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论文题目：Identifying a New Brain Region Involved in Regulating Liraglutide's Suppressive Effects on Alcohol Addiction

Identifying a New Brain Region Involved in Regulating Liraglutide's Suppressive Effects on Alcohol Addiction

Jialin Wang

1. Abstract

People are increasingly afflicted by a variety of addictive behaviors, including online gaming addiction among teenagers, social media and pornography addiction, as well as alcohol and drug addiction, all of which are confirmed as a serious and chronic brain disorders. To date, there are no effective medications to treat these disorders. Through my family life experiences, I observed that regular use of the antidiabetic drug, liraglutide, can effectively reduce alcohol-intake. From the medication guide, I learned that the target of liraglutide, glucagon-like peptide-1 receptor (GLP-1R), plays a key role in the process of blood sugar-lowering and anti-obesity. However, the underlying mechanism by which brain regions with high GLP-1R expression regulate alcohol addiction remains unclear.

In this study, I demonstrated that injection of liraglutide can indeed reduce alcohol addiction behavior in mice model. Subsequently, using the Allen Brain Atlas database, the lateral septal nucleus was identified as a region with high expression of the GLP-1R. Immunostaining revealed robust c-Fos expression in the dorsal lateral septum (dLS) following liraglutide treatment. Notably, Behavioral evaluations showed that chemogenetic activation of GLP-1R^{+} neurons led to a significant reduction in alcohol consumption and seeking behavior.

To the best of our knowledge, this study provides the first evidence that activation of dLS$^{GLP\text{-}1R}$ neurons is sufficient for the attenuation of alcohol-seeking behavior. Our findings highlight the critical role of dLS$^{GLP\text{-}1R}$ neurons in regulating alcohol-related behaviors and underscore the potential therapeutic value of targeting these neurons in the treatment of alcohol addiction.

Key words: Liraglutide, GLP-1R, Dorsal Lateral Septum, Alcohol Addiction

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2. Academic Integrity Statement

I hereby declare that all findings and conclusions presented in this research are based on my understanding and interpretation of the data, conducted under the guidance of my supervisor. To the best of my knowledge, this report does not include any results previously published. All sources, including research articles, data and conclusions, have been properly cited. Any experimental work involving animals was conducted with appropriate ethical approval.

By signing this statement, I affirm my commitment to upholding the standards of academic integrity and acknowledge my responsibility in doing so.

Signature of the contestants: Jialing Wang Date：2024.08.24

Signature of the supervisors: Yingjie Zhu Date：2024.08.24

3. Research background

3.1 The Abyss of Addiction---The brain's reward system

Addiction involves a series of stages, including attraction, temptation, compulsion, and suffering. Nowadays, substances like drugs, gambling, tobacco, alcohol, and even chocolate affect the brain in similar ways **(Figure 1)**. We only consider addiction to have occurred when something takes control of a person's life to the extent that its absence causes severe distress. What makes addiction particularly difficult for the brain to overcome is not only the pleasure derived from the drug, but also the withdrawal reactions experienced after stopping use.

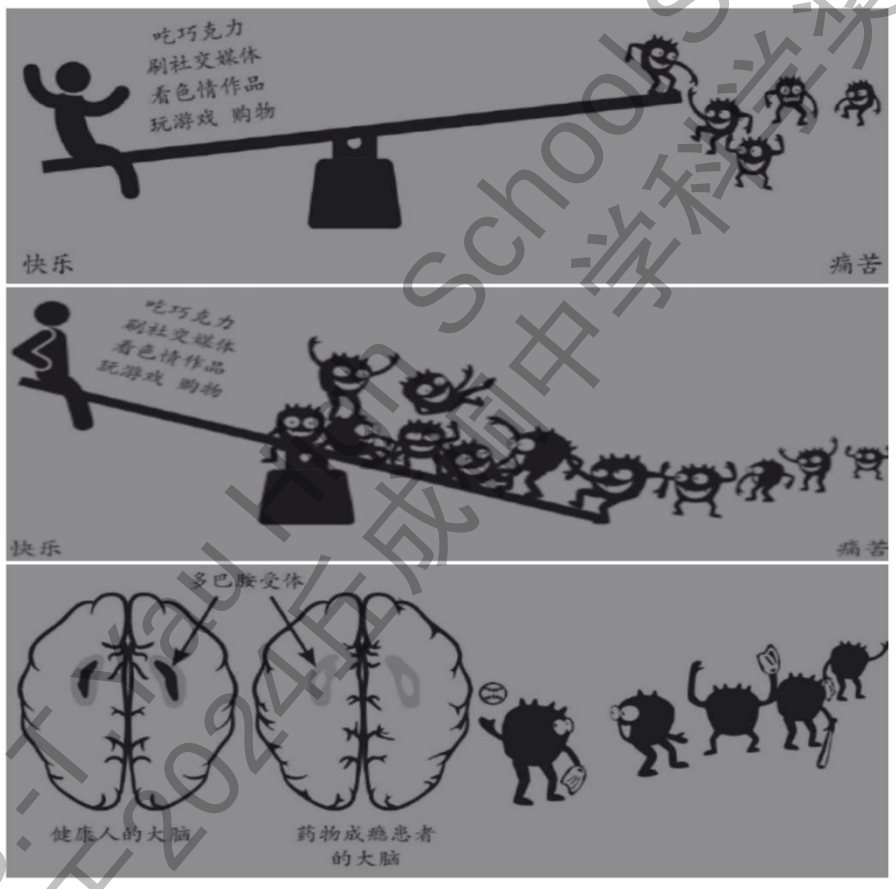


Figure. 1 Addictive behavior disrupt the balance between happiness and pain.

Picture from the book *Dopamine Nation* by Dr. Anna Lembke.

Many individuals with addiction report that they have tried to reduce or quit drug use but have failed, with withdrawal symptoms being a significant barrier to

overcoming addiction. For example, when my grandfather with alcohol addiction suddenly stops or reduces drinking alcohol, he often experience a range of physiological or psychological reactions include headaches, muscle pain, as well as insomnia, fatigue, and difficulty concentrating.

In the early 20th century, doctors and scientists began attempting to understand the mechanisms of addiction(*1*) **(Figure 2A)**. Neuroscientist have now confirmed that addiction is a serious, chronic, and relapsing brain disease(*2*). There is a region in the brain known as the "pleasure center" or "reward system". For example, when a person smokes or drinking alcohol which stimulates the central nervous system to release a chemical called dopamine. Once dopamine is released, the person experiences a pleasurable sensation. The main reward pathways for dopamine include the "mesolimbic pathway," which is the route where dopamine is transmitted from the ventral tegmental area (VTA) in the midbrain to the nucleus accumbens (NAc), and the "mesocorticolimbic pathway," where dopamine is transmitted from the VTA in the midbrain to the prefrontal cortex(*3*) **(Figure 2B)**. Last but not least, there are still some underlying nucleus in the brain that affect our dopamine secretion, which in turn controls our addictive behaviors.

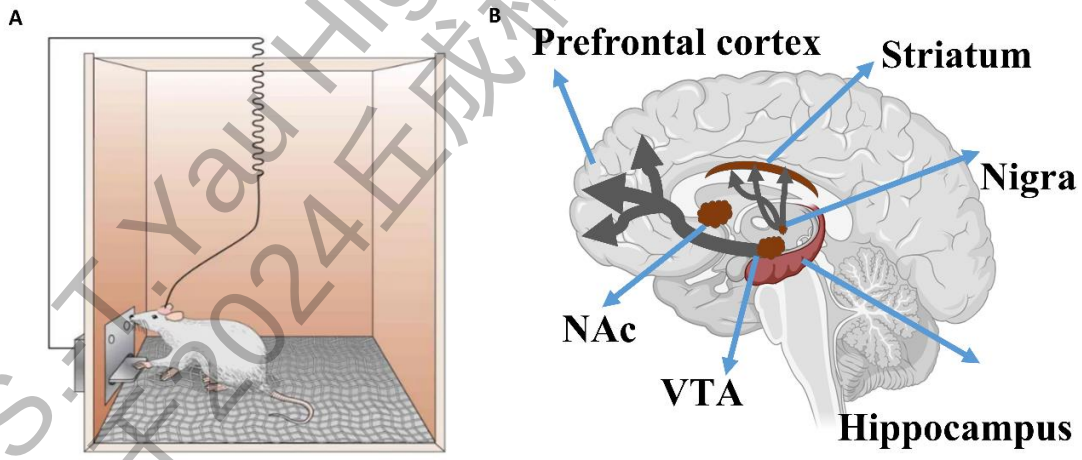


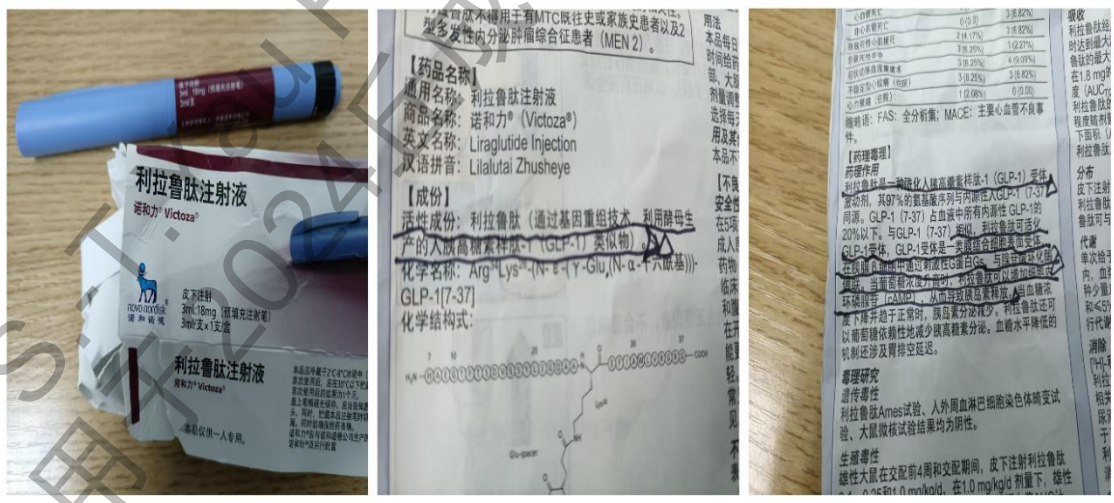
Figure. 2 (A), Electrical self-stimulate by rat. When the rat press the lever, it receive a brief electrical current to an electrode in its brain. Picture from the book *Neuroscience Exploring the Brain*; **(B), The CNS (central neural system) nucleus within the brain for alcohol addiction**, the main reward pathway for dopamine secretion. Picture depicting by myself.

3.2 Liraglutide Modulated Alcohol-addictive Behavior

Inspired by my grandfather's personal medication experience **(Figure 3)**, I learned that Liraglutide is a glucagon-like peptide-1 receptor (GLP-1R) agonists which have been approved in recent years for their role in anti-obesity and lowering blood glucose levels**(Figure 4)** (*4*, *5*). It functions by activating the G protein-coupled receptor GLP-1R. GLP-1 is a versatile peptide that also modulate alcohol related responses(*6*).

Numerous studies by diverse research teams have consistently demonstrated that acute administration of GLP-1R agonists leads to a significant reduction in alcohol preference, not only in rodent models but also in nonhuman primates (*7*-*9*). Moreover, a series of repeated liraglutide treatments, comprising of six injections over two weeks, has also been shown to significantly decrease in both alcohol consumption and preference in rats with a history of alcohol exposure (*10*, *11*). However, the specific functions of GLP-1R within brain associated with alcohol reinforcement remain largely unsolved.

These findings let me wonder whether liraglutide affect mesocorticolimbic pathway within the brain. In other words, the GLP-1 pathway may play a role in regulating the processes of dopamine system which contribute to alcohol addiction (*5*).



Fgure.3 The medication that my grandfather daily take. From the medication guide, I learned that the target of liraglutide, GLP-1R, which is a G protein-coupled receptor.

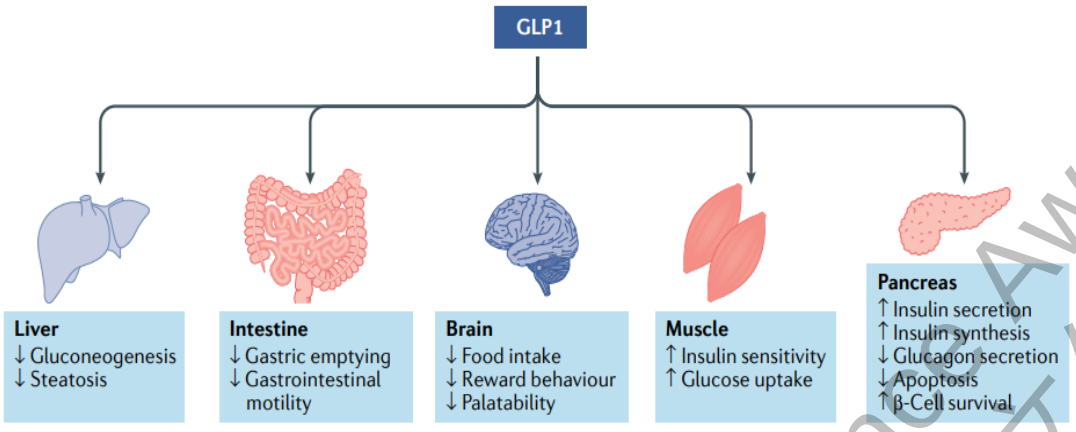


Figure.4 Regulation of body weight and glucose metabolism by GLP-1R agonist.

Picture from (Timo D. Müller et al; Nat Rev Drug Discov, 2022).

3.3 Novel brain region Relative to Alcohol addiction

The key brain region like VTA, NAc, prefrontal cortex, striatum, nigra and hippocampus play a main role in regulating alcohol addictive behavior. Giving the GLP-1R agonist has important function in regulating alcohol addiction, I attempt to search the brain regions with high GLP-1R expression related to alcohol addiction using Allen brain atlatls database. GLP-1R is widely expressed in the brain (*12, 13*). Notably, the functions of regions with a high density of GLP-1R, such as the lateral septum, have yet to be fully elucidated **(Figure.5A)** (*6, 14*).

The LS region has been implicated in reward-related behaviors since the 1950s(*1*).Its complex connections with midbrain dopaminergic system which is an important aspect in studies of drug addiction and alcohol-related behaviors(*15-17*). A recent study has revealed that directly delivering GLP-1R agonists into the LS significantly reduces alcohol self-administration, replicating the effects of systemic GLP-1R agonist administration (*18, 19*).

Although these studies provide evidence for the physiological significance of GLP-1R agonists in the LS in regulating alcohol consumption, little is known about how specific cell types within the lateral septum contribute to alcohol-seeking behavior. And the role of GLP-1R in the LS in mediating liraglutide's efficacy in lowering alcohol cravings remains unclear **(Figure.5B)** (*20*).

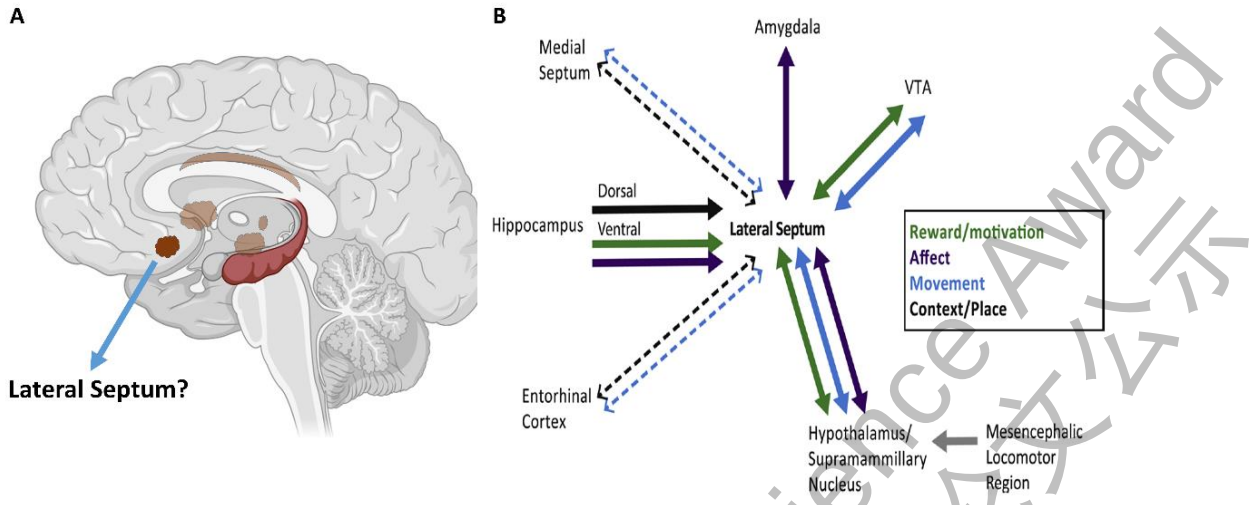


Fig. 5 The LS mediated neuronal networks in modulating reward behavior.

Picture from (Hannah S. Wirtshafter et al; Neuroscience and Biobehavioral Reviews, 2021)

4. Scientific Question and Research Objective

Traditional electrical stimulation and pharmacological approaches unable to distinguish precise neuronal types and traversing fibers that contribute to alcohol-related behavior. Given these methodological limitations, this study is designed to evaluate the effects of GLP-1R agonists: liraglutide, on alcohol consumption and alcohol-seeking behaviors in mice while study the specific processes by which GLP-1R^{+} neurons in the LS influence alcohol drinking behavior.

C57BL/6J mice will be used to establish a model of alcohol-seeking behavior, providing a basis for assessing the influence of liraglutide on these behaviors. The impact of systemic liraglutide administration on the excitability of dLS$^{GLP\text{-}1R}$ neurons will be quantified using c-Fos staining. Chemogenetic approaches will be employed to selectively activate dLS$^{GLP\text{-}1R}$ neurons in transgenic GLP-1R-ires-Cre mice, allowing for an examination of their role in modulating alcohol-seeking behaviors.

Collectively, these data will provide novel insights into the functional role of GLP-1R^{+} neurons in the dLS in reward-related behaviors associated with alcohol and further support GLP-1R as a potential treatment target for alcohol use disorder.

5. Results

5.1 Observation of Mice's Daily Behaviors and Proper Handling

Due to the need to design animal experiments involving mice for this study, my supervisor has instructed me to first carefully observe the normal daily behaviors of the mice and to familiarize myself with their habits. This is to avoid harming the mice and to prevent being bitten by them, as well as to guarantee animal welfare and the reliability of experimental data. Observing mice's daily behavior and handling them correctly are key to conducting animal experiments. I summary these observation in **Table1 and Figure.1.**

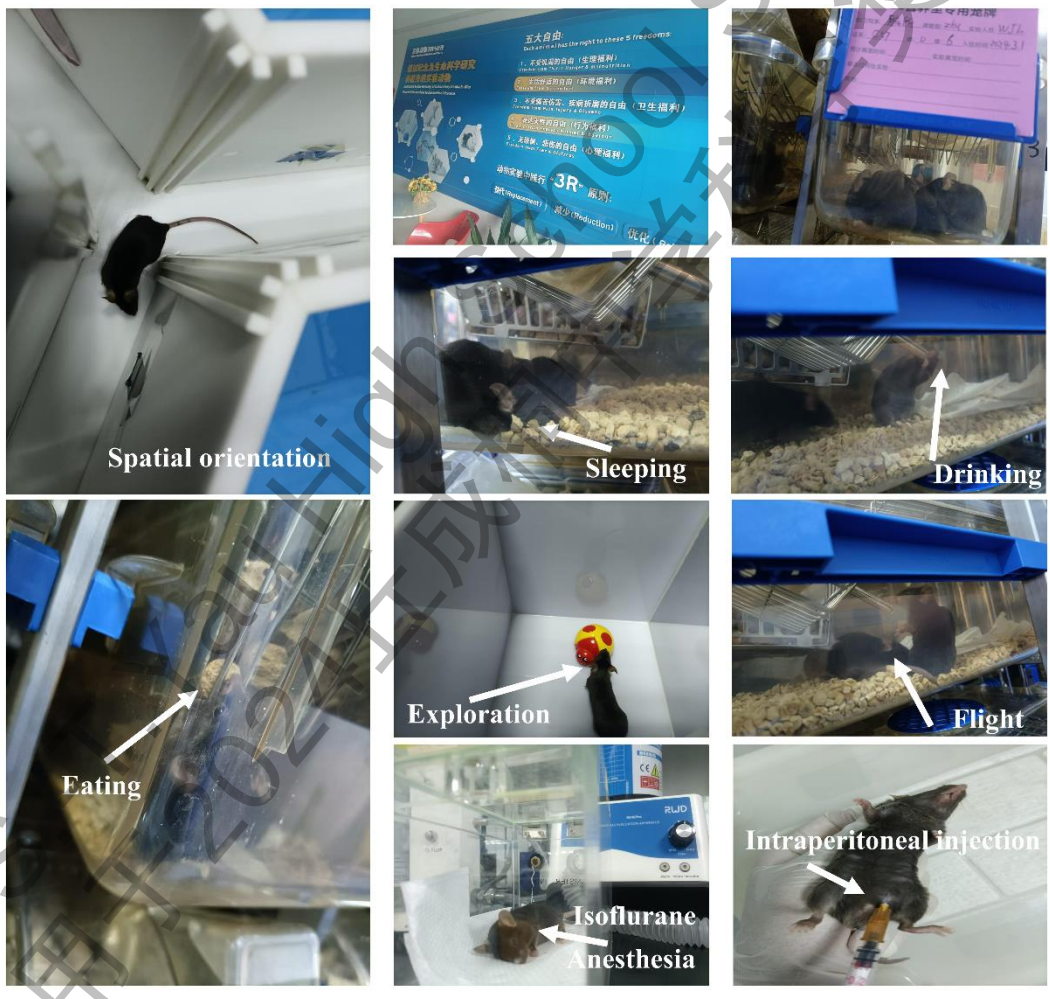


Figure. 1 Observing and becoming familiar with the basic behaviors and handling of mice. Picture are taken by myself from our laboratory animal center.

	Content	Details
Behavior	Eating and Drinking	Primarily during the night, nocturnal animals
	Activity and Exploration	Naturally curious and active at night. They explore their environment, sniffing and interacting with new objects
	Social Interaction	Grooming each other, play-fighting, and sniffing
	Self-Grooming	Mice frequently groom themselves to stay clean and healthy
	Sleeping	Sleep occurs during the day
	Chewing Behavior	Mice enjoy chewing on materials like wood shavings or cardboard
Handling	Holding Techniques	Gently grasp the base of the mouse's tail or use specialized equipment for transfer
	Suitable Environment	Appropriate temperature, humidity, lighting conditions, ample space
	Avoid Startling	Avoid sudden noises or abrupt movements
	Anesthesia	Isoflurane or Sodium Pentobarbital
	Euthanasia	Carbon Dioxide (CO2) Inhalation, Cervical Dislocation, Overdose of Anesthetic Agents

Table. 1 Summarize the basic habits of mice and the fundamental principles for handling them.

5.2 Effective Inhibition of Alcohol-Seeking Behaviors by Liraglutide Treatment

Mice, unlike humans, do not have an inherent preference for alcohol. However, when given access, they develop a preference for alcohol due to its rewarding effects **(Figure 2A)**. The drug self-administration paradigm is an extension of the animal

model used to investigate the determinants and correlates of drug-seeking behavior. This paradigm has proven useful in the development of medications for treating drug dependence.

We utilized a self-administration chambers to train mice. Each chamber contains two levers: an active lever, where presses result in a delivery of 10uL of the alcohol solution, and an inactive lever, where presses are recorded but no programmed events occurred **(Figure 2B)**. Mice were trained to self-administer a 20% alcohol solution in operant self-administration chambers as described (*19*) **(Figure 2C)**. Once a stable baseline of active lever presses was achieved, the animals underwent in vivo behavioral test. In this case, mice had achieved a consistent condition of alcohol consumption after several days of training. On the next day, they were subsequently given 200 μg/kg liraglutide intraperitoneal.

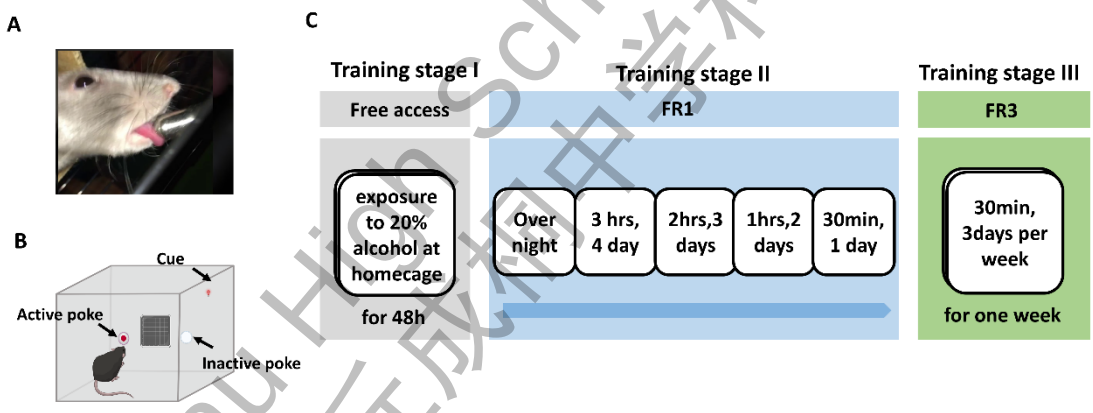


Figure.2 Establish self-administration alcohol delivery paradigm. (A), Representative photos showing mice lick the 20% EtOH solution; **(B),** Depictions of self-administration 20% EtOH solution delivery paradigm equipment ;**(C),** Schematic illustration of the self-administration 20% EtOH solution delivery experiment.

Within 30 minutes of liraglutide delivery, we found that both saline- and liraglutide -injected mice achieved a stable baseline of active lever presses. Importantly, the number of active nose-pokes in the saline group was significantly higher than that in

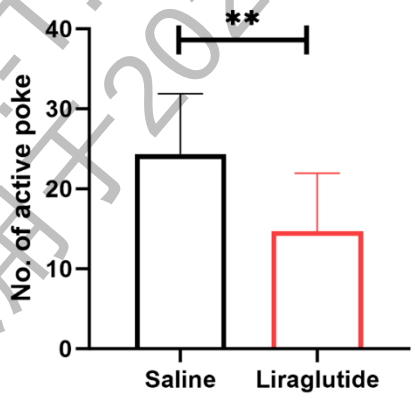
the liraglutide group **(Figure 3A-B)**. Additionally, liraglutide administration significantly decreased alcohol consumption compared to saline treatment **(Figure 3C)**.These results demonstrated that liraglutide medication plays a critical role in suppressing alcohol-seeking and alcohol intake behavior.

A

Mice: Saline i.p	No-active poke	Active poke	Consumption of alcohol (ul)
1	12	40	120
2	7	24	80
3	3	16	50
4	9	24	70
5	14	17	50
6	1	22	70
7	1	22	70
8	2	33	110
9	3	30	90
10	4	30	90
11	3	16	50
12	2	18	50

Mice: liraglutide i.p	No-active poke	Active poke	Consumption of alcohol (ul)
1	7	11	30
2	3	18	50
3	3	12	30
4	3	7	20
5	6	9	30
6	4	13	40
7	3	14	40
8	0	21	60
9	1	8	20
10	7	34	110
11	7	14	40
12	2	15	40

B



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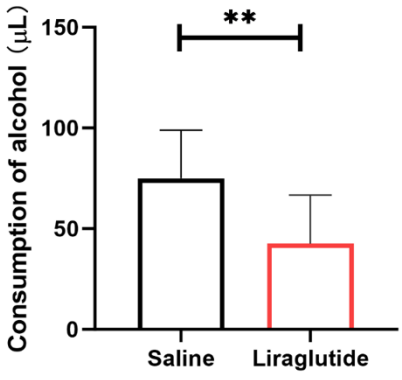

Figure.3 Liraglutide treatment significantly decreases 20% alcohol intake. (A), Recording of alcohol consumption volume and pokes at no-active and active ports; **(B-C),** Compared analysis of the number of alcohol consumption and pokes at active ports after liraglutide treatment. N=12 mice.

5.3 Liraglutide Administration Has No Side Effects on Thirst and Anxiety

Our data showed that liraglutide decreased 20% alcohol intake, prompting us to investigate whether systemic liraglutide injection might affect the mice's motivation to drink liquids, such as water intake. Thus, we evaluated their water intake in a self-administration situation. Interestingly, we found that during treatment condition, all mice exhibited similar water consumption **(Figure 4A-B)**, suggesting that liraglutide has no effect on mice's motivation for water intake.

Given that GLP-1 analogs such as semaglutide and Exendin-4 have previously been shown to increase anxiety-like behaviors in rodents(*21*). We aimed to rule out any side effects of our systemic liraglutide injection on the mice's emotional states, which might affect their appetite. We assessed their anxiety-related behaviors using the open-field test to determine whether the observed differences in drinking behavior were due to mood state changes. There were no significant differences between control and liraglutide administratied mice in terms of the distance of locomotor activity or the duration of time spent in the center zone **(Figure 5A-B)**, indicating our systemic liraglutide injection does not affect anxiety-related behaviors in mice.

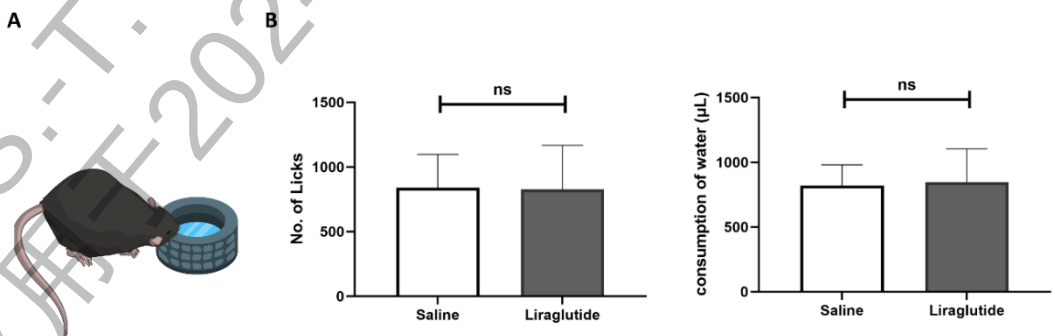

Figure.4 Liraglutide treatment would not affect water intake. (A-B), Liraglutide administration has no impact on either water intake or the number of licking behaviors of mice exhibiting thirst. N=5-7 mice.

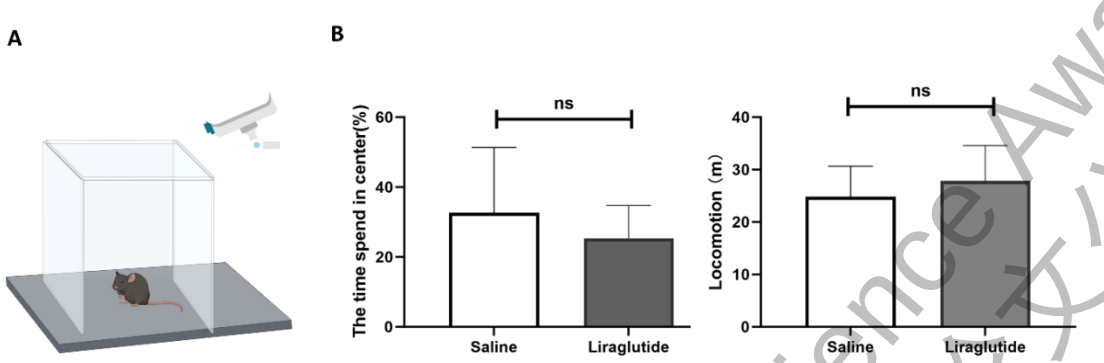

Figure.5 Liraglutide treatment would not affect anxiety levels. (A-B), Scheme depicting the open field test paradigm**,** liraglutide treatment does not influence mice's locomotion and duration in center.

Overall, these findings indicate that, under our experimental conditions, liraglutide's inhibitory effects on alcohol-seeking behavior are unlikely to be due to changes in basal thirst and anxiety states.

5.4 GLP-1R is Highly Expressed in the Dorsal Lateral Septum

Despite our findings that liraglutide treatment significantly suppresses alcohol-seeking behavior in alcohol self-administration paradigm, the exact neuronal mechanisms through which liraglutide affects brain function remain largely unknown. Given that GLP-1R is therapeutic target of liraglutide and its distribution is expressed not only in peripheral tissues but also highly in the central nervous system. Here, we aim to investigate the neuronal mechanism underlying liraglutide mediated alcohol-seeking behavior, with a focus on the functionality of GLP-1R in central neural system.

To explore this, I initial examined the GLP-1R expression within the mouse brain using Allen brain atlas database. We found that GLP-1R mRNA expression is widely distributed throughout the forebrain, striatum, hypothalamus, midbrain and hindbrain. The highest numbers of GLP-1R^{+} neurons were observed in regions associated with

autonomic and behavioral control of energy balance, such as the preoptic area (POA), paraventricular nucleus of hypothalamus (PVN), arcuate nucleus （ARC）, dorsomedial hypothalamus (DMH) and brainstem. Additionally, nucleus associated with addictive behavior, such as Ventral tegmental area (TVA), nucleus accumbens (NAc) and lateral septum (LS), also showed GLP-1R expression (*12, 13*) **(Table 2)**. Notably, highest density of GLP-1R expressing neurons were observed in the dorsal lateral septum (dLS) **(Figure 6)**.

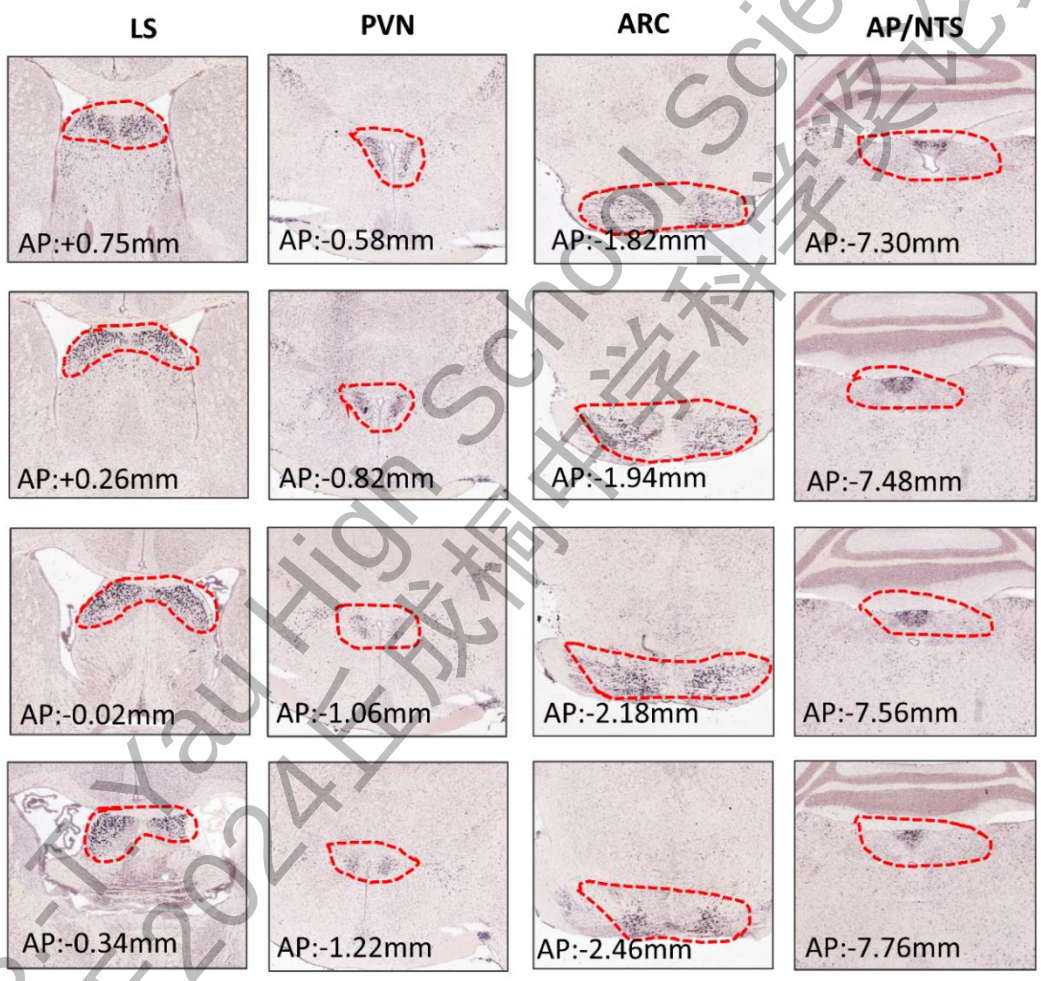

Figure.6 Distribution of GLP-1R expression in key brain region.

Pictures are collected from Allen brain atlas database. https://mouse.brainmap.org/search/show?page_num=0&page_size=26&no_paging=false&exact_match=true&search_term=GLP-1R&search_type=gene. GLP-1R mRNA expression pattern are encircled by red dashed line. **AP:** anterior and posterior; **LS**: Lateral septum; PVN: Paraventricular nucleus of hypothalamus; **ARC**: Arcuate nucleus; **AP**: Area postrema; NTS: Nucleus tracts solitaries.

The LS has recently emerged as a critical node in the neural network underlying drug addiction (*22, 23*). Therefore, we speculated that GLP-1R^{+} neurons within dLS might play a key role in regulating the process of alcohol additive behavior. Consequently, we selected the dLS$^{GLP\text{-}1R}$ neurons as our research target for further elucidation of the neuronal mechanism of liraglutide-mediated suppression of alcohol-seeking behaviors.

Area	Key nucleus	GLP-1R ISH from mouse	Addictive behavior
Forebrain	Hippocampus	+	++
	NAc	+	+++
Striatum	Globus Pallidus	-	+
	LS	+++	++
Hypothalamus	PVN	-	+
	DMH	++	+
	ARC	+++	+
Midbrain	VTA	++	+++
	PAG	++	NA
Hindbrain	NTA	++	NA
	AP	+++	NA

Table2: Overview of key brain region containing GLP-1R expression and involved in alcohol addiction. Data above are summarized from references (*13, 24*). NA, data not available.

5.5 Activation of dLS$^{GLP\text{-}1R}$ Neurons Effectively Diminishes Alcohol-Seeking Behavior

Genetically manipulating the activities of specific neuronal types allow us to dissect the causal relationship between specific neuronal subpopulations and particular behaviors in mice(*25*). Given liraglutide's significant inhibitory effect on alcohol consumption in alcohol self-administration paradigm, we hypothesized that manipulating the GLP-1R^{+} neuronal populations within lateral septum might mimic liraglutide's therapeutic effects. Fortunately, Dr. Zijun Chen told us that we can utilized GLP-1R-ires-Cre mice line to elucidate the specific role of dLS$^{GLP\text{-}1R}$ neurons in modulating alcohol-seeking behaviors. As we have a lot of this mice line in our animal library.

First, I practice stereotaxic injection technique with Evans blue **(Figure 7A-B)**. When the percentage of my correct injection reach above 70%, I started injected a cre-dependent adeno-associated virus (AAV) into the dorsal LS of *GLP-1R-ires-Cre* mice **(Figure 8A)**. AAV was designed to express the excitatory Gq-coupled designer receptor exclusively activated by the designer drug (DREADD), hM3D-mCherry. After three weeks of viral expression, successful and specific hM3D expression was confirmed by mCherry staining in dorsal LS area **(Figure 8B)**.

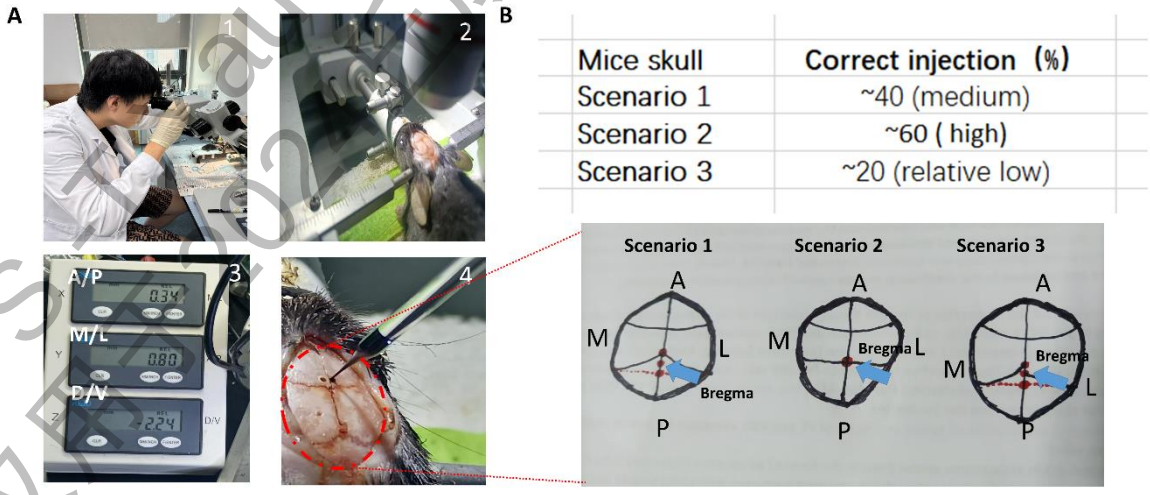

Figure.7 Practice stereotaxic injection technique in wild type mice. (**A),** Representative picture showed process of animal surgeries and stereotaxic injections; (**B),** Calculation of percentage of correct injection site in three different mice skull's scenarios.

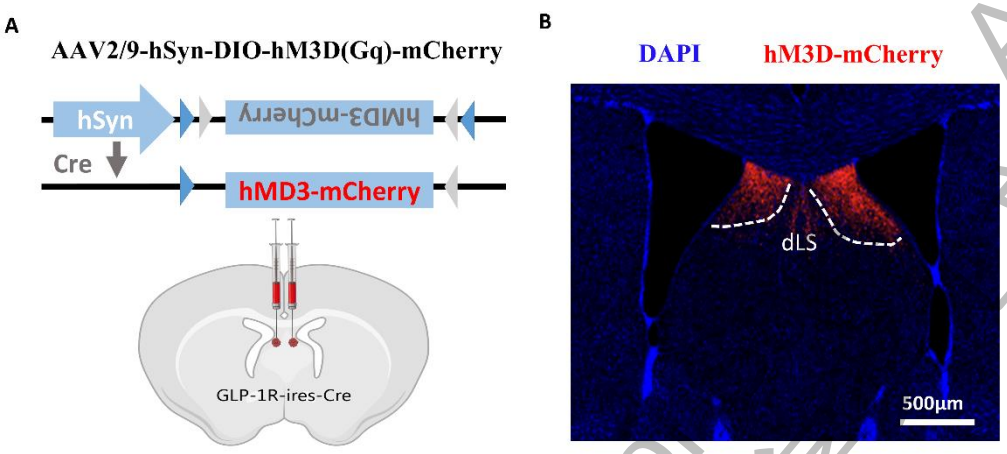

Figure.8 Schematic showing the viral-mediated strategy to express hM3D in dLS$^{GLP\text{-}1R}$ neurons. (A), Depiction of Cre-loxp mediated specific hMD3-mCherry expression in dLS$^{GLP\text{-}1R}$ neurons**; (B),** Representative image showing specific expression of hM3D-mCherry in LS$^{GLP\text{-}1R}$ neurons.

Next, intraperitoneal administration of the DREADD agonist clozapine-N-oxide (CNO), but not saline, resulted in robust c-Fos expression in GLP-1R^{+} neuronal population transduced with hM3D. This strongly indicated that chemogenetic activation indeed increased neuronal activity in dorsal LS **(Fig 9A-B)**.

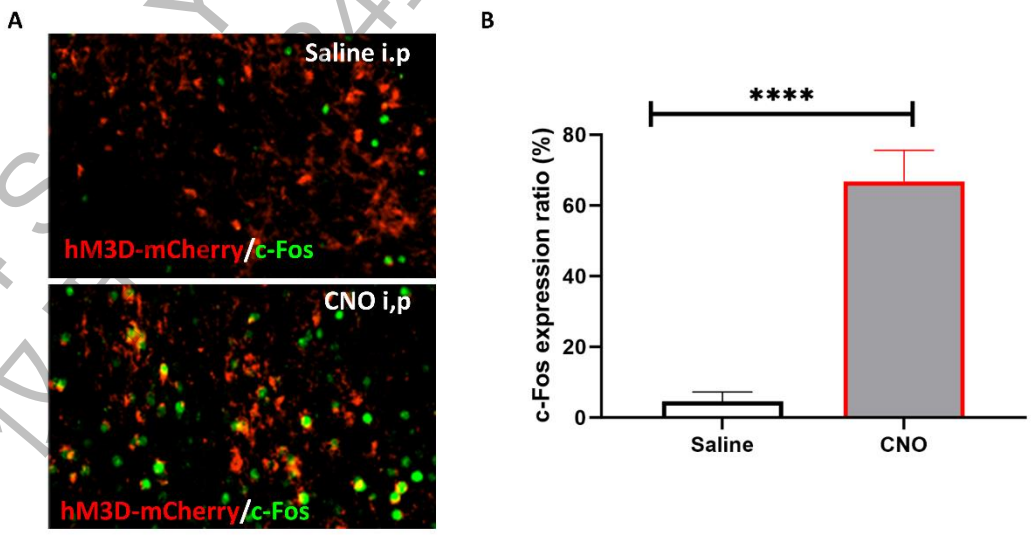

Figure.9 Robust c-Fos expression within LS$^{GLP\text{-}1R}$ neurons after CNO injection. (A), Representative depiction of robust c-Fos expression within dLS$^{GLP\text{-}1R}$ neurons in hM3D-expressing mice post CNO or saline injection; **(B),** A statistical comparison of the percentage of c-Fos^{+} cells among dLS$^{GLP\text{-}1R}$ neurons from hM3D-expressing mouse groups post saline (n=4) or CNO (n=4) administration.

Then, the mice were trained to self-administer a 20% alcohol solution and were randomly divided into two group. Following the FR 3 paradigm, once a stable baseline of active lever presses was established, we recorded baseline activity. The next day, mice received intraperitoneal (i.p.) injections of either saline or CNO (2 mg/kg). All mice showed similar baseline activity in terms of alcohol consumption and number of active nose pokes. Importantly, behavioral evaluations revealed that chemogenetic activation of GLP-1R^{+} neurons by CNO injection resulted in a reduction in both alcohol-seeking behavior and alcohol consumption in mice, with no significant behavioral change observed in saline injection group **(Figure 10A-B)**. These findings demonstrate that activation of GLP-1R^{+} neurons in dorsal LS is necessary for the attenuation of alcohol-seeking behavior. Altogether, GLP-1R^{+} neurons in dorsal LS could be a potentially novel target for developing clinical interventions to treat alcohol addiction.

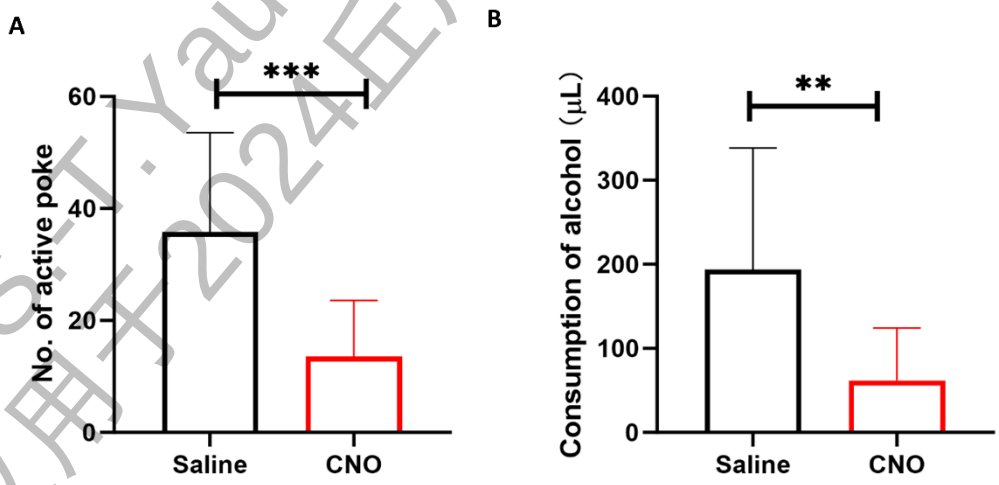

Figure.10 Activation of LS$^{GLP\text{-}1R}$ neurons decreases alcohol consumption. (A), Quantification of alcohol consumption and number of active poke in mice after CNO injection. N=12 mice.

6. Material and methods

6.1 Animals

The mice used in this work were adult male *C57BL/6J* mice (4 months old; Guangdong Medical Laboratory Animal Center, Guangzhou, China), GLP-1R-ires-Cre mice (Shanghai Model Organisms No. NM-KI-200134). Mice were kept at a constant temperature of 22-25°C with a 12-hour light/dark cycle. The Animal Care and Use Committees of the Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS), authorized all experimental protocols.

6.2 Drug and 20% alcohol

Liraglutide (GL Biochem)**,** 2 mg/kg of clozapine N-oxide (CNO, Enzo Life Sciences, BML-NS105), Diluted with pure ethyl alcohol (Sigma-Aldrich, 493511-1L), a solution with an alcohol concentration of 20% was obtained.

6.3 Virus injections

Pentobarbital (80 mg/kg) was used to anesthetize the mice. At a rate of 60 nL/min, stereotaxic injections delivered 200-300 nL of virus into the dorsal lateral septum (coordinates: AP: +0.75 mm, ML: +/- 0.30 mm, DV: -2.30 mm). Mice were given a three-week healing period after injection before beginning behavioral testing.

The information on the virus utilized is as follows: AAV2/9-hSyn-DIO-hM3D (Gq)-mCherry-WPRE-pA (Taitool Bioscience, S0192-9).

The procedure:

Preparation: Anesthetize the mouse using isoflurane. Place the mouse in a stereotaxic frame, securing the head with ear bars to ensure proper alignment.

Positioning: Adjust the mouse so that the skull is level, ensuring both bregma and lambda are on the same horizontal plane.

Site Identification: Identify the injection site using stereotaxic coordinates based on the mouse brain atlas. Measure the distance from the bregma point to determine the correct coordinates (A/P: anterior-posterior, M/L: medial-lateral, D/V: dorsal-ventral).

Skull Preparation: Make a small incision along the midline of the scalp to expose the skull. Using a drill, carefully create a small hole at the identified injection site on the skull.

Injection: Lower the injection needle or cannula to the predetermined depth using the dorsal-ventral coordinate. Slowly inject the AAV into the brain at the target site. The injection should be done gradually to prevent backflow and minimize tissue damage.

Post-Injection: Wait for 10 minutes after the injection to allow diffusion of the substance and to prevent leakage when retracting the needle. Gently remove the needle from the brain. Close the scalp incision using sutures or surgical glue.

Postoperative Care: Monitor the mouse closely for recovery from anesthesia. Provide analgesics if necessary and ensure the mouse is placed in a warm, safe environment until fully awake. Observe the mouse for any signs of distress or complications in the following days. Confirm the accuracy of the injection site using histological analysis if needed.

6.4 Behavioral assays

Mice were kept in housing with free access to food and water before the trials. Mice were housed in an operant conditioning box with a one-port nose-poke system (22 cm x 16 cm x 15 cm, AniLab) for the duration of the study. For the liraglutide treatment or chemogenetic activation experiments, once a stable baseline of active lever presses was established, the animals received intraperitoneal (i.p.) injections of either liraglutide (200 ug/kg) or CNO (2 mg/kg). Usually 30 minutes before the start of the test, 2 mg/kg of CNO was given intraperitoneally. All mice were treated with both CNO and saline vehicles in a counterbalanced approach. Liraglutide (GL Biochem) was administered intraperitoneally 30 minutes before test onset at doses of 200 μg/kg.

Self-administration 20% EtOH delivery paradigm

Mice were conditioned for self-administration of a 20% alcohol solution in specially designed operant self-administration chambers, following the protocol outlined in reference (*19*).

Each chamber was equipped with two types of pokes: an active poke, where nose pokes would dispense 10 µl of the alcohol solution, and an inactive poke, where nose pokes were recorded but did not trigger any programmed events. The training process began with a 48-hour exposure to a 20% alcohol solution in their home cages. This was followed by an overnight session in the chamber, during which pressing the active poke resulted in the delivery of 10 µl of water on a fixed ratio 1 (FR1) schedule.

The operant sessions were then conducted five days a week for two weeks, adhering to an FR1 schedule where an active lever press delivered 20% alcohol, with session durations gradually reduced from three hours to 30 minutes. After the initial two weeks, the sessions were reduced to three days a week for one week, and the schedule requirement was escalated to FR3. The collection of timestamps for both licks and nose pokes was conducted with precision, and the data obtained were analyzed using advanced, custom-developed MATLAB scripts.

In the liraglutide administration experiments, across each protocol, the mice were subjected to a three-day training regimen. This was succeeded by the acquisition of baseline data on the fourth day, and subsequently, the mice received an intraperitoneal injection of liraglutide on day five. Regarding the TeNT mice, these animals underwent a similar training phase spanning three days, with the collection of pertinent data being carried out on the fourth day.

Open field test

On the designated day for behavioral testing, the mice were transferred to the experimental setting and allowed a 3-hour acclimation period before the commencement of the experiment. To eliminate any residual odors from previous sessions, we meticulously cleaned the equipment with a 20% ethanol solution before each test. The behavioral assessment involved placing the mice in a 40 cm by 40 cm square open field arena constructed from plastic. Their movements within this space were precisely monitored and recorded in real-time using the ANY-maze software, which is specifically designed for such analyses. For our analysis, the arena was conceptually divided into two distinct zones: a central square measuring 20 cm by 20

cm, and the remaining peripheral area. Our primary focus was to evaluate the mice's overall locomotor activity patterns, alongside measuring the duration of time they spent in the central zone.

6.5 Preparation of brain sections

Mice were humanely euthanized with an overdose of pentobarbital sodium and subsequently underwent transcardial perfusion with phosphate-buffered saline (PBS, pH 7.4), followed by a 4% paraformaldehyde (PFA) solution. Post-perfusion, the brains were fixed in 4% PFA for 8-12 hours, then dehydrated in a 30% sucrose solution for 24-48 hours or until completely submerged. For preservation, brain tissues were embedded in the Tissue-Tek OCT compound (Sakura) and promptly frozen using dry ice. Brain sections of 40-μm thickness were prepared using a Leica cryostat, and the free-floating sections were stored in PBS.

6.6 Immunostaining procedures and cell counting

For the staining process, the sections initially underwent three 10-minute rinses in PBS. They were then blocked at room temperature with a solution containing 10% normal goat serum (GS) and 0.3% Triton X-100. This was followed by overnight incubation at 4°C with the primary antibody. After washing three times for 10 minutes each with PBST, the sections were incubated for 2 hours with a fluorophore-linked secondary antibody and subsequently counterstained with DAPI (1:3,000 dilution). Imaging was performed using an Olympus Virtual Slide Microscope (VS120-S6-W), and the captured images were analyzed by an evaluator who was blinded to the identities of the experimental groups.

The information on the antibodies used is as follows: anti-c-Fos (9F6) rabbit mAb (1:1,000, Cell Signaling Technology, Catalogue no. 2250), Anti-mCherry rabbit pAb (1:2,000, Abcam, Catalogue no. ab290), Alexa Fluor 488 goat anti-rabbit (1:500, Thermo Fisher Scientific, Catalogue no. A-11008).

To quantify cells expressing c-Fos, mCherry, and GLP-1R markers, we prepared 40 μm coronal sections from specific brain regions of each mouse. These sections were imaged using an Olympus Virtual Slide Microscope (VS120-S6-W). For cell counting,

we utilized a custom MATLAB script. Co-localization of these markers was then assessed through visual inspection.

6.7 Statistical analysis

Means ± SEM were used to summarize the data, as specified in figure legends. Statistical analyses use unpaired two-tailed t test. Significance levels are denoted as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant. Statistical analyses were performed using Prism version 10.1.2 or newer (GraphPad).

7. Conclusion:

Alcohol use disorder is a chronic, relapsing condition with a complex neurobiological mechanisms that are not yet fully understand.GLP-1R agonists like liraglutide, initially approval for obesity treatment, have recently been found to reduce alcohol intake(*11, 14, 26-28*), though the central neural mechanisms behind this effect remain unclear.

In this study, I found a novel brain region with high GLP-1R expression play an important role in regulating alcohol addictive behavior. My research has revealed that liraglutide treatment effectively reduces alcohol-seeking behavior by activating GLP-$1R^+$ neurons in dorsal lateral septum. Activating these neurons alone also decreases alcohol-intake behavior. This highlights the potential of targeting these neurons as a promising strategy for controlling alcohol consumption. GLP-1R not only regulates appetite for maintaining energy balance but also affects hedonic feeding by influencing brain areas related to reward, motivation, and addiction (*29-31*). Studies have shown that GLP-1R agonists reduce various reward-driven behaviors, such as lever pressing for food rewards and alcohol-seeking behavior (*28, 32*). Our findings, which align with other studies, revealed that systemic liraglutide administration reduced alcohol-seeking behaviors in mice without affecting their need for water intake and anxiety states.

Pharmacological approaches, including intracranial injections of GLP-1R agonists into brain areas like the VTA, LDTg, and NTS, have been shown to reduce alcohol intake, though they face challenges such as drug diffusion and limited GLP-1R expression in these regions (*7, 8, 33, 34*). Using transgenic mice and chemogenetic techniques offers a more precise method for study the functionality of GLP-1R subtypes. This strategy enhances the accuracy of research on how GLP-1R affects alcohol consumption behaviors at the neuronal level.

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9. Animal Experiment Ethics Statement

I hereby declare that during the experiment, I will make every effort to minimize harm and discomfort to the animals, strictly adhere to the 3R principles of animal welfare, and ensure the scientific and ethical integrity of the experiment. All experimental procedures will comply with national and local regulations on animal experimentation, and respect the intrinsic value of animal life.

Animal Experiment Principles:

Reduce: We will minimize the number of animals used in the experiment as much as possible, ensuring that the quantity of animals used is kept to a minimum while still achieving the research objectives.

Refine: All reasonable measures will be taken during the experiment to alleviate the animals' pain and stress. For instance, appropriate anesthesia methods will be used to ensure that animals do not experience unnecessary pain during the experiment.

Replace: We will prioritize the use of alternative methods to avoid animal experimentation. If the same educational objectives can be achieved through computer simulations, cell cultures, or other non-animal alternatives, these methods will be adopted as a priority.

Responsibility and Supervision:

This experiment has been reviewed and approved by the Animal Ethics Committee of the Shenzhen Institute of Advanced Technology. Chinese academic of Science Committee of our institution will supervise the entire experimental process to ensure compliance with ethical standards.

Declaration:

I have read and understood the above Animal Experiment Ethics Statement and hereby commit to abiding by all the regulations, respecting, and treating the experimental animals humanely.

Signature: _____

Date: _____

10. Acknowledgements

The idea for this research originated from my observation and reflection on my grandfather personal medication experience. My grandfather's long-term alcohol addiction has severely disrupted the harmony of my family, he also suffered from diabetes these years. To manage his diabetes, he frequently takes liraglutide. Since starting this medication, his alcohol cravings have noticeably decreased, which has been a relief for our family. This change made us curious about why his alcohol cravings diminished after taking this drug. Then I learned from reviewing the medication guide that liraglutide targets the GLP-1R receptor. Interestingly, I head the latest researches on the neural mechanisms of drug addiction from a lecture by Dr. Yingjie Zhu at our middle school's science symposium. This lecture greatly inspired my interest and motivated me to explore how liraglutide medications can influence my grandfather’s alcohol addictive behaviors.

Then I had the chance to establishing communication opportunity with Dr. Yingjie Zhu’s team. Under the thoughtful guidance of Dr. Yingjie Zhu and Teacher Nan Hu from Shenzhen Middle School. I received valuable advice on research ideas and direction. I would like to express my gratitude to Prof. Dr. Zhu for their guidance in designing the animal behavior experiments and preparing research report, as well as to Dr. Zijun Chen, the team membrane from Prof. Dr. Zhu, for their assistance with using Allen brain atlas database and suggest me utilized transgenic mice to study the function of GLP-1R neuron within brain. And many thanks for Dr. Tian Yu, the PhD student of Prof. Dr. Zhu, teach me how to use vibratome sectioning, stereotaxic injections and immunofluorescence staining techniques during which I face many challenges. Particularly, many thanks to my parents, who have consistently encouraged my interest in neuroscience research and supported the creation of a comfortable environment at home for my studies. From my view, this research experience like a journey of understanding the mysteries of the brain during which I identify novel brain region related to alcohol addiction. This study may providing essential foundational research for clarifying the novel targets for developing effective drug to treat alcohol addiction.

Brief introduction to the contestant:

Name: Jialin Wang

Education: Shenzhen Middle School (2022.09-until now)

Position and award:

- Shenzhen High School Artificial Intelligence community: **The president (2024)**
- The Shen Zhen Youth Science and Technology Innovation Competition: **The first prize (2023)**
- The 7th Asian Artificial Intelligence Technology Conference: **Best Student Paper (2023)**
- British Physics Olympiad: **BPHO global Top Gold (2023)**
- Mathematical Association of America: **AMC "Honor Role Distinction" (2023)**

Research and internship experience:

- The HRI Laboratory, Peking University Shenzhen Research Institute (2023-2024): Studied the basics of designing intelligent human-computer interaction control systems.
- The Nerve Plasticity Nobel Laboratory, Shenzhen Institutes of Advanced Technology (2023-2024): Learned techniques in the field of neuroscience to complete the content of this research report.
- The Xikang Welfare School (2023): Volunteered as a teacher, Raised funds and collected supplies for the school.

Brief introduction to the Supervisors:

1, Dr. Yingjie Zhu:

PI in Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (SIAT, CAS), and Recipient of the National Science Fund for Distinguished Young Scholars (2024)

Positions and awards

- Assistant Director of the Institute of Brain Cognition and Brain Diseases, SIAT, CAS
- Executive Director of the Nerve Plasticity Nobel Laboratory
- Director of the Shenzhen Key Laboratory of Drug Addiction
- Chair of the Addiction and Brain Science Branch of the Chinese Association of Drug Abuse Prevention and Control
- Board Member of the Chinese Neuroscience Society
- Vice Chair of the Synapse and Neural Plasticity Branch of the Chinese Neuroscience

- 14th batch of the National High-Level Overseas Talents Recruitment Program for Youth (2018)
- National Science Fund for Distinguished Young Scholars (2024)
- National Science Fund for Excellent Young Scholars (2020)
- Key Project of the Science and Technology Innovation 2030—"Brain Science and Brain-Inspired Intelligence" (2021, as the Project Leader)
- National Science and Technology Progress Award (Second Prize)
- Guangdong Youth Science and Technology Innovation Award, and Shenzhen Youth Science and Technology Award

Dr. Zhu has long been dedicated to studying the neural mechanisms and intervention strategies of drug addiction. His research has led to the discovery of key neural circuit mechanisms responsible for the onset and exacerbation of opioid withdrawal symptoms (Nature, 2016), important circuit mechanisms involved in the formation and maintenance of addiction memory (Science, 2018; Neuron, 2020), and the elucidation of the cellular architecture of the lateral septum and its role in the molecular, cellular, and circuit mechanisms underlying methamphetamine reward (Neuron, 2024).

2, Dr. Nan Hu:

Information technology teacher at Shenzhen Middle School, Host of the Shenzhen Middle School Doctoral Studio for Science and Technology Innovation.

Education: PhD in Electronics and Computer Science from the University of Southampton, UK (sponsored by Chinese Scholarship Council)

Positions and awards:

- Overseas High-Caliber Personnel (level C) in Shenzhen
- Guangdong Excellent Innovation and Entrepreneurship Instructor Award of the 8th China International "Internet+" Innovation and Entrepreneurship Sprout Track Project
- Excellent Instructor Award for the "National Primary and Secondary School Information Technology Innovation and Practice Competition" AI Creator Competition
- Silver Award for Outstanding Supervisor in GBASPC Greater Bay Area Science Project Competition
- Excellent Supervisor for *High School Student Newspaper*