitability of VTA and NAcLat, 2) synaptic plasticity, and 3) DA release. Since the heteromers formed by Kv7.3 channels are partially open at resting potential, and can be suppressed by various G protein coupled receptors such as substance P, opioid, and metabotropic glutamate receptors, they can effectively control the excitability of neurons. Synaptic plasticity is often studied through EPSCs. Chronic administration of MA produces reorganization of synaptic connections within the VTA and cellular states in the NAc dependent on changes in synaptic function of the VTA. We found that intra-VTA infusion of ICA069673 reduced excitement input in NAcLat and modified communications within the VTA-NAcLat circuit of MA-addicted mice. The hyperactivity of VTA neurons due to MA led to elevated levels of DA in the NAcLat which in turn activated NAcLat neurons. Activation Kv7.3 channels reduced DA levels in the NAcLat thereby reducing cellular excitability in the NAcLat of MA mice.

Overall, this study shows that the VTA-NAcLat circuit has a key role in MA addictive behavior. We identify that Kv7.3 channels can be a therapeutic target for MA addiction since their activation normalize the pathogenic hyperactivity of neurons in the circuit and the underlying addictive behavior. Overall, our data pave the way for a novel treatment strategy for MA addiction.

Methods

Mice

All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee at Shandong University. Male C57BL/6j mice (6-8 weeks old) were provided by the Department of Laboratory Animal Sciences, Shandong University. Mice were maintained under a 12-hour light/dark cycle with food and water ad libitum.

Viral vectors

pAAV-EF1a-DIO-hM4Di-mCherry-WPRE, pAAV-Ef1a-DIO-mCherry-WPRE, pAAV-hSyn-EGFP-P2A-NLS-Cre-WPRE (Retro), pAAV-CMV-DIO- KCNQ3-WPRE, pAAV-CMV-DIO-mCherry-WPRE, pAAV-CMV-GCaMP6s-P2A-nls-dtomato, pscAAV-Hsyn-Cre-tWPA, and pAAV-hSyn-mCherry-P2A-NLS-Cre-WPRE (Retro) viruses were purchased from Shanghai SunBio Biomedical technology Co. Ltd. (Shanghai). rAAV-NCSP-YFP-2E5 viruses were purchased from Brain Case Biotechnology Co., Ltd (Shenzhen). All viruses were aliquoted
and stored at −80 °C and the viral titers were more than 10^{12} viral particles per mL. Red retrobeads (Lumafluor, USA) was diluted by PBS into a 4-fold solution for injection.

pAAV-CMV-DIO-KCNQ3-WPRE were made by sub-cloning their sequences into pAAV-CMV-DIO-EGFP-WPRE vector with respective titers of 4.7×10^{12} virus molecules per mL (Obio Technology, Shanghai, China).

**Stereotaxic surgery for viral injections**

Mice (6-8 weeks old) were anesthetized with 5% chloral hydrate (10mL/kg) intraperitoneal (ip) and then placed on a stereotaxic instrument (Model 900, Kopf, USA). A craniotomy was performed with a drill at the bilateral stereotaxic coordinates of the NAcLat (from bregma: +1.2 mm A/P, ± 1.7 mm M/L) or the VTA (from bregma: -3.4 mm A/P, ±0.5 mm M/L). An Hamilton syringe (10 μL) was then slowly lowered into the NAcLat (4.7 mm D/V) or VTA (4.5 mm D/V) and viruses or red retrobeads (0.2 μl) were injected bilaterally at a speed of 0.04 μl/min. The syringe was slowly withdrawn 10 min after the injection, the scalp skin sutured over the holes, and mice were placed back into their original cages for recovery. AAV-injected mice were subjected to the experiments two weeks after surgery whereas red retrobeads-injected mice 4 to 7 days after surgery.

**Cannula implantation and microinfusion**

Mice were placed on the stereotaxic apparatus after anesthesia. Mice were bilaterally implanted with a stainless 26-gauge cannula directly above the VTA (from bregma: -3.4 mm A/P, ±0.5mm M/L and 3.9mm D/V) or NAcLat (from bregma: +1.2 mm A/P, ± 1.7 mm M/L and 4.0 mm D/V). The cannula was fixed with dental cement. After the surgery, mice were housed individually, and the experiments carried out after 5 days of recovery.

After recovery from surgery, the cannulated mice received an intra-VTA microinfusion of ICA069673 (10μM, 200 nl, Med Chem Express) and intra-NAcLat microinfusion of clozapine N-oxide (CNO) (3 μM, 200 nl, Med Chem Express) or filtered 1× PBS as vehicle control. We performed bilateral microinfusion 0.2 μl per side through the cannula with an Hamilton syringe (5 μL) at a rate of 0.04 μL/min, removing slowly the syringe 5 min after the injection.

**Conditioned place preference test**

A custom-made two-compartment apparatus (15 × 15 × 30 cm) was used to perform conditioned place preference (CPP) test. One chamber had white walls and the other one black walls, and the two chambers were separated by a doorway (5 × 5 cm). The behavioral activity of the mice was recorded with a camera on the chamber lid.

We placed the mice in the CPP apparatus and left free to explore it for 15 min to assess their baseline place preference. On the first day (day1), control mice were injected with saline (10 ml/kg, i.p.) and the MA-administered mice were injected with MA (2mg/kg, i.p.). ICA069673-administered mice received a microinfusion of ICA069673 (10μM, 200 nl) through the cannula into the VTA 15min before MA.
(2mg/kg,i.p.) injection. Mice injected with AAV-hM4Di-mCherry or AAV-mCherry received a microinfusion of CNO (3 μM, 200 nl) into the NAcLat 15min before MA (2mg/kg,i.p.) injection. Then, mice were confined in the white chamber for 30 min before returning to their home cage. This procedure was repeated on day 3 and 5. On day 2, 4 and 6, control mice and MA-administered mice were injected with saline (10 ml/kg, i.p.), ICA069673-administered mice received a microinfusion of saline (200 nl) into the VTA 15min before saline (10 ml/kg, i.p.) injection. Mice injected with AAV-hM4Di-mCherry or AAV-mCherry received a microinfusion of saline (200 nl) into the NAcLat 15min before saline (10 ml/kg, i.p.) injection. Then, mice were confined in the black chamber for 30 min before return to home cage. After each trial, the apparatus was cleaned with 75% alcohol. On day 7, we left the mice to freely explore both sides of the CPP apparatus for 15 min. We got CPP score by subtracting the time spent in the black chamber from the time spent in the white chamber. The movement of animals in the two chambers was monitored and analyzed by a video tracking system (DigBehav, Jiliang Software Technology, China) 67. For a scheme of the experimental protocol, please see Fig. 1A.

**Brain slice preparation and electrophysiology**

**Brain slice preparation**

We quickly removed the brains of the anesthetized mice after undergoing the CPP paradigm, and immediately placed them in the N-Methyl-D-glucamine (NMDG)-containing cutting solution containing the following (in mM): 2.5 KCl, 93 NMDG, 30 NaHCO₃, 25 glucose, 1.2 NaH₂PO₄, 5 sodium ascorbate, 20 HEPES, 2 thiourea, 3 sodium pyruvate, 0.5 CaCl₂, 10 MgSO₄, pH 7.4, 295-305 mOsm. We used a vibratome (VT1200, Leica Microsystems, USA) to cut brain slices with 300 μm thickness containing the NAcLat or VTA. We put the slices in the cutting solution to recover for 30 min at 36ºC saturated with 95% O₂ and 5% CO₂. Then, we left the brain slices at room temperature for 1 hour before use. We placed the NAcLat or VTA-containing slices in the perfusion chamber and continuously fed into a flowing oxygenated recording solution(in mM: 25 NaHCO₃, 25 glucose, 125 NaCl, 2.5 KCl, 2 CaCl₂, 1.25 NaH₂PO₄ and 1 MgCl₂, pH 7.4, 295-305 mOsm) 68.

**Currents and action potentials recording**

We observed neurons under a 40x water immersion lens and used a laser to excite fluorescence. Glass capillaries (World Precision Instruments, USA) with a resistance of 3-5 mΩ were pulled on a micropipette puller (P-1000, Sutter Instrument Co., USA). Evoked and spontaneous currents were recorded at -65 mV. The recordings all used a K-gluconate-based internal solution (in mM: 126 K-gluconate, 10 KCl, 2 MgSO₄, 4 NaCl, 0.3 Na-GTP, 4 Mg-ATP, 0.2 EGTA, 10 HEPES and 10 phosphocreatine, pH 7.3, 290 mOsm).

**EPSC recording**

EPSC was recorded at -65 mV with a pipette solution (in mM: 130 CsCl, 2 MgCl₂, 2 Mg-ATP, 10 HEPES and 0.2 EGTA, pH 7.2, 290 mOsm). When recording EPSCs, 100 μM Picrotoxin and 5 μM GP52432 were added.
to the recording solution to block GABA-A and GABA-B receptors, respectively.

**Patch-seq**

**Single DA cell isolation**

Brain slices were prepared as described above for electrophysiology. We placed VTA-containing slices in the perfusion chamber and continuously fed into a flowing oxygenated recording solution. We found the retrobeads-labeled VTA cells under the microscope, performed whole-cell recording with the pipette filled with internal solution, and gave -300pA current stimulation to determine whether they were DA neurons. We then used a new pipette with a tip that was about a quarter to a third the diameter of the cell body. We applied negative pressure to the pipette and could see the entire cell body entering the pipette. The pipette was discarded if extracellular contents entered the pipette. Otherwise, we used a positive pressure to eject the contents into a PCR tube which contained 4 µl of RNase-free lysis buffer.

**Library construction and sequencing**

The samples in the PCR tube were directly amplified using the Smart-Seq2 method, and the amplified product cDNA with a length of about 1-2 kb was obtained by the reaction. Qubit® 3.0 Fluorometer was used to measure the cDNA concentration of amplified products. The Agilent 2100 Bioanalyzer was used to detect the fragment distribution of amplified cDNA samples to ensure the quality. Library construction was performed using the amplified cDNA. The sample cDNA was disrupted into small fragments of approximately 350 bp by sonication using the Bioruptor® Sonication System (Diagenode Inc.). We perform end repair, 3’ ends A tailing and adapter ligation on the samples. After each step, Beckman Ampure XP magnetic beads were used for purification. The adaptor products were taken for PCR amplification, and each sample was introduced with different Index tags for distinguishing from each other during on-machine sequencing. The PCR amplification products were passed through the Pippin HT to construct the final library. After library construction, the library was tested for fragment length distribution and effective concentration using the Agilent 2100/LabChip GX Touch and Q-PCR. Eligible libraries were loaded on the HiSeq sequencing platform for PE150 sequencing program.

**Immunofluorescence staining and confocal imaging**

Mice were perfused transcardially with 4% paraformaldehyde after anesthesia 30 minutes after the CPP test. Mouse brains were removed and fixed overnight at 4°C before equilibration in 30% sucrose solution. We used a cryostat (Leica Biosystems, Germany) to prepare the sections (40 µm). Free-floating immunohistochemistry was performed on the obtained sections that were blocked with 0.3% Triton X-100 and 5% bovine serum albumin (BSA) for 1 h. Then, we incubated sections with primary antibodies (mouse anti-TH (1:500, Santa cruz, USA), mouse anti-Arc (1:500, Santa cruz, USA), rabbit anti-Kv7.3 (1:500, Alomone, Israel)) overnight at 4°C. We incubated sections with secondary antibody (donkey anti-rabbit IgG Alexa 647 (1:200, Proteintech, USA) and FITC goat anti-mouse IgG (1:200, Proteintech,
USA) for 2 hours. The slides were mounted after washing the sections with PBS. We obtained confocal images with a LSM880 confocal microscope (Zeiss, Germany).

Brains of the mice infected with rAAV-NCSP-YFP-2E5 were sectioned at 70 μm. Slices were slide mounted and images were obtained with a 63× oil objective on the LSM880. We measured spine density from YFP-expressing VTA neurons and used the software ImageJ to analyze spine types. The spine morphology was divided into 3 types: mushroom spine, thin spine and stubby spine.

RT-PCR

The brains of the anesthetized mice were removed and we used a stereomicroscope to dissect the VTA and stored the tissue at -80 °C. We extracted total RNA using RNA fast 200 kit (Fastagen, China). We detected the quality and quantity of RNA samples by using Nanodrop 2000 Spectrophotometer, and used PrimerScript RT reagent kit with gDNA Eraser (TAKARA, Japan) for reverse transcription of total RNA on the basis of the manufacturer’s protocol. We performed qRT-PCR with FastSYBR Mixture (CWbio, China), and run the PCR program as follows: denaturing at 95 °C for 120s, 40 cycles at 95 °C for 35s, 62 °C for 30s, and 72 °C for 40s, and a final extension at 72 °C for 300s.

Local field potential

Mice were anesthetized with 5% chloral hydrate (10mL/kg), fixed on a stereotaxic apparatus and after performing a craniotomy implanted with a single tungsten wire (diameter 50 μm) for recording local field potential (LFP) in the VTA (-3.4 mm A/P, ±0.5mm M/L, 4.5 mm D/V) or in the NAcLat (1.2 mm A/P, ±1.7mm M/L, 4.7 mm D/V). The reference electrode (miniature stainless-steel screw) was implanted over the cerebellum. The electrodes were fixed on the skull using dental acrylic and then connected to the amplifiers (AD Instruments, Australia) while the animal still anesthetized to process a recording for 5 min. We used LabChart software to record and analyze data for getting the power spectral density and spectrogram. We divide neuronal oscillations into five frequency bands: delta (1–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), beta (13–30 Hz) and gamma (30–100 Hz).

Electrochemistry

**Carbon fiber electrode preparation**

5 μm diameter carbon fiber (Tokai Carbon Co., Japan) with a length of about 10 cm was inhaled into a glass capillary (coreless, outer diameter: 1.5 mm, inner diameter: 0.89 mm, length: 8 cm), and the pipette was drawn by the P-1000 micropipette puller. Under the microscope, the exposed carbon fiber was cut to 100–300 μm with a surgery scalpel. We used silicone rubber to fill the tip of the glass capillary.

**DA release detection**

Brain slices containing the NAcLat were prepared as described above for electrophysiology. KCl (3M solution was added to the prepared carbon fiber electrode, and then the electrode was fixed on the
micromanipulator arm of the patch clamp, and the silver wire was immersed in the solution in the electrode tube. The NAcLat-containing slices were placed in the perfusion chamber and continuously fed into a flowing oxygenated recording solution. The NAcLat was found under the microscope, and the carbon fiber electrodes were inserted. In the voltage-clamp mode, a voltage of 780 mV was added, and the spike generated by the release of secretory DA could be observed. When the voltage returned to 0 mV, the spike disappeared. The addition of 70mM high K$^+$ solution to the extracellular fluid caused the depolarization of cells to release DA. The carbon fiber electrode can oxidize DA to dopamine quinone, and the current generated by the oxidation, which is proportional to the amount of DA content, can be detected by the carbon fiber electrode. A calibration curve with different standard concentrations of DA was performed to calculate the concentration of DA in the NAcLat$^{72,73}$.

**Statistical analysis**

We used GraphPad Prism 8.0.2 version software for the statistical analysis of the data. Data were checked for normality using Kolmogorov–Smirnov test. Depending on the experimental conditions, we used t-test (unpaired, two-tailed) and one-way or two-way ANOVA followed by Bonferroni test post-hoc comparisons when allowed. Cumulative frequency and amplitude plot were analyzed with the two-sample Kolmogorov-Smirnov test. All data are presented as mean ± SEM. A p-value <0.05 was considered to indicate statistical significance.

**References**


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Figure 1

Anatomical organization of VTA neurons projecting to NAcLat. a Scheme illustrating the MA-addiction experimental protocol. Mice were intraperitoneally injected with MA (2 mg/kg) or saline for 6 conditioning days after the pre-test. The post-test was carried out on day 7 after the conditioning. b Representative tracks of the MA-administered mouse in white chamber and black chamber. c Changes in CPP scores pre- and post-test (n=12, unpaired t test, ***p = 0.0001). d, e Schematic of red retrobeads injection and
representative images of red retrobeads injection in NAcLat.  

The distribution of the VTA neurons projecting to NAcLat.  

Representative images of TH staining in the slice containing VTA.  

Percentage of TH+ neurons and TH- neurons in VTA neurons labeled with red retrobeads.  

Schematic image of red retrobeads injection and electrophysiological recordings.  

Distribution of VTA neurons projecting to the NAcLat and representative fluorescence images of red retrobeads-tagged VTA neurons.  

-300pA current stimulation evoked sag specific to dopaminergic neurons.  

Percentage of sag+ neurons and sag- neurons in VTA neurons labeled with red retrobeads.
Figure 2

Chemogenetic inhibition of the VTA-NAcLat circuit attenuates addictive behaviors. **a, b** Schematic summary for chemogenetic stimulation and CPP test. pAAV-EF1a-hM4Di-mCherry-WPRE or a control vector (pAAV-EF1a-mCherry-WPRE) were bilaterally injected into the VTA of the mice which received bilateral cannulas into the NAcLat. Intra-NAcLat microinfusion of CNO was performed 15 min before MA injection. CPP test was conducted on day 7. **c** CPP scores in mice with VTA neurons tagged with dio-hM4Di or m-Cherry (m-Cherry, n=6; hM4Di, n=6. Two-way ANOVA, ***p = 0.0002). **d** Representative image of hM4Di-mCherry expression in the VTA. **e, f** Experimental process of the virus injection and CPP test. **j** Changes in CPP scores pre- and post-test (m-Cherry, n=6; Dio-hM4Di, n = 6. Two-way ANOVA, ****p = 0.0001). **h** Representative images of virus expression in the VTA after post-test. **i** Representative traces of the spontaneous discharge in hM4Di-expressing VTA neurons. **j, k** Average amplitude and frequency of spontaneous discharge recorded from dio-hM4Di-tagged or m-Cherry-tagged VTA neurons in MA mice (frequency (j): m-Cherry, n=6 cells/3 mice; Dio-hM4Di, n=6 cells/3 mice; amplitude (k): m-Cherry, n=6 cells/3 mice; Dio-hM4Di, n=6 cells/3 mice. unpaired t test, **P = 0.0024 in (k)).
Figure 3

Single-cell RNA-seq analysis of DA neurons in the VTA-NAcLat circuit of mice treated with MA.  

**a** Experimental scheme of Patch-seq analysis. **b** Heatmap (left) and volcano plot (right) of the differential genes expression following single-cell RNA-seq. MA, n=8 cells/3 mice; control, n=12 cells/4 mice. **c** 11 differential genes in MA mice were verified by RT-PCR (control, n=6; MA, n=6. Two-way ANOVA, *p < 0.05). **d** Representative images of Kv7.3 channels colocalized with red retrobeads injected into NAcLat in the slice containing VTA. **e** Fluorescence intensity for Kv7.3 channels in VTA neurons projecting to the NAcLat (control, n=6; MA, n=6. unpaired t test, ***p = 0.0002). **f** IM currents evoked by -60mv voltage stimulation...
in control and MA mice. g IM current amplitude (control, n=8 cells/3 mice; MA, n=8 cells/4 mice. unpaired t test, ****p < 0.0001).

Figure 4

Activation of Kv7.3 channels reduces addictive behavior and neuronal oscillations in MA-administered mice. a Experimental design of microinfusion into the VTA of the mice. b Experimental timeline of CPP
test. Intra-VTA microinfusion of retigabine or ICA069673 was performed 15 min before MA injection. LFPs of VTA and NAcLat were performed after the post-test. c CPP scores pre- and post-test in MA mice following the different treatments (n = 6 mice per group. Two-way ANOVA, ***p = 0.0003). d LFP power of the different frequency bands in the VTA (left) and NAcLat (right) (n = 6 mice per group. Two-way RM ANOVA, *p < 0.05). e Representative examples of LFP traces and spectrograms of VTA and NAcLat in control animals and in those treated with MA and MA+ICA069673.

![Image of LFP traces and spectrograms](image-url)

**Figure 5**

**Activation Kv7.3 channels reduces the levels of Arc and Ca^{2+} in the VTA and NAcLatof MA mice.** a Representative images of Arc expression in VTA neurons. Red retrobeads were injected into NAcLat to label the VTA neurons of the circuit. b Fluorescence intensity of Arc in VTA (n = 6 mice per group, one-way ANOVA, ****p < 0.0001). c Representative images of Arc expression in NAcLat neurons. pscAAV-Hsyn-Cre-tWPA were injected into VTA and pAAV-Ef1a-DIO-EGFP-WPRE were injected into NAcLat to label the NAcLat neurons of the circuit. d Fluorescence intensity of Arc in the NAcLat (n = 6 mice per group, one-
way ANOVA, ****p < 0.0001). e Representative images of GCaMP6s virus expression in VTA neurons. f Summary bar graph of fluorescence intensity of Ca²⁺ in VTA (n = 6 mice per group, one-way ANOVA, ****p < 0.0001). g Representative images of GCaMP6s virus expression in NAcLat. h Summary bar graph of fluorescence intensity of Ca²⁺ in NAcLat (n=7 mice per group, one-way ANOVA, ****p < 0.0001).

Figure 6
Activation of Kv7.3 channels modulates cellular excitability of VTA neurons and DA release in the NAcLat. 

a Schematic of red retrobeads injection and electrophysiological recordings. 
b Experimental timeline of CPP test and electrophysiology recordings. Intra-VTA microinfusion of ICA069673 was performed 15 min before MA injection. Electrophysiological recordings were conducted on the retrobeads-tagged VTA neurons after the post-test (Day 7). 
c Representative action potential traces of the red retrobeads-tagged VTA neurons in the different treatment groups. 
d Phase plots (mVms\(^{-1}\)/mV) of the representative action potential traces for different treatment groups. 
e, f Mean amplitude and frequency of the action potentials from the retrobeads-tagged VTA neurons in the different treatment groups (amplitude (e): n =8 cells/4 mice per group; frequency (f): n =8 cells/4 mice per group, one-way ANOVA, ****p < 0.0001 in (f)). 
g Representative action potential firing in response to current stimulation of 50-150pA in 50pA steps from the retrobeads-labeled VTA neurons. 
h Quantification of the induced spike number of the retrobeads-labeled VTA neurons (n =5 cells/3 mice per group, Two-way ANOVA, **p <0.01, ****p < 0.0001). 
i, j Schematic representation of the electrochemistry recordings. The carbon fiber electrode can oxidize DA to dopamine quinone, and the carbon fiber electrode can detect the current generated by the oxidation. The electrochemistry recording was performed on day 7 after the post-test. 
k Representative traces of current signal recorded by the carbon fiber electrode in the NAcLat. 
l Example of currents of the dopamine standard solutions at different concentrations detected by the carbon fiber electrodes. 
m Calibration curve for dopamine quantification. 
n Quantification of dopamine in the NAcLat (n=7 mice, one-way ANOVA, ****p < 0.0001).
Figure 7

Effects of the activation of Kv7.3 channels on the synaptic plasticity of the VTA-NAcLat circuit. 

Experimental timeline of CPP test. Representative confocal images of dendritic segments in mice injected with viruses rAAV-NCSP-YFP-2E5 into the VTA and treated with MA or MA+ICA069673. Total number of all the different morphologies of spines, and mushroom, thin, and stubby types of spine morphology per 80 mm section (n=7 slices/4 mice per group, one-way ANOVA, ** p <0.01, ****p < 0.0001).

Schematic cartoon of viruses injection and electrophysiological recordings. pscAAV-Hsyn-Cre-tWPA was injected into VTA and pAAV-Ef1a-DIO-EGFP- into NAcLat to trace the NAcLat neurons of the VTA-NAcLat circuit. EPSCs were directly measured on the EGFP-labeled neurons of the NAcLat-containing
slices after the CPP test. **Representative traces of EPSC recordings in the different treatment conditions. g, h** The cumulative probability distribution of (g) average amplitude and (h) frequency of EPSCs recorded from EGFP-labeled NAcLat neurons (n=8 cells/3 mice, Kolmogorov–Smirnov test). i, j Mean amplitude (i) and frequency (j) of EPSCs recorded from EGFP-labeled NAcLat neurons (n=8 cells/3 mice, one-way ANOVA, ****p < 0.0001).
Overexpression of Kv7.3 channels rescues behavioral and electrophysiological changes induced by MA addiction in mice. a-c Schematic picture of Cre-dependent AAV virus strategies used to selectively overexpress Kv7.3 channels in VTA neurons of the VTA-NAcLat circuit. CPP test was performed 2 weeks after the virus injection. d Effect of Kv7.3 overexpression on CPP scores (n=7 mice, Two-way ANOVA, ****p < 0.0001). e Representative images of Kv7.3 expression colocalized with Cre-mCherry-Retro in the VTA. f,g Schematic picture of viruses injection and electrophysiological recordings in MA treated mice. h Representative traces of IM currents evoked by -60mv voltage stimulation in mCherry-tagged VTA neurons. i IM current amplitude (n=9 cells/4 mice per group, unpaired t test, ****p < 0.0001). j Representative action potential traces of the mCherry-tagged VTA neurons. k Representative phase plots (mVms⁻¹/mV) of the action potentials in MA and Ma+Kv7.3 overexpression mice. l, m Mean amplitude and frequency of the action potentials from the mCherry-tagged VTA neurons (amplitude (l): n =8 cells/4 mice per group; frequency (m): n =8 cells/4 mice per group, unpaired t test, ****p < 0.0001).