The Role of SNr KCC2 in Opioid-Related Behavioral Adaptations

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August 2026

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INTRODUCTION

Opioid use disorder (OUD) is a chronic, compulsive disease characterized by persistent opioid use despite negative consequences. OUD affects over 16 million people globally,[1] and 76% of all drug overdose deaths in 2023 were attributed to opioids.[2] Despite this, the neural mechanisms underlying OUD are not fully understood. Recent investigations have identified potassium chloride cotransporter isoform 2 (KCC2) as a modulator of maladaptive inhibitory plasticity in substance use.[3] KCC2 is a neuron-specific anion transporter responsible for maintaining low intracellular chloride (Cl-). In the midbrain, KCC2 has only been detected in GABAergic neurons, therefore implicating changes specifically in inhibitory transmission. Previous investigations have shown that substance use downregulates KCC2 in GABA neurons, leading to irregular dopamine (DA) transmission across multiple reward circuits and ultimately promoting maladaptive behaviors which may perpetuate behaviors associated with OUD.[3, 4, 5] When upregulated, KCC2 has been shown to restore normal behavior, making it a promising target for OUD.[4, 5, 6]

Within the field of substance use disorder research, KCC2 has been characterized most heavily in the ventral tegmental area (VTA). Recent research, however, has highlighted the neighboring substantia nigra pars reticulata (SNr) as a promising new target area.[7] Consisting primarily of GABAergic neurons, the SNr serves as an inhibitory output nucleus for the basal ganglia,[8] and it's understood to be critical for movement control.[8] In particular, it regulates output of the adjacent substantia nigra pars compacta (SNc),[7,9] a dopamine-rich area increasingly implicated in drug reinforcement and relapse.[7,10,11,12] Recent studies have expanded on the SNr's role in addiction to implicate OUD; notably, its GABAergic neurons have been found to express a high concentration of mu opioid receptors, and their pharmacological blockage has a more robust effect on opioid self-administration (SA) than that of analogous receptors on VTA GABAergic neurons.[7] Such findings highlight the SNr as a powerful potential modulator of opioid reward and addiction.

In the current study, we investigated whether morphine self-administration downregulated KCC2 in SNr GABA neurons, and whether KCC2 downregulation in morphine-naive rats was sufficient to produce affective behaviors associated with OUD. Our preliminary *ex vivo* data

demonstrates that chronic morphine self-administration diminishes KCC2 expression in SNr GABA neurons. Additionally, we demonstrate that downregulation of KCC2 in SNr GABA neurons of drug-naive rats produces deficits in several behavioral tasks which may be associated with withdrawal. These findings help elucidate the potential of KCC2 in SNr GABA neurons as a novel target for therapeutic interventions aimed at OUD-induced maladaptive behaviors.

METHODS

Subjects

Male and female wild type and GAD-Cre Long-Evans rats (Harlan-Envigo) weighing 250–500 g were pair-housed in a temperature- and humidity-controlled facility under a 12 hr light/dark cycle. Rats had food and water available *ad libitum*. All animals were handled by experimenters prior to experimental testing. All procedures were carried out in compliance with guidelines specified by the Institutional Animal Care and Use Committee at Georgetown University.

Jugular Catheterization

Surgeries were performed under isoflurane gas anaesthesia. A polyurethane catheter was implanted subcutaneously over the shoulder blade, inserted into the jugular vein and secured with sutures. The catheter was attached to a mesh-back mount platform (Instech Laboratories), which was implanted and secured subcutaneously between the shoulder blades. The catheters were flushed with 0.2 mL of timentin (0.93 mg/mL, Fisher Scientific) dissolved in heparinised saline (1% heparin, Med‑Vet International) daily to maintain patency. When not in use, catheter ports were capped with aluminium obturators.

Morphine Self-Administration

Standard operant conditioning chambers (Med Associates Inc.) were used for all self-administration experiments. After recovery, rats were trained to self-administer morphine on a fixed ratio (FR1) schedule for 12 hours per day (18:00 to 06:00) for 11 days. Rats were able to self-select between an active and inactive lever. Upon pressing the active lever, rats received 5-second infusions of 0.75 mg/kg morphine, accompanied by a cue light illumination directly above the lever, and followed by a 20-second “time-out” period. There were no consequences upon pressing the inactive lever. Persistence of drug seeking despite increased requirements to

obtain an infusion was assessed using a progressive-ratio (PR) reinforcement schedule. Rats underwent 6-hour PR sessions on Days 12, 13 and 14 following the 11-day FR1-schedule self administration. The response requirement for successive morphine infusions within each PR session increased according to a preset breakpoint schedule (1, 1, 2, 2, 3, 3, etc.). The primary variable measured was breakpoint, defined as the highest ratio completed to obtain morphine during the session.

Stereotaxic KCC2 Manipulation

Stereotaxic injection of AAV vectors was used for modulation of KCC2 protein expression in GABAergic neurons within the SNr. GAD Cre rats were used for specific targeting of GABA neurons. Surgeries were performed using a stereotaxic apparatus (Stoelting Co.) under isoflurane anesthesia (2–3%), with body temperature maintained using an isothermal heating pad. To selectively downregulate KCC2 in SNr GABA neurons, GAD-Cre rats received bilateral SNr injections of AAV8-EF1-mCherry-SICO-GFP-KCC2-shRNA. Viruses were expressed for 10 days prior to additional behavioral testing. Control animals received bilateral SNr injections of scrambled shRNA.

Immunohistochemistry and microscopy

Transcardial perfusions were performed under deep isoflurane gas anaesthesia. Animals were transcardially perfused with 50 mL phosphate‐buffered saline (PBS; EMD Millipore), followed by 40 mL of 4% paraformaldehyde (PFA; Chembiotec). Brains were extracted and postfixed in 4% PFA for 2 hours, then immersed in sucrose-PBS solutions of increasing concentration (10% for 2 hours, 20% overnight and 30% until saturation) for cryoprotection. Prior to cryostation, brains were embedded in OCT glue, frozen and stored at –80°C. Coronal sections containing the SNr (40 µm) were cut using cryostation and maintained as free-floating slices in PBS.

After being washed thrice with PBS, slices were incubated with a blocking solution of 3% Normal Goat Serum (Abcam) and 0.3 % Triton X (Abcam) in PBS for two hours. Then, sections were incubated overnight at 4°C with a primary antibody against KCC2 (1:150; Cell Signalling Technology, host species mouse). After washing with PBS, slices were exposed to the fluorescent secondary antibody Goat anti-Mouse IgG Alexa 488 (1:1000; Thermofisher) for 3

hours at room temperature. After incubation, slices were mounted onto glass slides using VectaShield DAPI Mounting Medium, and stored at –20°C

Immunolabelled sections were imaged with a Mica Microhub (Leica Microsystems) confocal microscope at 20x magnification. All illumination settings were automatically adjusted using the OneTouch function in LAS X software (Leica Microsystems), and exported images were further processed with Fiji software (ImageJ).

Behavioral Tests

Sucrose Preference Test (SPT)

The Sucrose Preference Test (SPT) was used to compare natural reward sensitivity between control animals and those with downregulated SNr KCC2. To prevent neophobic behaviors on test day, animals were exposed to 1% sucrose solution for 2 hours on the preceding day. On test day, animals were allowed access to two pre-weighed bottles, one with water and one with 1% sucrose solution, for 2 hours. To account for side bias, the positions of the water and sucrose bottle were switched after 1 hour. At the end of the 2-hour period, both bottles were weighed, and fluid consumption for each bottle was calculated by subtracting its final weight from its initial weight. Sucrose preference was calculated as sucrose consumption out of total fluid consumption, expressed as a percentage.

Elevated Plus Maze (EPM)

The Elevated Plus Maze (EPM) was used to compare anxiety-associated behaviors between control animals and those with downregulated SNr KCC2. Animals were placed on an elevated platform consisting of two opposing exposed (open) arms and two opposing walled (closed) arms. Animals were allowed to explore the maze freely for 10 minutes. Their movement was recorded using OBS with an infrared (IR) camera, and behavior was analyzed for locomotion (total distance travelled), exploration (number of entries into open vs. closed arms) and anxiety-like behavior (time spent in open vs. closed arms). Increased entries and time spent in open arms indicates decreased anxiety-like behavior, while increased entries and time spent in close arms indicates increased anxiety-like behaviors.

Social Interaction

A three-chamber social interaction test was used to compare sociability between control animals and those with downregulated SNr KCC2. Animals were acclimated to the test room for at least 30 minutes before the task. Testing was conducted in an apparatus with three chambers separated by clear dividers, with the left- and right-most chambers each containing a cage for holding conspecific (age- and sex-matched) animals. The task was recorded from above using an IR camera and OBS. The experimental animal was placed into the central chamber and allowed to move freely within the apparatus for 10 minutes. Then, the experimental animal was removed, and an age- and sex-matched conspecific animal was placed into a small, wire cage within one of the peripheral chambers. The chamber holding the conspecific was counterbalanced between animals to control for side bias. The experimental animal was then returned to the central chamber and allowed to freely explore the apparatus for another 10 minutes. Task recordings were analyzed for locomotion (total distance travelled during habituation and test phases), exploration (number of entries into each chamber), sociability (time spent in the conspecific-containing chamber vs. the empty chamber), and social investigation (time spent sniffing or otherwise interacting with the conspecific). Increased time in the conspecific chamber and/or increased interaction with the conspecific qualified greater sociability, total increased time in the empty chamber and/or decreased interaction with the conspecific qualified lower sociability. Social preference index (SPI) was calculated as (time spent in social chamber - time spent in empty chamber) / (time spent in social chamber + time spent in empty chamber).

Looming Task

A looming task was used to compare innate defensive responses (i.e. freezing) to a perceived threat between control animals and those with downregulated SNr KCC2. Animals were placed in a large, transparent cylindrical tube and recorded using OBS. An imminent overhead threat was simulated by a monitor displaying a dark, expanding disk above the tube. The animal was placed in the tube and allowed to habituate for 2 minutes, with the overhead screen displaying a solid grey background. After two minutes, stimulus presentation began, with the screen displaying a black disk extending from 2 to 20 degrees of visual angle over 250 ms. After reaching 20 degrees, the disk remained stable for another 250 ms, and then disappeared. This stimulus presentation was repeated 15 times, separated by 500 ms intervals, over a 22

second period. After the stimulus presentation concluded, the monitor returned to displaying a grey background until the end of the 3rd minute of the experiment. Behavioral Observation Research Interactive Software (BORIS) was used to analyze the duration of freezing before, during and after the looming stimulus.

Pavlovian Reward Learning

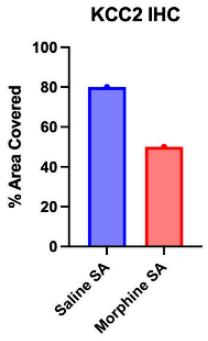
A Pavlovian Reward Learning task was used to compare conditioned cue-reward learning between control animals and those with downregulated SNr KCC2. Prior to behavioral testing, animals underwent mild food restriction to reach 90% of their standard free-feeding weight. Standard operant chambers (MedAssociates) were used for testing. Prior to the experiment, animals were acquainted with the chambers and food retrieval system through a training session, in which 15 sugar pellets (BioServ) were delivered at variable intervals of 30, 45, and 60 seconds. Then, the animals began the conditioning period, in which they completed one Pavlovian session of 50 trials per day for up to 13 days. During each trial, a sugar pellet (the Unconditioned Stimulus, US) was delivered following a 5-second audio cue (the Conditioned Stimulus, CS), alongside a 5-second illumination of a light above the food port. The audio cues and sugar pellets were delivered at variable intervals of 30, 45 and 60 seconds. The control, unpaired conditioning sessions presented conditioned and unconditioned stimuli in random order with large time differences (12-30 seconds) between the two, so as to prevent strong cue-reward associations. Conditioned responding was assessed through Rate Difference, quantified as the difference between port entries during the 5-s CS and 5-s period preceding it.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 10). Two‑tailed t‑tests were conducted to compare group means for IHC data. Repeated‑measures two‑way ANOVA analyses were used to evaluate data from morphine self‑administration and Pavlovian reward learning. ANOVAs were followed with appropriate post hoc tests (with correction for multiple comparisons) when warranted. Statistical significance was defined as $p < 0.05$ for all comparisons. All data are presented as mean ± SEM.

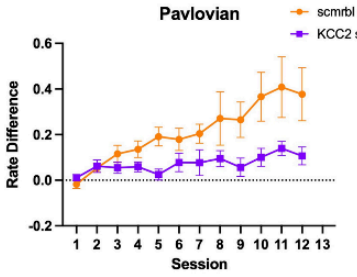
RESULTS

Fig. 1. Preliminary data suggests that SNr KCC2 was downregulated in response to chronic morphine self-administration.



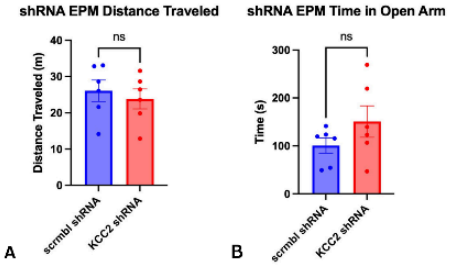
Immunochistochemical analysis shows that a morphine-administering subject has a lower area of KCC2 than a morphine-naive subject. Individaul points represent individual animals (n = 1). Statistical significance cannot yet be calculated; this data represents a preliminary trend.

Fig. 2. Downregulation of SNr KCC2 significantly decreased conditioned responding in the Pavlovian Reward Learning task.



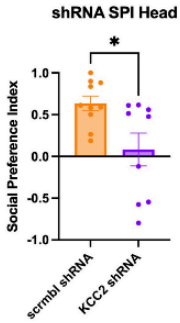
Conditioned responding was quantified as the difference in food-port head-entry rate during the 5-s conditioned stimulus (CS) period and the 5-second pre-CS period (rate difference). Animals receiving KCC2-shRNA showed reduced conditioned responding across training sessions compared with scrambled-shRNA controls. Data are presented as mean + SEM and were analyzed using a two-way repeated-measures ANOVA with treatment and session as factors.

Fig. 3. Downregulation of SNr KCC2 did not significantly affect Elevated Plus Maze behaviors.



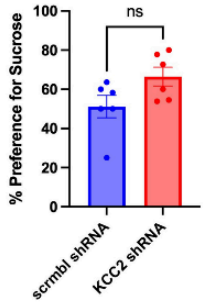
A Animals receiving KCC2-shRNA did not show a statistically significant difference from animals receiving scrambled-shRNA controls in total distance travelled during the Elevated Plus Maze task.
B Animals receiving KCC2-shRNA did not show a statistically significant difference from animals receiving scrambled-shRNA controls regarding time spent in the open arm of the Elevated Plus Maze.
Bars represent mean ± SEM, and individual points represent individual animals (n = 6). ns = not significant ($p > 0.05$).

Fig. 4. Downregulation of SNr KCC2 significantly decreased social preference.



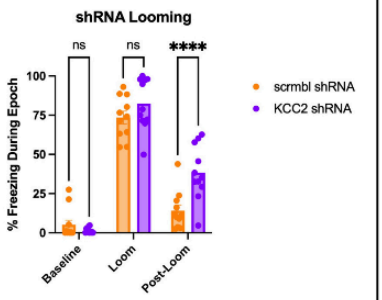
Animals receiving KCC2-shRNA showed a statistically significant decrease in head entries into the conspecific chamber compared to animals receiving scrambled-shRNA controls, suggesting decreased social preference. Social Preference Index (SPI) was calculated as (time spent in social chamber - time spent in empty chamber) / (time spent in social chamber + time spent in empty chamber). A value of -1 indicated a complete preference for the empty chamber, and value of +1 indicated a complete preference for the social chamber. Bars represent mean ± SEM; individual points represent individual animals (n = 10). $p < 0.05$.

Fig. 5. Downregulation of SNr KCC2 did not significantly affect sucrose preference.



Animals receiving KCC2-shRNA did not show a statistically significant difference in sucrose preference compared to animals receiving scrambled-shRNA controls. Bars represent mean ± SEM; individual points represent individual animals (n = 6). ns = not significant ($p > 0.05$).

Fig. 6. Downregulation of SNr KCC2 significantly increased freezing post-loom.



Animals receiving KCC2-shRNA did not show a statistically significant difference in freezing before or during the looming stimulus, but showed a significant increase in freezing after the looming sitmulus, as compared to animals receiving scrambled-shRNA controls. Bars represent mean ± SEM; individual points represent individual animals (n = 11). $p > 0.0001$.

Chronic Morphine Self Administration Downregulates KCC2 in the SNr

To determine the effect of chronic morphine self-administration on KCC2 expression in the SNr, animals underwent 14 days of morphine self-administration. Following the self-administration period, animals' brains were extracted and sliced, and KCC2 protein was labelled immunohistochemically with a primary and secondary antibody. Immunolabelled sections were then imaged and analyzed to determine the percentage of the SNr area including KCC2 protein for saline-administering and morphine-administering animals. Based on preliminary data from a

sample size of n = 1 rat per group (Fig. 1), animals that did not self-administer morphine expressed KCC2 in ~80% of the imaged SNr area, while animals that self-administered morphine had expressed KCC2 in ~45% of the imaged SNr area. Although the insufficient sample size prevents determining statistical significance, this preliminary data strongly suggests that chronic morphine self-administration substantially downregulates KCC2 in the SNr.

KCC2 Downregulation in the SNr Does Not Affect Anxiety-Like Elevated Plus Maze Behaviors

To determine the behavioral effects of isolated KCC2 downregulation, morphine-naive animals received bilateral SNr injections of either KCC2-targeting shRNA or scrambled control shRNA. Following a 10-day expression period, animals underwent a battery of behavioral tests. Elevated Plus Maze testing was used to assess anxiety-like behaviors by measuring total distance travelled and entries into the open vs. closed arms. Animals receiving KCC2-downregulating shRNA did not show a significant difference in either total distance travelled (Fig. 3A) nor time spent in open vs. closed arms (Fig. 3B). An increase in distance travelled or time spent in the open arms would indicate reduced anxiety-like behaviors; this data suggests that KCC2 downregulation neither reduced nor increased anxiety-like behaviors.

KCC2 Downregulation in the SNr Increases Duration of Post-Loom Freezing

The looming test was performed to assess defensive threat responses, particularly freezing, in KCC2- and scrambled-shRNA animals. For mean freezing duration before or during the presentation of the looming stimulus, the KCC2-shRNA animals did not differ from scrambled-shRNA animals (Fig. 6). However, KCC2-shRNA animals exhibited significantly prolonged freezing after the looming stimulus presentation than scrambled-shRNA animals (Fig. 6). This suggests that while KCC2 downregulation may not affect the initiation of defensive responses, it affects the duration of defensive responses to a threat.

KCC2 Downregulation in the SNr Reduces Social Preference

A 3-chamber social preference test was performed to assess sociability in KCC2- and scrambled-shRNA animals. Social preference was quantified by head entries into the chamber containing the conspecific animal. Animals with downregulated KCC2 had a significantly lower

mean social preference index (SPI) of 0-0.1 as compared to scrambled-shRNA animals' mean SPI of 0.5-0.6 (Fig. 4). This suggests that KCC2 downregulation significantly reduces social preference.

KCC2 Downregulation in the SNr Alters Reward-Related Learning

A sucrose preference test was performed to assess natural reward sensitivity in KCC2- and scrambled-shRNA animals. There was no statistically significant difference in percent sucrose consumption and thus sucrose preference between KCC2-downregulated and control animals (Fig. 5). This indicates that KCC2 downregulation does not affect natural reward sensitivity.

Additionally, reward-related learning in KCC2- and scrambled-shRNA animals was assessed using a Pavlovian Reward Conditioning test. While scrambled-shRNA control animals showed a progressive increase in conditioned responding across Pavlovian Reward Learning sessions, KCC2-shRNA animals exhibited consistently lower conditioned responding, ultimately achieving a maximum mean rate difference of ~0.05 as compared to scrambled-shRNA animals' maximum mean rate difference of ~0.4. (Fig. 2). Considering that KCC2 downregulation does not affect reward sensitivity (Fig. 5), the KCC2-shRNA animals' reduction in conditioned responding indicates that KCC2 downregulation specifically impaired animals' ability to learn cue-reward associations.

DISCUSSION

KCC2 has been shown to play a critical role in modulating Cl- homeostasis, inhibitory signalling, and addiction-related behaviors across several substances. Previous studies have established that KCC2 is downregulated in VTA GABAergic neurons following morphine administration,[4] and that KCC2 downregulation in morphine-naive subjects is sufficient to alter cue-reward acquisition in Pavlovian Reward Learning tasks.[13] However, KCC2's role in reward circuitry and addiction as a component of GABAergic neurons within the SNr, an increasingly implicated area in addiction biology, remain underexplored. The present study presents preliminary data that suggests SNr KCC2 is downregulated by chronic morphine self-administration. Moreover, it demonstrates that independent SNr KCC2 downregulation is sufficient to induce behavioral changes including impaired cue-reward association in Pavlovian

Reward Learning tasks, but also decreased social preference and prolonged defense responses, suggesting that SNr KCC2 downregulation can contribute to OUD's alteration of learning and affective behaviors. These findings situate KCC2's established role in modulating chronic opioid use via inhibitory signalling within the new context of the SNr and its downstream effects on movement.

Notably, we employed a self-administering model of morphine exposure rather than passive drug administration models that have been used previously.[14, 15] Volitional drug use has been known to elicit neural adaptations distinct from passive drug administration[16, 17]; moreover, the self-administration model more accurately resembles the dynamic patterns of opioid use in humans, including initial habit development, maladaptive changes in reward processing, and withdrawal states. Such a model gives our study unique translational significance. As such, our findings around SNr KCC2 downregulation following morphine SA and in affective- and reward-related behavioral alternations posits basal ganglia KCC2 as a new, promising area of investigation for OUD mechanisms and therapeutics.

Previous studies have shown that downregulated KCC2 produces a depolarizing shift in $GABA_A$ receptor reversal potential (E_{GABA}), ultimately leading $GABA_A$ receptor ($GABA_AR$) activation to produce an excitatory effect rather than an inhibitory one: in VTA GABA neurons, the reduction in Cl- extrusion following KCC2 downregulation produces intracellular Cl- accumulation, leading $GABA_AR$ activation to have a depolarizing rather than polarizing effect and a causing paradoxical hyperexcitability of neurons normally inhibited by $GABA_AR$ activation. This is significant as GABA neurons in both the VTA and SNr normally act as inhibitory regulators of downstream DA neuron activity, and disrupted DA signalling is a hallmark of substance abuse disorders including OUD. Future studies employing slice electrophysiology are necessary to assess the excitability of DA neurons in the SNr-adjacent SNc, as well as identify additional downstream targets affected by GABAergic SNr signaling, and elucidate the molecular consequences of SNr KCC2 downregulation.

Importantly, we demonstrated that even independently of morphine SA, KCC2 downregulation in the SNr is sufficient to alter behaviors related to social preference, defense responses, and

cue-reward learning. Previous studies observed an acceleration in cue-reward associative learning in the Pavlovian Reward Learning task following VTA KCC2 downregulation.[13] Our findings present a contradictory effect following SNr KCC2 downregulation, which reduced conditioned responding and suggested an impairment in associative cue-reward learning. This indicates that SNr KCC2 may be distinctly necessary for forming cue-reward associations, including those that trigger reward-seeking behaviour in addiction and withdrawal states. Additionally, we demonstrate that SNr KCC2 downregulation decreases social preference. Reduced sociability is a well-described characteristic of negative affective states during withdrawal following chronic morphine exposure[18], which suggests a parallel between reduced social preference following KCC2 downregulation and behaviors seen in morphine withdrawal. Such a connection would be significant: socialization has been shown to reduce drug-seeking behaviors in heroin withdrawal[19], meaning that a KCC2-induced reduction in social preference may diminish an important alternative source of reward during opioid withdrawal. Lastly, our study showed that SNr KCC2 downregulated selectively increased freezing duration following exposure to a looming stimulus, indicating a prolonged defensive response following the introduction and disappearance of a threat. This finding parallels previous observations of impaired fear extinction following chronic morphine exposure,[20] which potentially implicates KCC2 in broader investigations of affective behavioral alterations in opioid use. However, further studies are necessary to establish our behavioral results following SNr KCC2 downregulation as analogous to those observed in OUD. Our future directions include assessing whether KCC2 downregulation following morphine SA produces similar alterations in behavior. If so, additional studies should assess the bidirectionality of SNr KCC2's modulation of behavioral alterations by testing behavior following independent KCC2 upregulation. We aim to investigate this by bilaterally injecting a KCC2-expressing viral vector (AAV5-syn-FLEX-HA.KCC2) into the SNr after a period of chronic morphine SA, and analyzing whether SNr KCC2 upregulation helps rescue behavioral alterations associated with chronic morphine SA. Ultimately, continued research furthering our understanding of SNr KCC2's role in circuit-level and behavioral alterations following chronic morphine use may provide significant insights for developing targeted, sustainable therapeutics rescuing maladaptive adaptations underlying opioid use disorder.

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