THE UNIVERSITY OF CHICAGO

COMPREHENSIVE ASSESSMENT OF CHOLINERGIC RECEPTOR MRNA EXPRESSION IN CAUDAL NTS GLP-1 NEURONS WITH IMPLICATIONS IN COCAINE REWARD

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ABSTRACT

Cocaine use disorder (CUD) is a chronic and highly relapsing disorder with no FDA-approved therapeutics. Acetylcholine powerfully modulates drug-mediated behaviors, most notably cocaine. The cholinergic system, therefore, has long since been identified as a potential target for pharmacological manipulation in the treatment of CUD. Given the complex neurocircuitry, widespread expression of a myriad of cholinergic receptor subtypes, and confluence of excitatory and inhibitory systems, effective treatment would require precise targeting. There is growing evidence of cholinergic drive to glucagon-like peptide 1 (GLP-1) neurons within the caudal portion of the nucleus tractus solitarius (cNTS) which significantly modulate cocaine reward. The present study uses florescence *in situ* hybridization to provide a full characterization of nicotinic (nAChR) and muscarinic (mAChR) cholinergic receptor mRNA expression on GLP-1 neurons and explores its potential role in modulating cocaine reward. α7 and β2 nAChR subunits were found to be densely co-expressed in nearly all GLP-1 neurons with little to no expression of additional nAChR subunits, suggesting putative α7β2 heteromers. Local infusion of an α7 agonist into the cNTS significantly attenuated the acquisition of cocaine conditioned place preference. Pharmacologically, α7β2 nAChRs are effectively modified α7 nAChRs with highly restricted expression patterns in the mammalian brain. These results implicate α7 expression in the cNTS in the attenuation of cocaine reward with α7β2 nAChRs expression on cNTS GLP-1 neurons potentially serving as a novel site of targeted manipulation of the cholinergic system in the treatment of CUD. All GLP-1 neurons were also found to densely express inhibitory M_2 mAChRs. Cocaine is known to serve as a competitive antagonist of M_2 mAChRs. As such, cocaine mediated disinhibition of GLP-1 neurons via M_2 antagonism may serve to further potentiate GLP-1 driven attenuation of cocaine reward.

“We are mirrors whose brightness, if we are bright, is wholly derived from the sun that shines upon us.” – C.S. Lewis

To the family and friends that have been my sun.

It is because of you that I have, am, and will continue to shine.

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INTRODUCTION

Cocaine use disorder and the need for medically assisted therapeutics

Cocaine use disorder (CUD), the compulsive seeking and use of cocaine despite significant adverse physical, psychological, and societal consequences, remains a severe public health threat. In a recent 2023 study, cocaine was the most misused central nervous system stimulant with 5 million past year users in the United States alone, as compared to prescription stimulant misuse and methamphetamine use[1]. Among people aged 12 or older, 1.3 million people met the Diagnostic and Statistical Manual for Mental Disorders (DSM-5) criteria for CUD[1]. Drug overdose deaths involving cocaine have increased over 300% from 2015 to 2022, in large part due to the adulteration of cocaine with synthetic opiates such as fentanyl[2]. There remains no FDA-approved pharmacological treatment for CUD. Behavioral and psychosocial interventions, such as cognitive behavioral therapy and contingency management, are moderately effective at promoting abstinence, however, nearly 85% of users relapse within a year post-treatment[3]. Given the chronic and highly relapsing nature of CUD and complete lack of pharmacological treatments, critical advancements in therapeutic options are needed.

Brief overview of the neurobiological mechanisms of cocaine reward

The mesolimbic dopamine system fundamentally mediates the rewarding effects of addictive drugs[4]. Dopamine is classically considered the primary active signaling molecule underlying the rewarding and reinforcing properties of addictive drugs[5]. Though they achieve it through distinct pharmacological engagement of the circuity, rewarding substances converge on a common mechanism of increasing forebrain dopamine, thereby enhancing reward and driving addictive behaviors. This circuitry is primarily comprised of dopamine neurons in the ventral

tegmental area (VTA) that project to the nucleus accumbens (NAc)[4]. A dopamine transporter (DAT) is present on dopamine terminals and critically mediates the reuptake of extracellular dopamine. Cocaine acts on the mesolimbic reward system primarily through direct inhibition of DAT, resulting in halted dopamine reuptake and significant increases in dopaminergic signaling[6,7]. As such, further elucidating modulators of the mesolimbic dopamine system is foundational to the development of novel therapeutics for CUD.

The role of cholinergic signaling in drug reward, with an emphasis on cocaine

The ability to engaged in goal-directed motivated behavior is crucial to meeting the basic needs of survival, including food, water, sex, and social interaction. Reward processing, the ability to recognize, evaluate, and respond to stimuli or experiences that are pleasurable or beneficial, fundamentally drives motivated behavior[8]. While mesolimbic dopamine signaling is a cornerstone of reward processing, it relies on a vast and highly conserved set of neuronal circuits beyond the mesolimbic dopamine system that underly the complex relationship between reward, motivation, learning, and memory[9]. Drug misuse is often described as a disease of the brain's reward system wherein drugs hijack this endogenous circuitry leading to aberrant reward processing that drives maladaptive drug mediated behaviors[10].

Acetylcholine acts throughout this circuitry as a key neuromodulator of reward, motivation, arousal, attention, learning, and memory, effectively connecting the essential functions underlying motivated behavior. Unsurprisingly, acetylcholine has a well-documented role in mediating the development and maintenance of drug use, including cocaine[11] (Table 1). Central cholinergic signaling modifies cocaine acquisition and locomotor sensitization through its role in reward-guided decision making, enables cocaine procurement through its effects on

Psychological Function	Site of ACh action	Role of endogenous ACh	Relevance to cocaine reward
Reward	VTA	ACh activation of VTA DA neurons is rewarding[12,13]	M_5 receptors modulate rewarding effects of cocaine[14–17] α7 subunit modulates cocaine-induced potentiation of ICSS[18] β2 subunit mediates cocaine-induced behavioral sensitization[19]
	Striatum	Reward (& potentially satiety) increases NAc ACh[20–23]	Cocaine increases striatal ACh[22,23,20] ACh modulates cocaine-induced locomotor sensitization[24,25] NAc ACh interneuron ablation increase sensitivity to cocaine[25]
	Lateral Habenula	mAChRs modulate LHb neurons[26–28] & LHb signaling mediates negative feedback of reward seeking behaviors[29,30]	ACh implicated in suppression of impulsive responding for cocaine[37]
Associative Learning	VTA	VTA ACh receptor activation modulates conditioned learning[31,32]	M5-deficient rats have attenuated cocaine CPP[15]
	Striatum	ACh implicated in habit learning[33] NAc mAChRs modulate appetitive learning[34] 7/29/25 10:45:00 PM	NAc ACh interneuron ablation enhances cocaine CPP[24,35] mAChR inhibition blocks cocaine induced cue reactivity[36] α7 subunit on ACh neurons mediate cocaine reinforcement[37]
	Amygdala	mAChRs modulate conditional learning[38,39]	mAChRs modulate cue-induced cocaine seeking[40]
Explicit Memory	Hippocampus	ACh involved in the encoding of new information[41–44]	Cocaine increases hippocampal ACh levels[20,45]
Attention & Arousal	PFC	ACh regulates attention and arousal[41]	Human cocaine users have attention deficits[46,47]
Neuroadaptations	Striatum		Chronic cocaine alters mAChR expression and function[48,49]
	Lateral Habenula		Cocaine-evoked synaptic potentiation in the LHb[50]

Table 1. Literature review: role of cholinergic signaling in reward with an emphasis on cocaine

attention and arousal[41], and facilitates cocaine-associated cues and context-dependent sensitization as a mediator of conditional learning. Central cholinergic signaling, particularly in the hippocampus, underlies explicit memory which is critical for the learning of new drug-related information. Cocaine has been shown to increase hippocampal acetylcholine levels, which may strengthen the persistence of drug-related behaviors through enhanced learning and memory[20]. Furthermore, chronic cocaine results in persistent neuroadaptations to the mesolimbic dopamine system which likely contributes to the highly relapsing nature of CUD[48,51–53]. Together these results clearly indicate that the central cholinergic system may serve as a target for pharmacological manipulation in the treatment of CUD. Given the complexity of the system and widespread cholinergic receptor expression, effective treatment would require precise targeting.

Acetylcholine exerts its effects through two primary receptor families: fast-acting nicotinic AChRs and slow-acting muscarinic AChRs providing a complex and flexible modulation of diverse neuronal circuits[54,55].

Nicotinic acetylcholine receptor mediated modulation of cocaine reward

Nicotinic acetylcholine receptors (nAChRs) are fast-acting ligand-gated ion channels. Neuronal nAChRs are pentamers made up of heteromeric or homomeric combinations of alpha (α2-7, α9, and α10) and beta (β2-4) subunits. α7 homomers and α4β2 heteromers are the most common nAChR formations in the mammalian brain[55]. Interestingly, in recent years, there have been growing reports of functional α7β2 heteromers that have distinct pharmacological properties from the typical α7 homomers and β2-containing heteromers that predominate central nAChR expression[56–59]. As such, α7β2 nAChRs may serve as a novel and precise pharmacological target of the central cholinergic system.

Central nAChRs have a well-established and integral role in mediating the rewarding effects of drugs of misuse, including cocaine, primarily through their influence on dopaminergic pathways. Macamalamine, a non-selective nAChR antagonist, attenuates cocaine reward sensitivity as measured by low-dose cocaine-induced CPP[60], cocaine-induced behavioral sensitization[61], and sensitization of cocaine-induced increases in extracellular striatal dopamine levels.[62] Mecamalamine also attenuates cocaine behavioral reinforcement as measured by a reduction in cocaine self-administration[63]. Furthermore, mecamalamine has been shown to prevent cue-elicited cocaine cravings in humans[64].

Modulation of α7 and β2 nAChR subunits specifically, has been shown to significantly contribute to these effects. Inhibition of α7 nAChRs in the VTA has been shown to modulate the reinforcing effects of cocaine as measured by intracranial self-stimulation[18]. More recently, global and striatal cholinergic neuron knock-out (KO) of the α7 subunit in mice was shown to attenuate cocaine-induced CPP and cocaine self-administation[37]. Global β2 KO in mice attenuates low dose cocaine-induced CPP[60]. β2-selective antagonist, dihydo-betaerythroidine (DHβE) but not α7-selective methyllycaconine (MLA) to the VTA prevented the induction of behavioral sensitization to cocaine. Interestingly, co-administration of systemic DHβE and MLA but neither alone replicated mecamalamine induced attenuation of sensitization of cocaine-induced increases in extracellular striatal dopamine levels. Furthermore, striatal dopamine levels were unaffected in global β2 KO mice, but MLA alone was able to reinstate the attenuated striatal dopamine levels in the β2 KO mice[62]. Finally, α7 KO mice are hyporeactive to cocaine while β2 KO mice are hyperreactive to cocaine[65]. These results suggest a complex and region-specific role of α7 and β2 nAChR subunit activation in the modulation of cocaine sensitization and behavioral reinforcement. Given the widespread distribution of nAChR expression

throughout the mammalian brain, further studies on their expression patterns and role in cocaine reward are needed.

Muscarinic acetylcholine receptor mediated modulation of cocaine reward

Muscarinic acetylcholine receptors (mAChRs) are slow acting G-protein coupled receptors and are classified as five distinct mAChR subtypes (M_1-M_5). M_1, M_3, and M_5 are excitatory G_q coupled receptors, and M_2 and M_4 are inhibitory G_i coupled receptors[54]. mAChRs are present throughout the mesostriatal pathway with M_5 receptors primarily in the midbrain[66] and M_1, M_2, and M_4 expression in the striatum[67]. Most notably, the central muscarinic system mediates the rewarding effects of cholinergic signaling in the VTA that critically contributes to the rewarding effects of cocaine and other positive reinforcers[14]. This effect has been shown to be primarily regulated by M_5 mAChRs in the VTA[15–17,68]. Furthermore, M_5-deficient rats show attenuated cocaine-induced CPP[15]. While other mAChR subtypes have been implicated in cocaine reward, reinforcement, and drug related associative learning, their effects are far less characterized[69,70].

Cocaine itself has direct effects on M_1 and M_2 mAChRs where it functions as a competitive antagonist[71–73]. Interestingly, chronic cocaine has been shown to cause persistent neuroadaptations in the central cholinergic system, including altered striatal mAChR receptor density and function, most notably in the M_1 and M_2 subtypes[48,49,52,53]. This cocaine-induced downregulation of the muscarinic system is consistent with adaptive responses to increases in striatal cholinergic signaling in the presence of cocaine. Further studies exploring cocaine-induced neuroadaptations to mAChRs in non-striatal reward relevant brain regions are needed. M_2 mAChRs may be of particular and underexplored interest as human studies have shown

polymorphisms in the M_2 gene (CHRM2) are linked to phenotypes predictive of substance-use disorder[74].

Internal homeostatic state as an understudied modulator of reward

The combined action of nicotinic and muscarinic systems provides a sophisticated regulatory mechanism that finely tunes the reward sensitivity and behavioral reinforcement associated with cocaine exposure. These receptors are widely distributed throughout the brain, however, studies on their role in reward have been largely focused to the striatum, VTA, hippocampus, amygdala, and prefrontal cortex (Table 1). While cholinergic signaling in these regions profoundly connects the reward, learning, memory, attention, and arousal processes needed to drive motivated behavior, there remains a critically underexplored contributor – the organism's physiological internal state. Homeostatic signals like hunger, thirst, and satiety, are critical in guiding motivated behaviors by informing the brain about physiological needs[75]. These internal cues dynamically modulate motivational systems, by adjusting the rewarding value assigned to stimuli and driving behaviors that restore physiological balance[76,77]. A common hallmark of severe addiction occurs when the pursuit of a drug overrides these homeostatic needs, leading the organism to neglect essential physiological requirements in favor of drug procurement and use.

Research exploring the interplay between satisfying homeostatic needs, such as the consumption of food, and the reward sensitivity of drugs of misuse has yielded mixed results. Some studies, however, have shown that addressing nutritional deficiencies and managing food cravings may reduce drug-seeking behaviors[78]. The relationship between satiety and drug mediated behaviors is complex and may vary depending on individual differences and the

specific substances involved. Further research, particularly in brain regions that modulate both adaptive motivated behaviors, such as feeding and body weight maintenance, and maladaptive behaviors, such as drug misuse, is needed.

The cNTS as a potential site of cholinergic modulation of reward

One relatively understudied but potentially critical region for cholinergic modulation of reward is the nucleus tractus solitarius (NTS) in the hindbrain. The NTS is the site of first central synapse of visceral sensory afferents and receives descending inputs from a vast network of higher brain regions and local circuits making it a key integrative relay center in the brain[79]. The NTS is functionally divided into rostral (rNTS) and caudal (cNTS) portions. The rNTS primarily integrates gustatory visceral input and serves as a facilitator of motor responses like chewing and swallowing. The cNTS integrates cardiovascular, respiratory, and gastrointestinal visceral input and projects to a wide variety of brain regions to broadly modulate autonomic homeostasis, emotional responses to internal bodily states, and motivated behaviors, such as feeding[77,79]. The cNTS has direct projections to several key regions in the brain's reward circuit, including the VTA, NAc, hypothalamus, and amygdala[79] (Figure 1). As such, the cNTS is uniquely positioned to serve as a critical hub for integrating interoceptive input and cognitive information in the regulation of motivated behavior, including maladaptive drug mediated behaviors.

Evidence of cholinergic drive to the cNTS

There are a variety of studies that suggest cholinergic drive to and modulation of the NTS, including the cNTS. Putative cholinergic terminals[80,81] and acetylcholinesterase (AChE), the enzyme responsible for the breakdown of acetylcholine, expression[82,83] suggests cholinergic

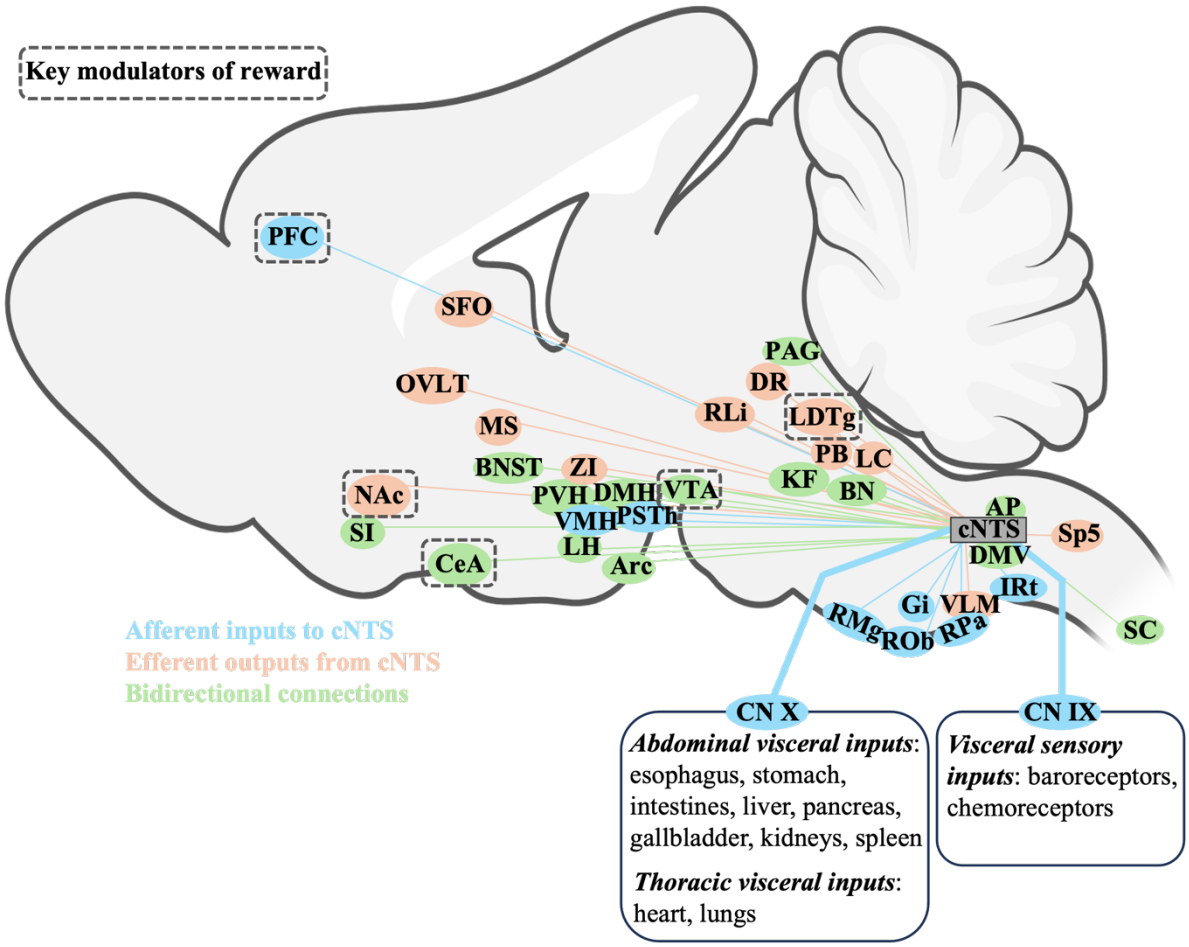


Figure 1. The cNTS is a critical hub for integrating interoceptive input and cognitive information. AP: Area Postrema**;** Arc: Arcuate Nucleus; BN: Barrington's Nucleus**:** BNST: Bed Nucleus of the Stria Terminalis**;** CeA: Central Nucleus of the Amygdala; cNTS: caudal portion of the Nucleus Tractus Solitarius; DMH: Dorsomedial Hypothalamus; DMV: Dorsal Motor Nucleus of the Vagus**;** DR – Dorsal Raphe Nucleus**;** Hipp: Hippocampus**;** Irt: Intermediate Reticular Nucleus**;** KF: Kölliker-Fuse Nucleus**;** LC: Locus Coeruleus**;** LDTg: Laterodorsal Tegmental Nucleus**;** LH: Lateral Hypothalamus**;** MS: Medial Septum**;** NAc: Nucleus Accumbens**;** OVLT: Organum Vasculosum of the Lamina Terminalis**;** PAG: Periaqueductal Gray**;** PB: Parabrachial Nucleus**;** PFC: Prefrontal Cortex**;** PSTh: Parasubthalamic Nucleus PVH: Paraventricular Hypothalamus; RLi: Rostral Linear Nucleus of the Raphel; Rob: Raphe Obscurus; RPa: Raphe Pallidus; RMg: Raphe Magnus; SC: Superior Colliculus; SFO: Subfornical Organ; SI: Substantia Innominata; Sp5: Spinal Trigeminal Nucleus; VLM: Ventrolateral Medulla; VMH: Ventromedial Hypothalamus; VTA: Ventral Tegmental Area; ZI: Zona Incerta.

signaling within the NTS. Cholinergic modulation is further supported by evidence of functional nicotinic and muscarinic receptor expression throughout the NTS[84–90]. Within the cNTS, there is evidence of a wide variety of nAChR subunits, including α7, α5, and non-α7 subunits thought to be either β2 or β4[84,91]. The specific mAChR subtypes present in the cNTS are far less characterized, but thought to consist of primarily M_1 and M_2 with mild M_4 mAChRs[88,90]. Taken together, these results strongly suggest that cholinergic signaling, mediated through both nicotinic and muscarinic receptors, modulates the activity of cNTS neurons.

Given the well-established role of cholinergic signaling in modulating reward-mediated behaviors, including cocaine, evidence that the NTS itself influences reward processing, and evidence of cholinergic drive to the NTS, the NTS emerges as a potential site of cholinergic modulation of reward. A thorough characterization of nicotinic and muscarinic receptor expression and its potential role in modulation of cocaine reward is needed.

cNTS GLP-1 neurons as an emerging therapeutic target for cocaine use disorder

A particularly promising cellular population within the cNTS as a mediator of cholinergic modulation of reward is the glucagon-like peptide-1 (GLP-1) neuron population. GLP-1 neurons are the primary cell type within the cNTS with which the visceral sensory afferents synapse, have a well-documented role in modulating reward, and have been shown to mildly express nAChRs.

Brief overview of the endogenous GLP-1 system

GLP-1 is a peptide hormone that is produced centrally, primarily by a distinct population of neurons in the cNTS and peripherally by enteroendocrine L-cells in the intestinal tract.

Peripheral GLP-1, secreted in response to nutrient ingestion, acts via vagal afferent pathways and through direct endocrine signaling to stimulate insulin release from pancreatic beta cells in a glucose-dependent manner, thereby regulating insulin homeostasis. This peripheral GLP-1 also slows gastric emptying and suppresses glucagon secretion, further contributing to tight control of blood glucose levels following food consumption[92]. Central GLP-1 is the primary satiety signal that regulates both homeostatic need-based feeding as well as hedonic reward-based feeding[93]. As such, GLP-1 receptor agonists are widely used to effectively treat Type 2 diabetes mellitus (T2D) and promote weight loss in individuals with obesity. While the peripheral effects of GLP-1 are profound with the widespread use of GLP-1 drugs rapidly increasing, they are not the focus of the present work. Peripherally released GLP-1 is rapidly broken down with only about 10-15% reaching general circulation, even less of which is thought to cross the blood brain barrier and act centrally[94]. Furthermore, GLP-1 drugs are administered systemically, and growing evidence has indicated some may have poor blood brain barrier penetration[95]. GLP-1 modulation of reward, however, has been robustly shown to be mediated through central GLP-1 receptor activation[96,97]. cNTS GLP-1 neurons are the source of nearly all centrally acting GLP-1[98], and, therefore, will be the focus of the current work seeking to characterize the connection between central cholinergic signaling, GLP-1, and cocaine reward.

GLP-1 as a powerful modulator of drug reward

Central GLP-1 modulation of reward extends far beyond hedonic-reward based feeding. Activation of GLP-1 neurons and central GLP-1 receptors (GLP-1R) has been shown to significantly attenuate drug reward across a variety of drug classes, including cocaine. Substance use disorder is a complex cycle that begins with acute exposure to the drug eliciting psychomotor

activation and drug-induced reward. With repeated exposure, the user develops tolerance, leading to diminished effects and increased motivation to seek out higher doses or more frequent use. This progression marks the transition to chronic drug intake, during which lasting neuroadaptations in reward and motivation pathways reinforce compulsive drug-seeking behaviors. If users achieve a period of abstinence, they typically face withdrawal symptoms, heightened stress sensitivity, and drug-associated environmental cues, factors that significantly increase vulnerability to relapse, thereby perpetuating the addiction cycle[99]. As such, the treatment of substance use disorder is highly complex and effective treatments must address multiple physiological mechanisms that are constantly adapting with ongoing drug use. This is of particular importance for substance with high relapse rates, such as cocaine. Central GLP-1 signaling has been shown to profoundly attenuate drug-mediated behaviors across the entire cycle of addiction and is, therefore, emerging as a promising new therapeutic for substance use disorder (Table 2). The ability of central GLP-1 to ameliorate rewarding and reinforcing effects of cocaine and thereby attenuate cocaine-mediated behaviors is well summarized in the literature[100–102]. Briefly, various systemic and region-specific manipulations of central GLP-1 signaling have been shown to attenuate cocaine intake, psychomotor activation, reward sensitivity, the acquisition and expression of cocaine-induced conditional learning, and the reinstatement of cocaine-conditioned behaviors[100,102] (Table 2).

Possible mechanisms underlying GLP-1 mediated attenuation of cocaine reward

GLP-1 mediated attenuation of reward has been shown to be primarily through modulation of dopaminergic neurotransmission, however, the exact mechanisms are less clear. GLP-1Rs are abundantly expressed in the mesolimbic reward system, including the VTA, NAc,

	Stimulants			Depressants	Opiates	
	Cocaine	**Amph.**	**Nicotine**	**Alcohol**	**Morphine**	**Oxycodone**
Voluntary intake	↓	NA	↓	↓	NA	↓
Reward sensitivity	↓	↓	↓	↓	✗ ↓	NA
Tolerance	NA	NA	NA	↓	NA	NA
Motivation and craving	NA	NA	↓	↓	↓ *unspecified opiate(s)	
Withdrawal symptoms	NA	NA	NA	↓	✗	NA
Relapse	↓	NA	↓	↓	NA	↓

Table 2. Literature Review: GLP-1 attenuates key components of the addictive cycle across multiple drug classes. Green arrow indicates GLP-1 mediated attenuation. Arrows do not indicate degree of attenuation. Red x indicates no GLP-1 mediated effect. NA indicates no known studies. *Unpublished, preliminary data presented at AAAS Annual Conference from clinical trial in patients with opiate use disorder

and lateral septum (LS)[100]. A number of studies have shown that excendin-4, a GLP-1R agonist, attenuates cocaine-induced increases in NAc and LS dopamine levels[103–105]. Excendin-4 has also been shown to attenuate cocaine-induced locomotor hyperactivity, a dopamine-associated behavior[103]. Furthermore, GLP-1 has been shown to positively regulate the surface expression and function of DAT[105]. Inhibition of DAT-mediated dopamine reuptake is the primary mechanism by which cocaine increases dopaminergic signaling[6,7], and DAT expression is inversely related to these cocaine-mediated effects[106,107]. Together these studies suggest that GLP-1 may modulate cocaine reward by regulating cocaine-induced increases in dopamine in part by enhancing DTA expression and function.

Voluntary cocaine intake has been shown to increase activity in cNTS GLP-1 neurons[108], which may suggest an intriguing cocaine-induced GLP-1 dependent negative feedback loop limiting further cocaine intake. This could be interpreted as a form of GLP-1-potentiated cocaine 'satiety' serving to promote termination of cocaine consumption similar to GLP-1-mediated meal termination. One study has demonstrated that cocaine-mediated activation of stress circuits and increased levels of circulating corticosterone contributes to cocaine's ability to activate GLP-1 neurons and potentiate the negative feedback loop[108]. Cocaine has been shown to act directly on both nicotinic and muscarinic receptors[71,73,109]. Most notably, cocaine is a competitive antagonist of inhibitory M_2 receptors which have been shown to be expressed in the cNTS where the GLP-1 neurons are located. These results reinforce the need for a full characterization of cholinergic receptor expression on cNTS GLP-1 neurons, as they may contribute to cocaine-mediated activation of cNTS GLP-1 neurons and subsequent potentiation of a negative feedback loop regulating cocaine intake.

Evidence of cholinergic drive to cNTS GLP-1 neurons

Low and high doses of nicotine, which acts on nAChRs, increases activity in GLP-1 neurons[91]. While it is unknown if this is through direct and/or indirect mechanisms, there is also modest evidence that GLP-1 neurons express α5 containing (α5*) nAChRs. The α5 subunit does not form homomeric nAChR complexes and, therefore, must exist in receptor complex with other nAChR subunits[55]. These results suggest the GLP-1 neurons may be part of an unexplored cholinergic circuit. A full characterization of nAChR subunits as well as potential mAChRs present on the cNTS GLP-1 neurons and the role that these may play in cocaine reward is needed.

Thesis overview and critical knowledge gaps that will be addressed

There is a well-established role of central cholinergic signaling in the development and maintenance of drug addiction. Robust pre-clinical and preliminary clinical studies demonstrate cNTS GLP-1 neurons and central activation of GLP-1Rs attenuates all key components of the cycle of addiction across drug classes, including cocaine. There is also evidence that the cNTS and the GLP-1 neurons specifically are part of an unknown endogenous cholinergic circuit. As such, this thesis aims to anatomically and functionally examine the interplay between cholinergic signaling systems and GLP-1 neurons within the cNTS in regulating reward-related behaviors.

Aim 1: Fully characterize nicotinic and muscarinic receptor expression on cNTS GLP-1 neurons

The purpose of this aim is to provide the first comprehensive characterization of both nicotinic and muscarinic receptor systems on cNTS GLP-1 neurons. This will elucidate a prominent neurotransmitter class of receptors likely modulating GLP-1 neurons that has yet to be

characterized. Given the rapidly growing interest in manipulating the central GLP-1 system for the treatment of substance use, this will provide foundational evidence for potential novel pharmacologic targets by which to modulate cNTS GLP-1 neurons. We hypothesize that cNTS GLP-1 neurons will express robust nicotinic and muscarinic receptor systems.

Aim 2: Investigate the role cNTS cholinergic receptor expression may play in GLP-1mediated attenuation of cocaine reward

The purpose of this aim is to functionally investigate the role that specific cholinergic receptors may play in GLP-1 mediated attenuation of cocaine reward. α7 is the primary nAChR subunit shown to be expressed on cell bodies in the cNTS. α7-containing nAChRs have been implicated in all key functions of GLP-1, including satiety, glucose homeostasis, and food reward. Furthermore, α7-containing nAChRs have been shown to modulate cocaine reward sensitivity and behavioral reinforcement. As such, we hypothesize that engagement of α7-containing nAChRs in the cNTS will modulate cocaine reward.

MATERIALS AND METHODS

Animals

All procedures followed National Institutes of Health Guide for the Care and Use of Laboratory Animal and were approved by the University of Chicago Institutional Animal Care and Use Committee. Mice were group housed on a standard 12 hr:12 hr light:dark cycle under controlled temperature and humidity conditions. Food and water and standard chow available ad libitum. C57BL/6J male mice aged 10-12 weeks were purchased from Jackson Labs (stock number: 000664). Mice weighed 25-30 g at the start of behavioral experiments.

Florescence *in situ* hybridization

RNAscope™ florescence *in situ* hybridization (FISH) was used to detect cell type-specific nACh and mACh receptor expression in preproclucagon $(Gcg)^{+}$ cells in the cNTS.

Fresh frozen tissue preparation

Mice were deeply anesthetized with isoflurane. The whole brain was extracted and immediately flash-frozen on dry ice and stored at -80°C for up to one week. The time between decapitation and freezing of the tissue was approximately 90 seconds to minimize mRNA degradation. On the day of sectioning, brains were removed from -80°C freezer and placed in the -20°C cryostat (Leica Cs3030 S) for 60 minutes to acclimate. Brains were embedded in optimal cutting temperature (OCT) compound (Tissue-Tex, Cat. No. 4583) and sliced into 20µm thick coronal sections. Slices were taken from the cNTS + 0.25mm anterior and posterior (approximately -8.25 to -7.0, relative to bregma) to ensure all GLP-1 neurons were included. Slices were immediately transferred to Superfrost Plus™ Microscope Slides (fisherbrand, Cat.

No. 1255015), and slides were kept in the -20°C cryostat throughout the sectioning process. Upon completion of sectioning, slides were left in the -20°C cryostat for 30 minutes to fully dry and stored at -80°C for approximately 12-24 hours until the hybridization protocol was performed the following day. All surfaces and tools used for extraction and sectioning were treated with RNase Away (MβP, Cat. No. 7002) to minimize RNAse contamination during tissue preparation.

Probe and opal dye preparation

cNTS GLP-1 neurons were labeled using the mRNA probe for preproglucaton (Gcg), the gene that encodes for the polyprotein that ultimately gets cleaved into the GLP-1 peptide and other fragments[110]. Table 3 describes the nAChR subunit and mAChR mRNA probes that were used to test for cholinergic receptor expression in Gcg^{+} cells. One day prior to conducting the hybridization assay, the probes were removed from the 4°C fridge and warmed in the HybEZ II Hybridization System oven at 40°C for 10 minutes then allowed to cool to room temperature (RT) for 10 minutes. Probes were mixed in a 50 (channel 1):1 (channel 2):1 (channel 3) ratio. Each round included Gcg in channel 3 and receptor probe(s) in channel(s) 1 and/or 2. If a channel 1 receptor probe was not used, RNAscope™ Probe Diluent (Cat. No. 300041) was used to dilute the channel 2 and 3 probes. Probe mixtures were stored at 4°C overnight. Probes were visualized using Opal 520, Opal 570, and Opal 690 (Akoya Biosciences, Cat. No. OP-00100, OP-001003, OP-001006, respectively). One day prior to conducting the hybridization assay, the Opal dyes were diluted to a 1:750 concentration using RNAscope™ TSA Buffer (Cat. No. 322809). Diluted dyes were stored at 4°C overnight.

Protein	Abbrev.	Gene name	ACD Cat. No.
Preproglucagon	Gcg	*Mm-Gcg*	400601
α3 nAChR subunit	α3	*Mm-Chrna3*	449191
α4 nAChR subunit	α4	*Mm-Chrna4*	429871
α5 nAChR subunit	α5	*Mm-Chrna5*	312571
α7 nAChR subunit	α7	*Mm-Chrna7*	465161
β2 nAChR subunit	β2	*Mm-Chrnb2*	449231
β3 nAChR subunit	β3	*Mm-Chrnb3*	449201
β4 nAChR subunit	β4	*Mm-Chrnb4*	452971
M_1 mAChR	M_1	*Mm-Chrm1*	495291
M_2 mAChR	M_2	*Mm-Chrm2*	495311
M_3 mAChR	M_3	*Mm-Chrm3*	437701
M_4 mAChR	M_4	*Mm-Chrm4*	410581
M_5 mAChR	M_5	*Mm-Chrm5*	495301

Table 3. mRNA probes used in RNAscope™ florescence *in situ* hybridization experiments

Hybridization assay

The hybridization assay was conducted according to the Advanced Cell Diagnostics (ACD) RNAscope™ Multiplex Florescent Reagent Kit v2 (Document Number UM 323100) with slight modifications. The assay was run using the RNAscope™ Multiplex Florescent Detection Reagents V2 (Cat. No. 323110). All reagents in the kit were brought to RT prior to using. All wash buffer washing steps consisted of gently agitating the tissue for 2 minutes at RT in RNAscope™ wash buffer (Cat. No. 310091) and then repeating a second time with fresh wash buffer. (1) *Fixation*: Slides were removed from the -80°C freezer and immediately placed in a Tissue-Tek slide holder and submerged in pre-chilled 4% PFA and placed at 4°C for 30 minutes. (2) *Dehydration*: The tissue was then dehydrated at RT in a series of EtOH dilutions for 5 minutes per dilution: 50% EtOH, 70% EtOH, and twice in 100% EtOH. Slides were removed from Tissue-Tex holder and allowed to airdry on paper towel for 2 minutes. (3) *Hydrophobic barrier*: A hydrophobic barrier was drawn around the tissue using a hydrophobic pen (Vector Laboratories, Cat. No. H-4000) and dried for 2 minutes. (4) *Hydrogen peroxide*: The hydrogen peroxide step described in the instruction manual was skipped as it appeared to impact tissue quality and adherence to the slide. (5) *Protease*: The slices were placed in the ACD EZ-Batch™ Slide Holder and treated with a protease (Protease IV) to permeabilize the cells allowing for better probe penetration and incubated for 25 minutes at RT followed by 2 washes in deionized H_2O. (6) *Probe hybridization*: Probe mixtures were warmed at 40°C for 10 minutes then allowed to cool to RT for 10 minutes. Probe mixtures were pipetted onto the slides until tissue was fully covered (approximately 50µL per slide). The slides were placed in the HybEZ Humidity Control Tray (Cat. No. 321721) and incubated for 2 hours at 40°C in the HybEZ oven, followed by wash buffer washes. (7) *Signal amplification*: The slides then went through a series of signal

amplification steps where the tissue was treated with Amp 1, Amp 2, and then Amp 3 and incubated at 40°C in the oven for 30 min, 30 min, and 15 min, respectively. The slides were washed with wash buffer after each amplification step. Following the post-Amp-3 washes, the tissue underwent a series of horseradish peroxidase (HRP) steps to achieve customizable channel-specific labeling. The channel 1 HRP (HRP-C1) was applied to the tissue and the slides were incubated at 40°C in the oven for 15 min, followed by wash buffer washes. The Opal dye selected for labeling of the channel 1 probe was then applied to the tissue and the slides were incubated at 40°C in the oven for 30 min, followed by wash buffer washes. The HRP blocker was then applied to the tissue to quench the HRP and the slides were incubated at 40°C in the oven for 15 minutes, followed by wash buffer washes. The HPR-Opal dye-HRP blocker series was then repeated for channels 2 and 3, using the corresponding HRP and desired Opal dye for each channel. (8) *DAPI and coverslip*: Following the washes after the final HRP-blocker step, the slides were removed from the ACD EZ-Batch™ Slide Holder and placed on a paper towel. DAPI was applied to each slide for 30 s and then knocked off the slide. A dropper was used to immediately put 2-3 drops of ProLong Gold Antifade Mountant (Thermo Fischer Scientific, Cat. No. P36930) on the slide before placing a coverslip (fisherbrand, Cat. No. 12541023) over the tissue. Slides were stored in the dark at 4°C until imaging the subsequent day.

Imaging

Images were taken from approximately 8 slices evenly spaced along the anterior posterior axis of the cNTS from 2-3 mice for each probe combination. Images were taken approximately 12 and no more than 24 hours after the completion of the hybridization assay to ensure maximal and consistent signal intensity across independent FISH assays. All images were collected on a

Marianas spinning disk confocal microscope with 3i software. Images used for quantification of Gcg and receptor co-expression were taken with using a 40x oil objective (Zeiss Plan-Apochromat NA 1.3 oil). Images used to illustrate Gcg expression patterns in the cNTS were taken with a 20x dry objective (Zeiss Plan-Neofluar NA 0.5 air). All images across independent rounds of FISH were taken with similar microscope setting, mostly notably, intensity and exposure times. Each round of FISH included a positive control for each channel (C1: *Polr2a*, C2: *Ppib*, C3: *Ubc;* RNAScope™ Cat. No. 320881) and a negative control (*Dapb;* RNAScope™ Cat. No. 320871) which were used to verify signal and set microscope setting such that signal was present in the positive controls and absent from the negative control. Florescence was detected using the following filter sets: DAPI (ex 358 nm/em 461nm), GFP (ex 488 nm/em 525 nm) for Opal Dye 488, Texas Red (ex 561 nm/em 595 nm) for Opal Dye 561, and Cy5 (ex 680 nm/em 700nm) for Opal Dye 690.

Quantifying co-localization of receptor mRNA and cNTS GLP-1 neurons

FISH images were analyzed using QuPath-0.5.1-x64. Nuclei were identified using the DAPI/405nm channel and the QuPath *Cell Detection* feature which performs a threshold and size-based nuclear detection. The detection threshold was empirically optimized to distinguish true nuclear signal from background. Nuclear size detection parameters were set to a minimum nuclear area of 10 μm^2 and maximum nuclear area of 400 μm^2. A Gaussian smoothing sigma of 1.5 μm was used to reduce high-frequency noise and improve nuclear shape detection. Closely packed nuclei were resolved based on intensity peaks using a distance-based watershed algorithm. Following nuclear detection, cell boundaries were estimated by expanding each nucleus outward by 10 μm, resulting in an average total cell diameter of approximately 20 μm.

Following cell detection, the QuPath *Subcellular Detection* tool was applied within each previously segmented cell to detect individual mRNA puncta based on local intensity maxima and size constraints in the 488nm, 561nm, and/or 638nm channels. Signal intensity detection thresholds were empirically determined for each channel based on negative control tissue and overall background signal, typically falling within the range of 200–300 arbitrary units. The expected spot size was set to 1 μm^2 with a min spot size of 0.5 μm^2 and maximum spot size of 2 μm^2. Small-radius Gaussian smoothing with a prefixed sigma was used to reduce high-frequency noise and improve the separation of discrete puncta. Closely packed puncta were resolved based on both intensity and shape. Detected puncta were automatically assigned to individual cells based on spatial overlap with the previously defined cell boundaries. Puncta counts per cell in the 488nm, 561nm, and/or 638nm channels were exported.

In each individual round of FISH, GLP-1 neurons were identified using the Gcg probe in channel 3 and visualized with the 488nm channel. When one nAChR subunit or mAChR mRNA probe was multiplexed with the Gcg probe, it was assigned to the 638nm channel. When two nAChR subunit probes were multiplex with the Gcg probe, the 561nm and 638nm channels were used. Gcg^+ cells were easily identifiable, as Gcg puncta filled nearly the entire area of the cell with very low background. For receptor expression, if a Gcg^+ cell had 4 or more mRNA puncta for a given receptor probe, it was classified as positive for that corresponding nAChR subunit or mAChR.

MERFISH spatial transcriptomic Allen Brain Cell Atlas

To assess the external validity of our FISH-based findings, we queried the publicly available MERFISH spatial transcriptomic Allen Brain Cell Atlas[111]. We identified cNTS GLP-1

neurons by filtering for cells expressing *Gcg* mRNA. We then extracted expression levels for all available nAChR subunits (*Chrna3*, *Chrna4*, *Charna5*, *Chrna7*, *Charnb3*, *Chrnb4*) and mAChR subtypes (*Chrm1*, *Chrm3*, *Chrm3*, *Chrm5*) from the Gcg^{+} cells, allowing for targeted evaluation of cholinergic receptor expression in GLP-1 neurons. The gene expression values reported in the MERFISH interactive atlas reflect log-normalized imputed expression levels, calculated by integrating spatial transcriptomic data with single-cell RNA sequencing profiles using a nearest-neighbor approach. For each MERFISH cell, the expression of non-measured genes is estimated as a weighted average from the 30 most similar cells in the scRNA-seq dataset, and values are $\log_1$p-transformed (i.e., log(1 + normalized count)).

Drugs and reagents

For cocaine conditioned place preference, Cocaine hydrochloride C-II (Sigma-Aldrich, CAS No: 53-21-4) was dissolved in sterile saline. For local infusion, selective α7 agonist, PHA 543613 hydrochloride (Tocris, CAS No: 1586767-92-1) was dissolved in sterile saline.

Intra-cNTS infusions

Surgical implantation of local infusion cannula

For local delivery of the α7 nAChR agonist PHA-543613, mice were chronically implanted with a unilateral guide cannula (PlasticsOne, 26GA, 6 mm, C315GS-5-SP) targeting the cNTS. Surgeries were performed under isoflurane anesthesia (1–2% in oxygen), and cannulas were stereotaxically positioned to terminate 1 mm dorsal to the cNTS. Guide cannulas were affixed to the skull with dental acrylic and miniature screws. Dummy internal cannulas

(PlasticsOne, 33GA, C315DCS-5-SPC) fitted with screw caps were inserted into the guide cannula between infusions to maintain patency.

α7 agonist injection

On infusion days, the dummy internal cannula was removed and an internal injector (PlasticsOne, 33GA, C3151S-5-SPC) extending 1 mm beyond the guide cannula was inserted to reach the cNTS (final depth –5.0 mm). PHA-543613 (0.3 µL per infusion) or vehicle was delivered over 60 s using a microsyringe pump (Chemyx Inc.) with the injector left in place for an additional 60 s post-infusion to minimize backflow. The internal injector was removed, and dummy internal cannula replaced. Mice were immediately given i.p. injection of cocaine (20mg/kg) and proceeded with cocaine conditioned place preference (CPP) conditioning sessions.

Cocaine conditioned place preference

Prior to testing, mice we handled and habituated to injections for 3 days. A three-chamber apparatus (Med Associates) was used to assess CPP in a real-time assay. Two larger chambers (16.8x12.7x12.7 cm) were connected by a smaller intermediate chamber (7.2x12.7x12.7 cm). Each chamber had a unique combination of visual and tactile properties. One of the large chambers had black walls and a rod floor, and the other large chamber had white walls and grid mesh floor. The small intermediate chamber had gray walls and solid smooth floor and served as a neutral pathway between the two larger test chambers. Chambers were connected by vertical sliding doors that were used to confine the mouse to the larger compartments on conditioning days. Each chamber had a light embedded in a clear, Plexiglas lid. Time spent in each chamber

was measured via photobeam breaks as the mouse passed between chambers.

Mice were divided into two groups: (1) mice that received local infusion of selective α7 agonist PHA-534613 to the cNTS immediately prior to each conditioning session and (2) mice that received local infusion of saline to the cNTS immediately prior to each conditioning session. The behavioral paradigm consisted of three sequential phases: pre-test, conditioning, and test. *Pre-test phase*: on day 1, mice were allowed to freely explore the entire apparatus for 20 min to habituate to the chambers. Five hours later mice were again allowed to freely explore the entire apparatus for 20 minutes. Mice that spent more than 500 s in the grey intermediate compartment or more than 800 s in either of the large compartments during the second free exploration session were excluded from the study. *Conditioning phase*: on days 2-5 mice underwent twice daily conditioning sessions separated by five hours. During the morning session, mice received local infusion of PHA-534613 or saline to the cNTS (as described above) immediately followed by an i.p. injection of saline and were confined to the black chamber for 30 min. Mice were then returned to their home cage for five hours. During the afternoon session, mice received local infusion of PHA-534613 or saline to the cNTS (as described above) immediately followed by an i.p. injection of cocaine (20 mg/kg) and confined to the white chamber for 30 min. *Test phase*: on day 6, mice were again allowed to freely explore the entire apparatus for 20 min. Change in preference score was calculated as time spent in cocaine paired side – time spent in saline paired side on test day.

Local infusion cannula placement verification

Upon completion of the cocaine CPP behavioral paradigm, placement of the local infusion catheter was verified. Mice were deeply anesthetized with isoflurane and decapitated.

Headcaps were carefully removed, and brains were extracted and flash frozen. Brains were embedded in OCT and 50μm coronal sections spanning the cNTS plus 400μm posterior and anterior to the cNTS were collected using a cryostat (Leica CS3050 S). Slices were transferred to slides and 2-3 drops of Fluoromount-G™ (SouthernBiotech, Cat. No. 0100-20) were added on the slide before placing a coverslip over the tissue. Slides were imaged on a Marianas spinning disk confocal microscope and 3i software with a 20x dry objective (Zeiss Plan-Neofluar NA 0.5 air) and the DAPI filter set. Local infusion cannula placement criteria for exclusion were: lack of penetration into the brainstem or below the central canal along the dorsal-ventral axis and >100μm off target in the anterior-posterior and/or medial-lateral axis.

Data analysis

Frequency distributions of receptor mRNA puncta counts per GLP-1 neuron were plotted for each nAChR subunit and mAChR. Gaussian distribution curves were fit to the data using nonlinear regression. For statistical comparison of mean puncta counts and percent co-expression, a one-way analysis of variance (ANOVA) was conducted followed by Tukey's multiple comparisons test to assess pairwise differences. For statistical comparison of change in time spent in drug paired champed, Wilcoxon matched-pairs signed rank test was used.
For statistical comparison of change in preference score between control and experimental groups, a parametric t-test was used. A Pearson correlation analysis was performed to assess the linear relationship between mRNA expression levels measured by the present FISH study and Allen Institute MERFISH spatial transcriptomics dataset. All data presented as mean ± S.E.M. All data analysis was performed using GraphPad Prism 10 (GraphPad Prism Software).

RESULTS

Distribution of Gcg$^+$ GLP-1 neurons in the cNTS

We first used FISH to confirm Gcg mRNA expression throughout the cNTS (Figure 2). Gcg mRNA signal was restricted to cells in the cNTS and the interior reticular nucleus (IRt) according to expected distribution patterns of GLP-1 neurons. Gcg$^+$ cells, henceforth referred to as GLP-1 neurons, were present in the cNTS from approximately -8.00 to -7.48 relative to bregma. IRt GLP-1 neurons (images not shown) were not used for subsequent receptor expression studies.

cNTS GLP-1 neurons exhibit robust subtype-specific nAChR subunit mRNA expression

To characterize nAChR expression on GLP-1 neurons in the cNTS, we performed FISH to assess mRNA expression of all key nAChR subunits expressed in the mammalian brain (α3-5, α7, β2-4) alongside Gcg mRNA. Representative images and frequency distribution histograms of mRNA puncta per GLP-1 neurons are shown in Figure 3. β2 exhibited a unimodal distribution with a peak at 10 puncta and no cells lacking expression (Fig. 3E, L), suggesting that nearly all GLP-1 neurons robustly express β2 mRNA. Similarly, α7 exhibited a unimodal distribution with a peak at 8 puncta with very few GLP-1 neurons lacking expression (Fig 3D, K). α4 exhibited a unimodal distribution centered at zero, however, relatively low levels of expression were seen on a small number of GLP-1 neurons (Fig. 3I, B), suggesting a small population of GLP-1 neurons may express the α4 subunit. α3 (Fig. 3A, H), α5 (Fig. 3C, J), β3 (Fig 3F, M), and β4 (Fig. 3G, N) displayed sharply skewed distributions centered at zero, consistent with minimal or absent expression.

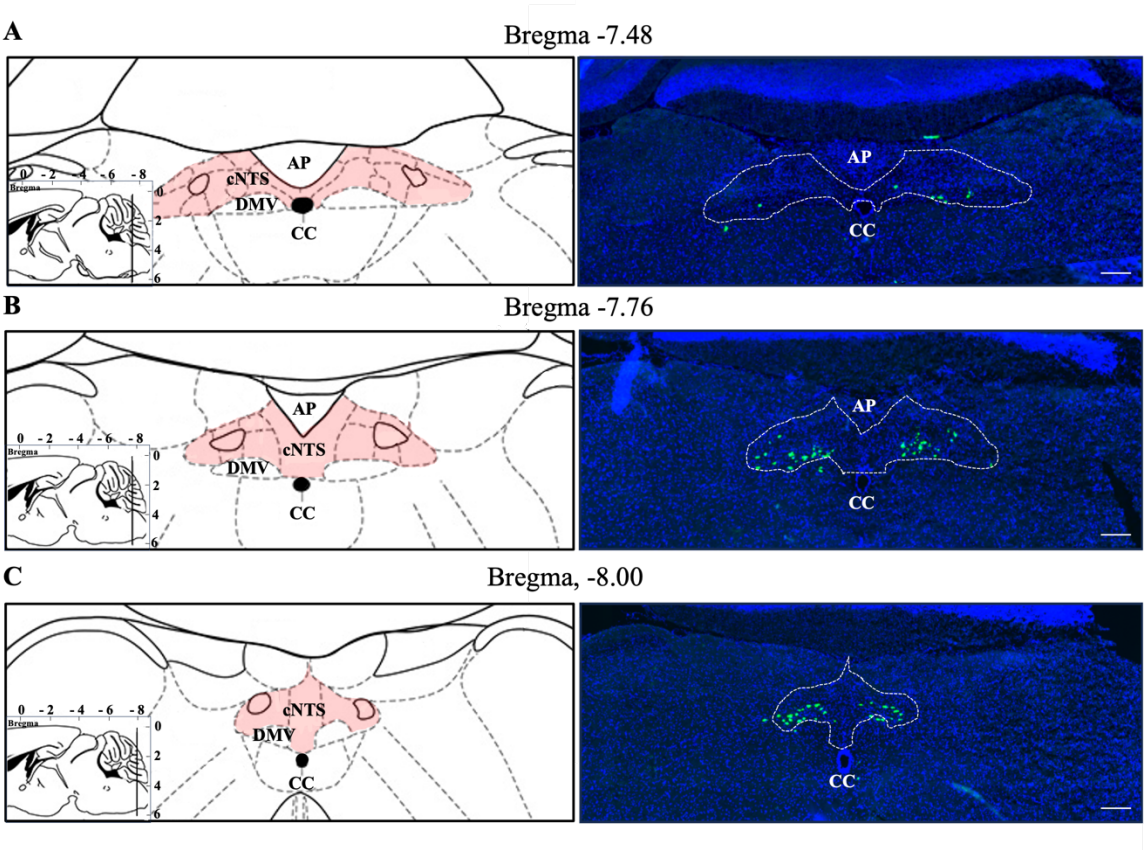
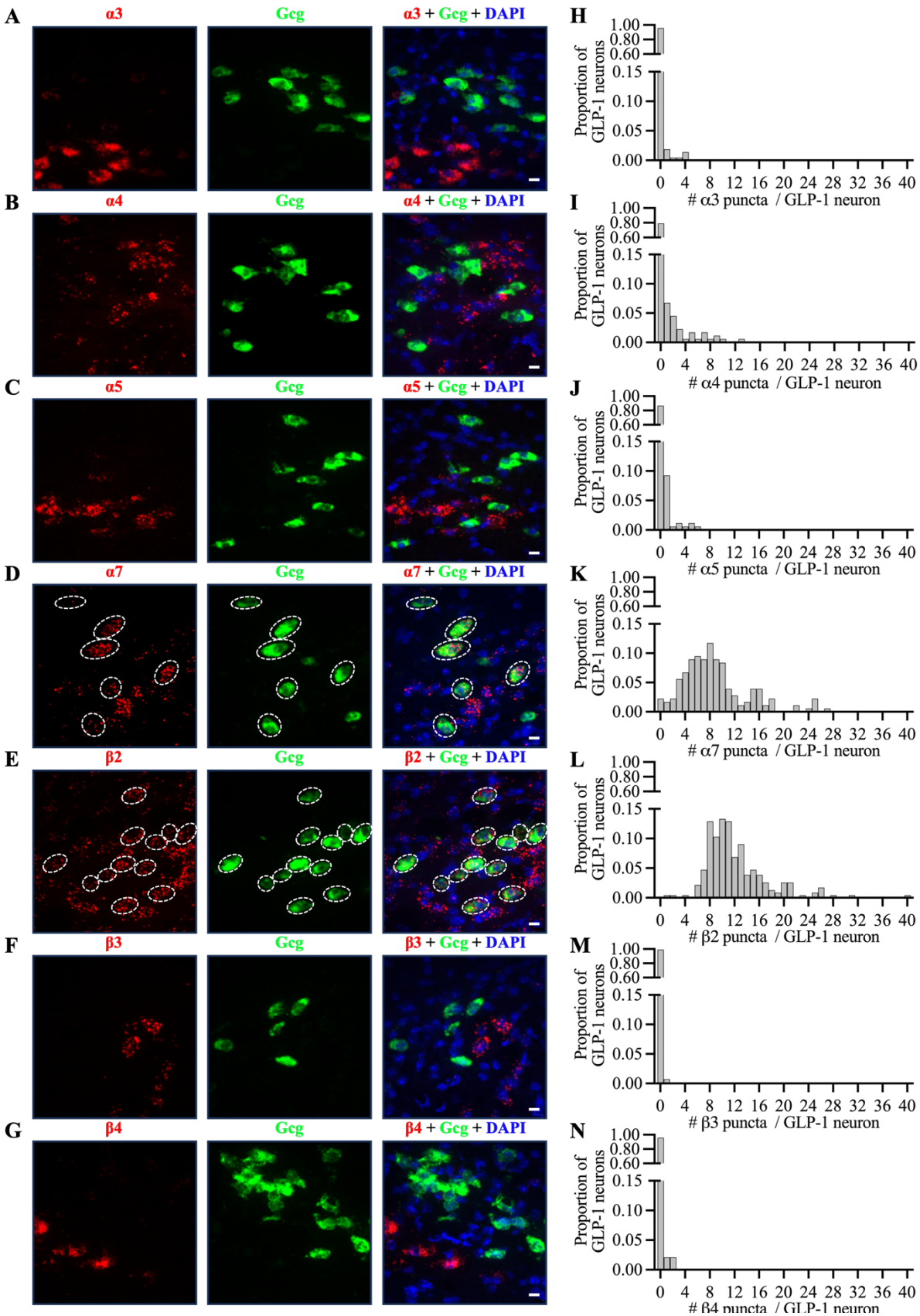


Figure 2. Spatial distribution of Gcg^{+} GLP-1 neurons within the cNTS. (Left) Schematic of coronal mouse brain slices with stereotaxic coordinates modified from the Paxinos atlas highlighting the cNTS region and (right) representative *in situ* hybridization images illustrating Gcg mRNA (green) expression in the cNTS depicting the approximate (A) anterior most, (B) relative middle, and (C) posterior most distribution of cNTS GLP-1 neurons. Scale bar: 200 μm.



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Figure 3. Robust expression of nAChR subunit mRNA in cNTS GLP1 neurons. (A-G) Representative in situ hybridization images illustrating nAChR subunit mRNA (red) and Gcg (green) in the cNTS. White dashed circles indicate GLP-1 neurons co-expressing nAChR subunit mRNA. 40x. Scale bar: 10μm. (H-N). Frequency distributions of normalized nAChR subunit mRNA puncta count per GLP-1 neuron in the cNTS. Data from 6 coronal tissue slices/mouse evenly spaced from approximately AP -8.0 to -7.48 (relative to bregma) from 2-3 mice per nAChR subunit.

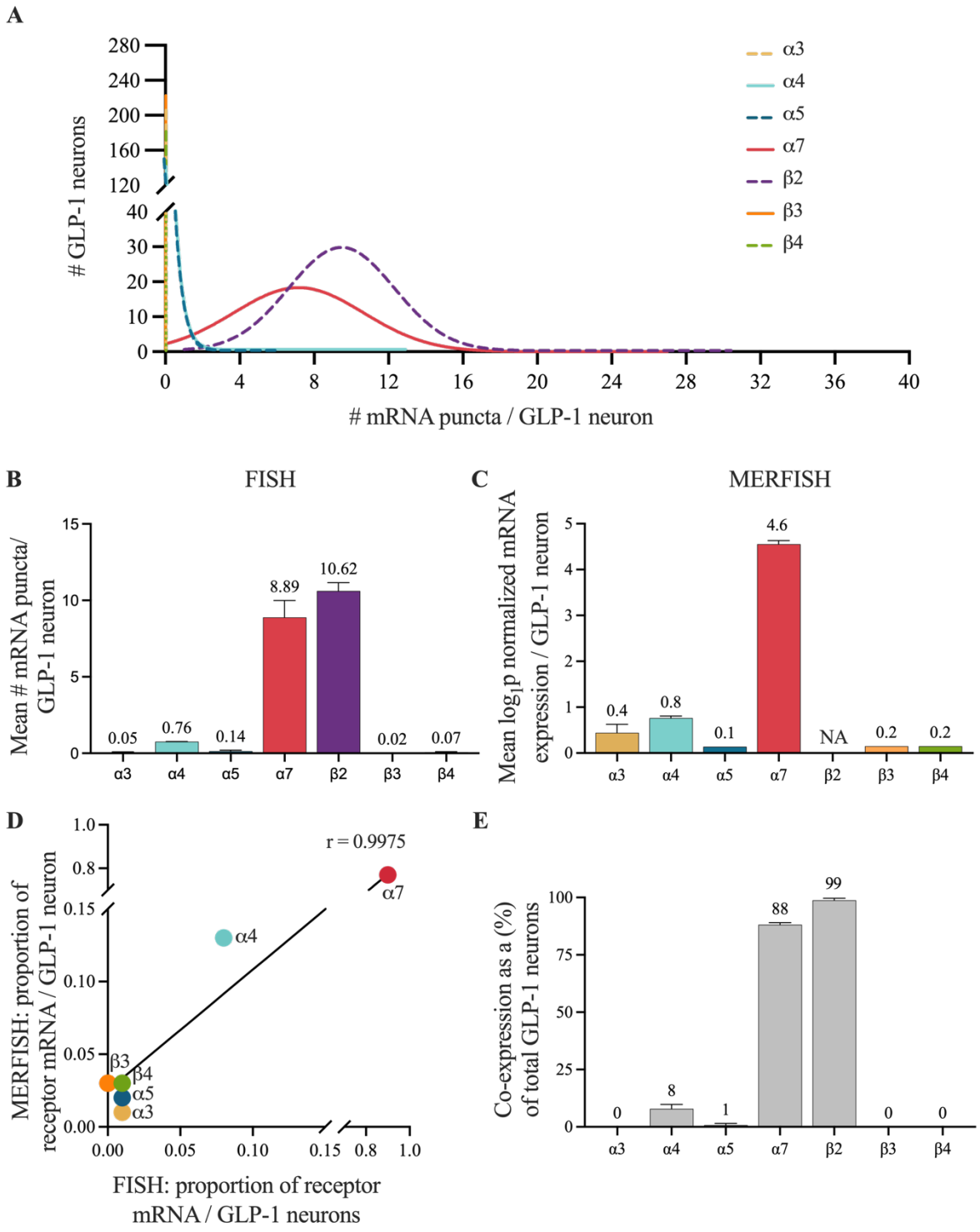
β2 and α7 are the most commonly expressed nAChR subunits on cNTS GLP-1 neurons

To estimate the proportion of GLP-1 neurons expressing each nAChR subunit, we applied a threshold of 4 puncta per cell to define receptor positivity. This threshold reflects a consistent inflection point seen in the individual nAChR subunit mRNA puncta distributions (Fig. 3 H-N) and overlayed smoothed frequency distributions across all tested nAChR subunits (Fig. 3A). We found that β2 is the most prevalent subunit with nearly all (99.8 ± 0.9%) GLP-1 neurons expressing β2 followed by 88.2 ± 0.9% expressing α7 (see Table 4 for all one-way ANOVA Tukey's multiple comparisons). In contrast, a small population of GLP-1 neurons (7.9 ± 1.9%) were found to express α4. GLP-1 neurons did not significantly express α3 (0.0 ± 0%), α5 (0.8 ± 0.8%), β3 (0.0 ± 0%), or β4 (0.0 ± 0%) subunits.

β2 and α7 are the strongest expressed nAChR subunits on cNTS GLP-1 neurons

To quantify overall nAChR subunit abundance, we compared the mean number of mRNA puncta per GLP-1 neuron for each subunit (Fig. 4B). A one-way ANOVA with Tukey's multiple comparisons revealed a significant main effect of receptor subtype on transcript abundance ($F_{6,8} = 4.889$, $p = 0.0217$). In line with the percent overlap data, β2 and α7 mRNA levels were significantly higher (10.62 ± 0.53, 8.89 ± 1.10 puncta, respectively) than all other receptors ($p < 0.0001$ for all pairwise comparisons) and not significantly different from each other ($p = 0.2313$). α4 showed very low overall mRNA expression (0.76 ± 0.2 puncta), consistent with the small proportion of GLP-1 neurons expressing the α4 subunit. α3 (0.05 ± 0.03 puncta), α5 (0.14 ± 0.7 puncta), β3 (0.01 ± 0.01 puncta), and β4 (0.07 ± 0.03 puncta) had near zero mRNA expression.

While a significantly higher proportion of GLP-1 neurons were positive for the α4



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Figure 4. β2 and α7 are the primary nAChR subunits expressed on cNTS GLP-1 neurons. (A) Frequency distribution curves showing nAChR subunit mRNA puncta counts per GLP-1 neurons. (B) Mean number of nAChR subunit mRNA puncta per GLP-1 neuron. One-way ANOVA revealed a significant main effect of receptor subtype on transcript abundance ($F = 102.7$, $p < 0.0001$). See table 5 for Tukey's multiple comparisons. (C) Mean $\log_1 p$ normalized imputed expression levels of nAChR mRNA expression per GLP-1 neuron from the MERFISH transcriptomic Allen Brain Cell Atlas[111]. All nAChR subunit data from the same 36 GLP-1 neurons, n=1 mouse. NA: not applicable due to *Chrmb2* exclusion from the MERFISH dataset. (D) Pearson correlation between the relative receptor expression patterns observed in our FISH dataset and those extracted from the MERFISH atlas ($r = 0.9975$, $p < 0.0001$). Expression values were normalized within each dataset to allow comparison of relative distribution across receptors. (E) Co-expression of Gcg and nAChR subunit mRNA within the cNTS as a percent of total GLP-1 neurons. Co-expression determined by at least 4 nAChR subunit mRNA puncta per GLP-1 neurons. One-way ANOVA revealed a significant main effect of receptor subtype on percent co-expression ($F = 2686$, $p < 0.0001$). See table 5 for Tukey's multiple comparisons. (A, B, E) α3: n=214 cells, 2 mice; α5: n=174 cells, 2 mice; α7: n=175 cells, 2 mice; β2: n=231 cells, 3 mice; β3: n=274 cells, 2 mice; β4: n=191 cells, 2 mice. All error bars represent mean ± S.E.M.

	Mean # mRNA puncta per GLP-1 neuron (Figure 4B)			Co-expression as a % of total GLP-1 neurons (Figure 4E)		
Comparison	**Mean diff.**	**95.00% CI**	**Adj. p-value**	**Mean diff.**	**95.00% CI**	**Adj. p-value**
β2 vs. α7	1.73	-4.240 - 0.7796	0.2313	10.65	5.979 - 15.32	0.0003
β2 vs. α4	9.865	7.355 - 12.37	<0.0001	90.9	86.23 - 95.57	<0.0001
β2 vs. α5	10.48	7.97012.99	<0.0001	98.05	93.38 - 102.7	<0.0001
β2 vs. α3	10.57	8.060 - 13.08	<0.0001	98.8	94.13 - 103.5	<0.0001
β2 vs. β3	10.61	8.095 - 13.11	<0.0001	98.8	94.13 - 103.5	<0.0001
β2 vs. β4	10.55	8.040 - 13.06	<0.0001	98.8	94.13 - 103.5	<0.0001
α7 vs. α4	8.135	5.386 - 10.88	<0.0001	80.25	75.13 - 85.37	<0.0001
α7 vs. α5	8.75	6.001 - 11.50	<0.0001	87.4	82.28 - 92.52	<0.0001
α7 vs. α3	8.84	6.091 - 11.59	<0.0001	88.15	83.03 - 93.27	<0.0001
α7 vs. β3	8.75	6,126 - 11.62	<0.0001	88.15	83.03 - 93.27	<0.0001
α7 vs. β4	8.82	6.071 - 11.57	<0.0001	88.15	83.03 - 93.27	<0.0001
α4 vs. α5	0.615	-2.134 - 3.364	0.9705	7.15	2.033 - 12.27	0.0078
α4 vs. α3	0.705	-3.454 - 2.044	0.9455	7.9	2.783 - 13.02	0.0042
α4 vs. β3	0.74	-2.009 - 3.489	0.9331	7.9	2.783 - 13.02	0.0042
α4 vs. β4	0.685	-2.064 - 3.434	0.9519	7.9	2.783 - 13.02	0.0042
α5 vs. α3	0.09	-2.8392.659	>0.9999	0.75	-4.367 - 5.867	0.9965
α5 vs. β3	0.125	-2.6242.874	>0.9999	0.75	-4.367 - 5.867	0.9965
α5 vs. β4	0.07	-2.6792.819	>0.9999	0.75	-4.367 - 5.867	0.9965
α3 vs. β3	0.035	-2.7142.784	>0.9999	0	-5.117 - 5.117	>0.9999
α3 vs. β4	-0.02	-2.7692.769	>0.9999	0	-5.117 - 5.117	>0.9999
β3 vs. β4	-0.055	-2.8042.694	>0.9999	0	-5.117 - 5.117	>0.9999

Table 4. One-way ANOVA Tukey's multiple comparisons for Figure 4.

nAChR subunit (7.9 ± 1.9%) as compared to α3, α5, β3, or β4, the mean puncta count per GLP-1 neuron for α4 was not significantly higher from these other low expressing subunits (see Table 4 for all pairwise comparisons). This likely reflects both the low proportion of α4-positive GLP-1 neurons and the fact that α4 expression is modest even within the subset of GLP-1 neurons that do express it resulting in an overall low abundance of α4 mRNA. The high degree of GLP-1 neurons with dense β2 and α7 expression, coupled with minimal to no expression of other nAChR subunits and an inability of β2 to form homomeric receptor complexes[112], strongly suggests that β2 is forming heteromeric receptor complexes with α7 on majority of cNTS GLP-1 neurons.

MERFISH spatial transcriptomics demonstrates strong concordance with FISH-based nAChR subunit expression profiles

To compare our FISH-based findings with an independent dataset, we examined nAChR subunit expression from the MERFISH transcriptomic Allen Brain Cell Atlas[111]. We analyzed relative expression patterns of all available nAChR subunit genes (*Chrma3*, *Chrma4*, *Chrma5*. *Chrma7*, *Chrmb3*, *Chrmb4*) in Gcg-expressing cells in the cNTS. β2 (*Chrmb2*) was not included in the MERFISH imputed gene list and, therefore, could not be assessed. The MERFISH values represent log-normalized, imputed expression estimates derived from integration with single-cell RNA-sequencing data and are not direct transcript counts. Accordingly, this comparison focused on the relative distribution of expression across receptor subunits rather than absolute expression levels. Consistent with our FISH results, α7 showed the highest expression on GLP-1 neurons as compared to the other available nAChR subunits (Fig 4C). GLP-1 neurons were found to have low levels of α4 and little to no α5, β3, or β4 expression. In slight contrast to our FISH data,

MERFISH data revealed very low non-zero α3 expression. Overall, there is strong concordance (r = 0.9975) in the relative expression hierarchy of the nAChR subunits (Fig 4D), providing independent validation for our FISH-based characterization of nAChR subunit expression in GLP-1 neurons.

Majority of cNTS GLP-1 neurons express co-express β2 and α7 mRNA

We then performed triple-labeling FISH in the cNTS to independently examine Gcg-β2-α7 (Fig 5A, D) and Gcg-β2-α4 (Fig 5B, E) colocalization (Figure 5). In concordance with our double-labeling results, all GLP-1 neurons expressed β2 mRNA, and majority of β2-expressing GLP-1 neurons (87.5 ± 2.5%) co-expressed α7 (Fig. 5D), further supporting the presence of α7β2 nAChRs in cNTS GLP-1 neurons. In contrast, only a minority of β2-expressing GLP-1 neurons (9%) co-expressed α4 (Fig 5E), again in concordance with expression patterns seen in double-labeling experiments. Representative images demonstrate strong and typical co-expression of β2 and α4 on non-GLP-1 neurons, however, GLP-1 neurons primarily express β2 without α4. To determine whether putative α7β2 and α4β2 nAChRs are expressed in overlapping GLP-1 neuronal populations, we examined Gcg-α7-α4 colocalization (Fig 5C, F). In concordance with double labeling experiments, 9.5 ± 1.3% of GLP- neurons expressed α4 and 87.0 ± 3.0% of GLP-1 neurons expressed α7. Interestingly, all α4 expressing GLP-1 neurons were also positive for α7 (Fig 5F), suggesting a subset of α7β2-expressing GLP-1 neurons also co-express α4β2 nAChRs. While this data may suggest that the small subset (11.8 ± 0.2%) of non-α7 expressing GLP-1 neurons, only express β2, nearly all (97.8 ± 0.8%) GLP-1 neurons had non-zero α7 expression, and may, therefore, may still be in receptor complex with β2.

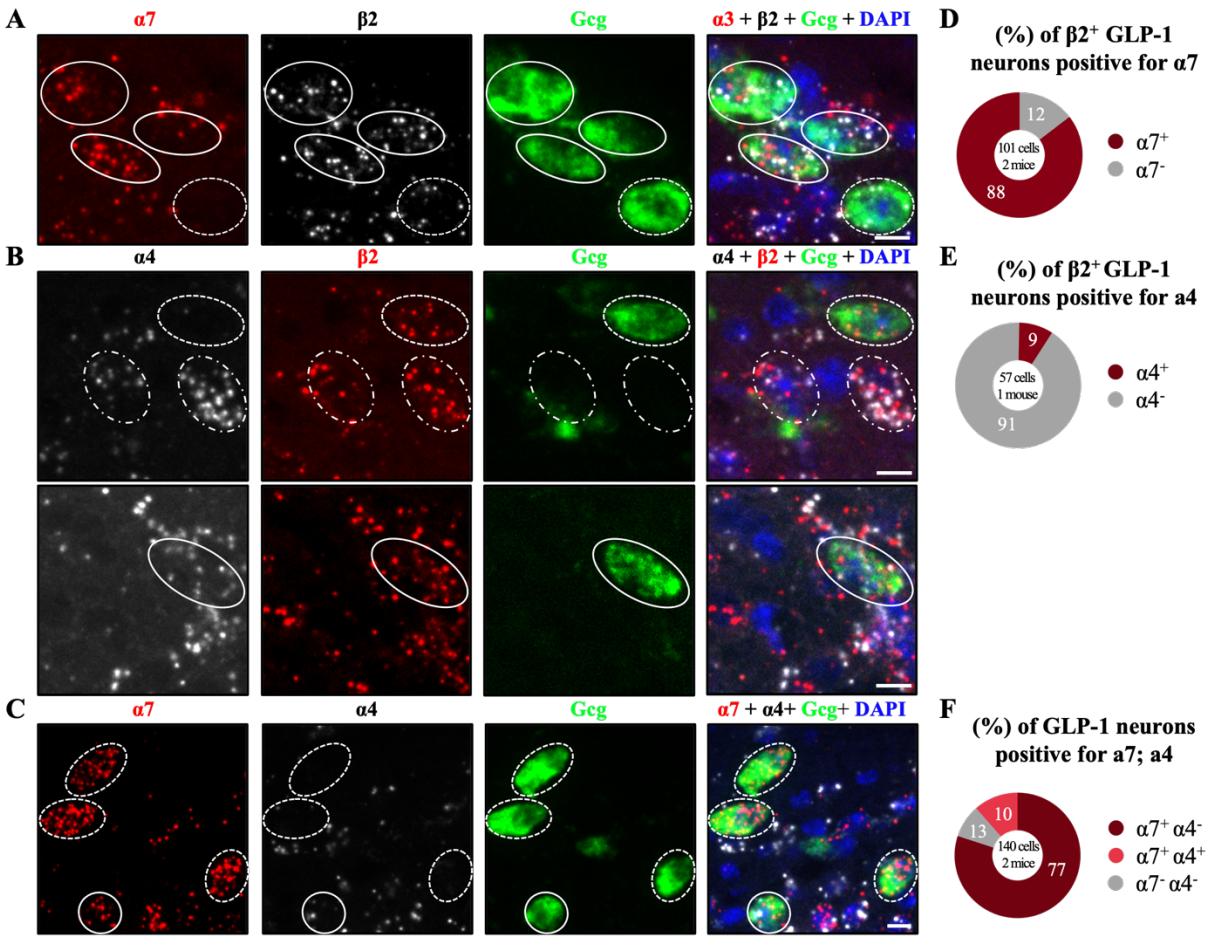


Figure 5. Majority of cNTS GLP-1 neurons co-express β2 and α7 mRNA. (A-C) Representative *in situ* hybridization images illustrating co-expression of nAChR subunit mRNA on GLP-1 neurons in the cNTS. 40x. Scale bar: 10μm. (A) α7 (red) – β2 (white) – Gcg (green) triple labeled cells marked with solid white circle. β2 (white) – Gcg (green) double labeled cell marked with solid white circle. (B-top) β2 (red) – Gcg (green) double labeled cell marked with solid white circle. α4 (white) – β2 (red) non-Gcg double labeled cells marked with dashed/dotted white circle. (B-bottom) α4 (white) – β2 (red) – Gcg (green) triple labeled cell marked with solid white circle. (C) α7 (red) – Gcg (green) double labeled cells marked with solid white circle. α7 (red) – α4 (white) – Gcg (green) triple labeled cell marked with solid white circle. (D-E) Quantification of percent overlap of α7 (D) and α4 (E) nAChR subunit mRNA on β2 expressing GLP-1 neurons in cNTS. (F) Quantification of percent overlap of α4 and α7nAChR subunit mRNA on GLP-1 neurons in cNTS. Co-expression determined by at least 4 nAChR subunit mRNA puncta per cell. Data from 6 coronal tissue slices evenly spaced from AP: -8.0 to -7.48 (relative to bregma) from 2 mice per nAChR subunit combination.

α7 activation in the cNTS attenuates cocaine-induced reward learning

There is a well-established role of nAChRs in cocaine-mediated behaviors (Table 1), with growing evidence for a specific involvement of the α7 subunit[18,37,62,65]. Given the robust α7 nAChR expression we found to be expressed on cNTS GLP-1 neurons, which are significant modulators of cocaine reward[100], we next examined whether α7 activation within the cNTS influences cocaine-induced reward learning as measured by cocaine CPP. We local infused an α7 agonist, PHA-534613, or saline to the cNTS directly prior to the conditioning sessions of our CPP paradigm (Fig. 6A, B). Mice that received local infusion of the α7 agonist, did not exhibit a significant increase in time spent in the cocaine-paired chamber on test day as compared to pre-test day (Wilcoxon matched-pairs signed rank test, $p = 0.3203$, Fig 6C). Control mice demonstrated a robust acquisition of cocaine CPP, spending 378.1 ± 83.0 more second in the cocaine paired chamber on test day as compared to pre-test day (Fig. 6D). In comparison, mice that received local infusion of the α7 agonist, demonstrated a significant reduction in the acquisition of cocaine CPP ($p = 0.0101$), spending only 73.4 ± 73.6 more second in the cocaine paired chamber on test day as compared to pre-test day (Unpaired t test, $p = 0.0125$), Fig. 6D). These results indicate that α7 expression within the cNTS is implicated in cocaine-induced reward learning.

cNTS GLP-1 neurons exhibit robust subtype-specific mAChR mRNA expression

To provide a comprehensive profile of cholinergic receptor expression, we next characterized mAChR expression on cNTS GLP-1 neurons. We performed FISH to assess mRNA expression of each mAChR (M_1 – M_5) alongside Gcg mRNA. Representative images and frequency distribution histograms of mRNA puncta per GLP-1 neurons are shown in Figure 7.

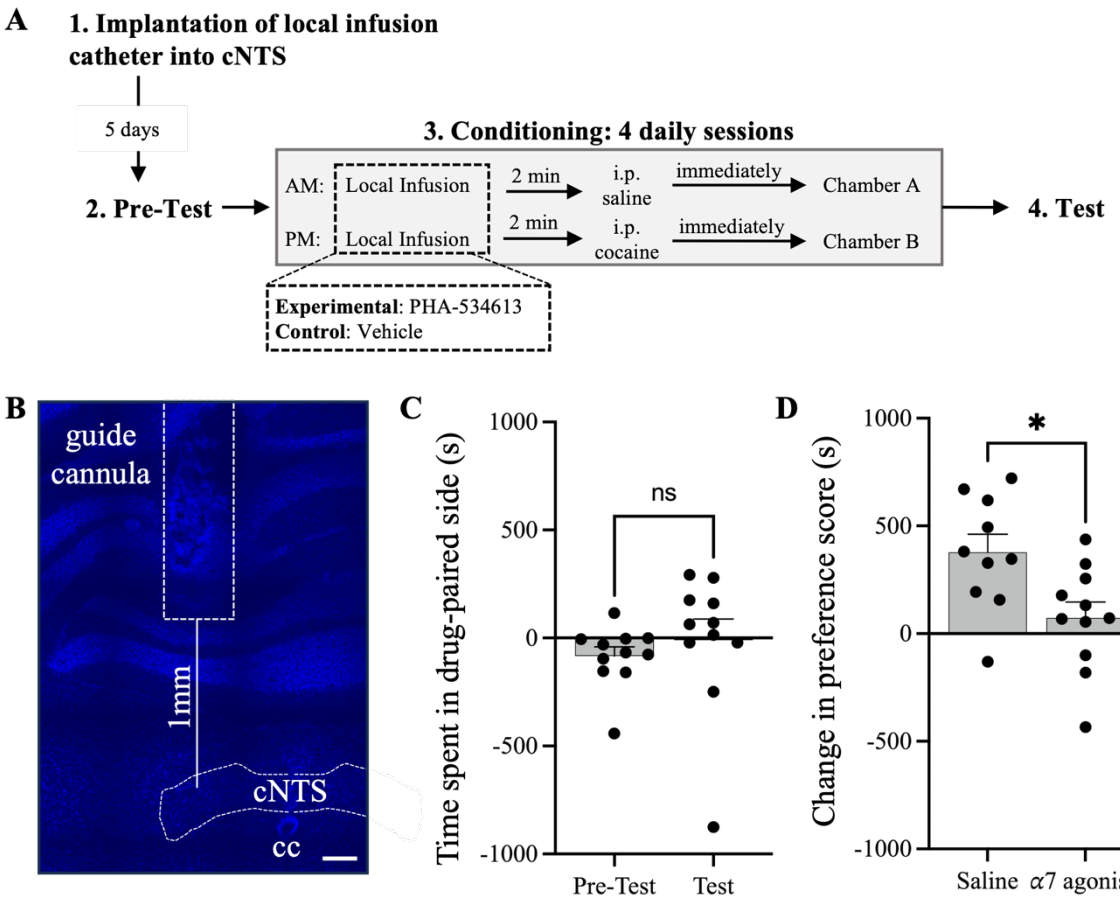


Figure 6. Local infusion of an α7 agonist to the cNTS attenuates cocaine-induced reward learning. (A) Timeline of cocaine CPP with local infusion of α7 agonist (PHA-534613; 0.3µL/infusion) or saline (0.3µL/infusion) into the cNTS prior to conditioning sessions. Cocaine 20 mg/kg. (B) Representative image of local infusion catheter track. Injection needle extends 1mm past end of cannula, depicting relative injection site. cc = central canal. Scale bar: 200µm. (C) Time spent in drug-paired side on pre-test and test day for α7 local infusion mice (n=11) (Wilcoxon matched-pairs signed rank test, p = 0.3203). (D) Change in preference score as measured by [time in cocaine paired side (s) on test day – time in cocaine paired side (s) on pre-test day] for mice that received local infusion of saline (n = 10) or α7 agonist (n = 11) (Unpaired t test, p = 0.0125). Error bars represent mean ± S.E.M.

M_2 exhibited a unimodal distribution with a sharp peak at 17 puncta and no cells lacking expression (Fig. 7 B, I), suggesting that nearly all GLP-1 neurons robustly express M_2 mRNA. In contrast, M_1 (Fig. 7 A, H) and M_3 (Fig. 7 C, J) showed bimodal distributions, each with a peak at zero and a second peak at 7 puncta, indicative of expression in only a subset of GLP-1 neurons. M_4 (Fig 7 D, K) and M_5 (Fig. 7 E, L) displayed sharply skewed distributions centered at zero, consistent with minimal expression.

M_2 are the most commonly expressed mAChR on cNTS GLP-1 neurons

To estimate the proportion of GLP-1 neurons expressing each receptor, we again applied a threshold of 4 puncta per cell to define receptor positivity (Fig. 8A). Consistent with the nAChR subunit expression profiles, this threshold reflects a consistent inflection point in the receptor mRNA puncta distributions (Fig. 7 H-L, Fig. 8A). Using this criterion, we found that 100.0 ± 0.0 % of GLP-1 neurons expressed M_2, while 62.0 ± 0.6% expressed M_3 and 55.4 ± 7.5% expressed M_1. Only 6.9 ± 0.9% of GLP-1 neurons expressed M_4 and 6.9 ± 0.3% expressed M_5. A one-way ANOVA with Tukey's multiple comparisons revealed that significantly more GLP-1 neurons express M_2 as compared to all other mAChR subtypes ($p < 0.0001$ for all comparisons). M_1 and M_3 did not differ significantly from one another ($p = 0.4832$) but were each expressed in significantly more GLP-1 neurons than M_4 and M_5 ($p < 0.0001$ for all comparisons). M_4 and M_5 did not differ significantly from each other ($p > 0.9999$). These proportions align with the qualitative impressions from the frequency distributions, further supporting the presence of muscarinic receptor-specific subpopulations among GLP-1 neurons.

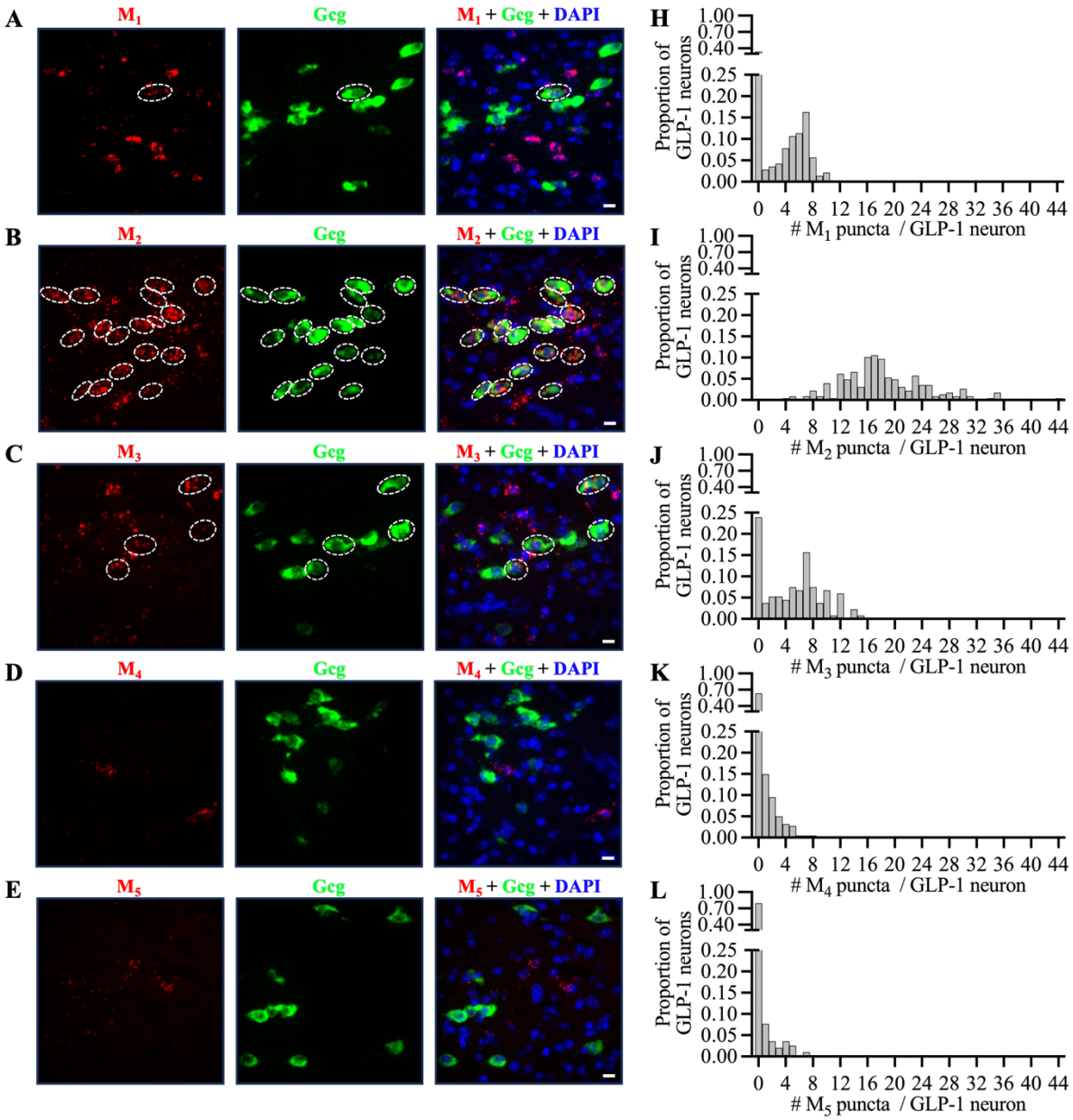


Figure 7. Robust expression of mAChR subunit mRNA in cNTS GLP-1 neurons. (A-E) Representative in situ hybridization images illustrating mAChR subtype mRNA (red) and Gcg (green) in the cNTS. White dashed circles indicate GLP-1 neurons co-expressing mAChR mRNA. 40x. Scale bar: 10µm. (H-L). Frequency distributions of normalized mAChR mRNA puncta counts per Gcg^{+} cells in the cNTS. Data from 6 coronal tissue slices/mouse evenly spaced from approximately AP -8.0 to -7.48 (relative to bregma) from 2-3 mice per mAChR subtype.

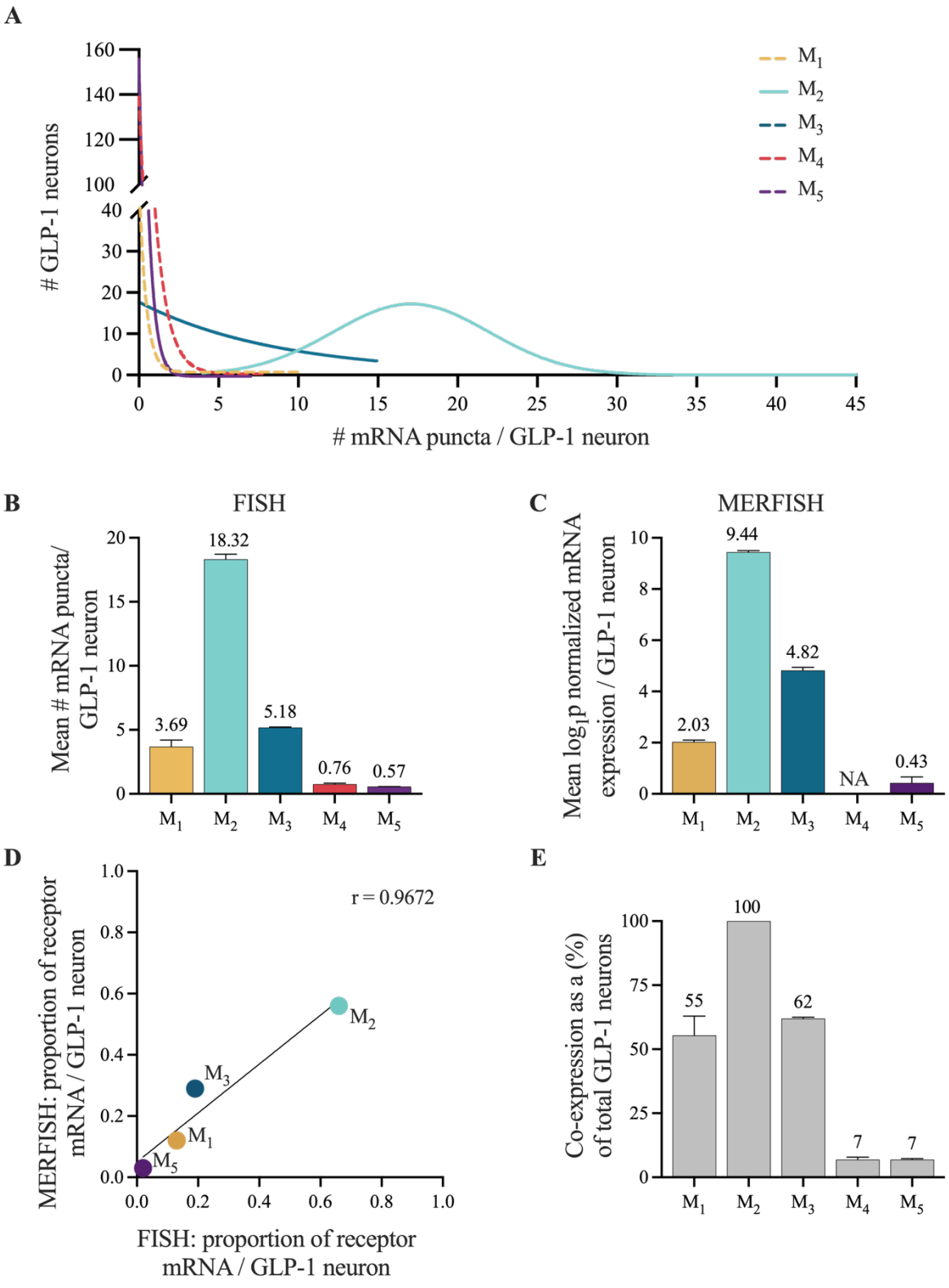
M_2 are the strongest expressed mAChR on cNTS GLP-1 neurons

To quantify overall mAChR abundance, we plotted a combined frequency distribution across all five receptors (Fig. 8A) and compared the mean number of mRNA puncta per GLP-1 neuron for each subtype (Fig. 8B). A one-way ANOVA with Tukey's multiple comparisons revealed a significant main effect of receptor subtype on transcript abundance ($F_{4,7} = 2.34$, $p < 0.0001$). In line with the percent co-expression data, M_2 mRNA levels were significantly higher (18.32 ± 0.4 puncta) than all other receptors ($p < 0.0001$ for all pairwise comparisons). M_3 and M_1 were expressed at moderate levels (5.18 ± 0.1 and 3.69 ± 0.5, respectively), and were not significantly different from each other ($p = 0.0853$). M_4 and M_5 showed low expression (0.76 ± 0.1 and 0.57 ± 0.0, respectively), consistent with the low proportion of GLP-1 neurons expressing these receptors. Together, these findings demonstrate that M_2 is the predominant mAChR subtype expressed by cNTS GLP-1 neurons, with more selective and heterogeneous expression of M_1 and M_3, and minimal expression of M_4 and M_5.

MERFISH spatial transcriptomics demonstrates strong concordance with FISH-based mAChR expression profiles

We next used the MERFISH transcriptomic atlas to examine relative expression patterns of mAChR genes in Gcg-expressing cells to externally validate our *in situ* hybridization studies (Fig 8C). We analyzed relative expression patterns of all available mAChR genes (*Chrmm1*, *Chrmm2*, *Chrmm3, Chrmm5*). M_4 (*Chrmm4*) was not included in the MERFISH imputed gene list and, therefore, could not be assessed. As with the nAChR subunit analysis, this comparison focused on the relative distribution of expression across receptor subunits rather than absolute expression levels. Consistent with our FISH results, M_2 showed the highest expression, while M_3

and M_1 exhibited moderate and comparable levels. M_5 expression was near zero. When expression values from both datasets were normalized and compared, a Pearson correlation analysis revealed a strong concordance ($r = 0.9672$) in the relative expression hierarchy (Fig 8D), providing independent validation for our FISH-based characterization of mAChR expression in GLP-1 neurons.



Legend on next page

Figure 8. M_2 is the primary mAChR subtype expressed on cNTS GLP-1 neurons. (A) Frequency distribution curves showing mAChR mRNA puncta counts per GLP-1 neurons. (B) Mean number of mAChR mRNA puncta per GLP-1 neuron. One-way ANOVA revealed a significant main effect of receptor subtype on transcript abundance ($F = 679.4$, $p = <0.0001$). See table 5 for Tukey's multiple comparisons. (C) Mean log_1p normalized imputed expression levels of mAChR mRNA expression per GLP-1 neuron from the MERFISH transcriptomic Allen Brain Cell Atlas[111]. All mAChR data from the same 36 GLP-1 neurons, n=1 mouse. NA: not applicable due to Chrmm4 exclusion from the MERFISH dataset. (D) Pearson correlation between the relative receptor expression patterns observed in our FISH dataset and those extracted from the MERFISH atlas ($r = 0.9672$, $p = 0.0328$). Expression values were normalized within each dataset to allow comparison of relative distribution across receptors. (E) Co-expression of Gcg and mAChR mRNA within the cNTS as a percent of total GLP-1 neurons. Co-expression determined by at least 4 mAChR mRNA puncta per GLP-1 neuron. One-way ANOVA revealed a significant main effect of receptor subtype on percent co-expression ($F = 312.7$, $p <0.001$). See table 5 for Tukey's multiple comparisons. (A, B, E) M_1: n=141 cells, 2 mice; M_2: n=227 cells, 3 mice; M_3: n=134 cells, 2 mice; M_4: n=221 cells, 3 mice; M_5: n=180 cells, 2 mice. All error bars represent mean ± S.E.M.

	Co-expression as a % of total GLP-1 neurons (Figure 8B)			Mean # mRNA puncta per GLP-1 neuron (Figure 8E)		
Comparison	**Mean diff.**	**95.00% CI**	**Adj. p-value**	**Mean diff.**	**95.00% CI**	**Adj. p-value**
M_2 vs M_3	38.05	25.90 - 50.20	<0.0001	13.14	11.59 - 14.68	<0.0001
M_2 vs M_1	44.6	32.45 - 56.75	<0.0001	14.63	13.08 - 16.17	<0.0001
M_2 vs M_4	93.07	82.20 - 103.9	<0.0001	17.56	16.18 - 18.94	<0.0001
M_2 vs M_5	93.07	82.20 - 103.9	<0.0001	17.75	16.21 - 19.30	<0.0001
M_3 vs M_1	6.55	6.7656 - 19.86	0.4832	1.49	0.2012 - 3.181	0.0853
M_3 vs M_4	55.02	42.87 - 67.16	<0.0001	4.42	2.876 - 5.964	0.0001
M_3 vs M_5	55.02	42.87 - 67.16	<0.0001	4.62	2.924 - 6.306	0.0002
M_1 vs M_4	48.47	36.32 - 60.61	<0.0001	2.93	1.386 - 4.474	0.0016
M_1 vs M_5	48.47	36.32 - 60.61	<0.0001	3.125	1.434 - 4.816	0.0019
M_4 vs M_5	0	-10.86 - 10.86	>0.9999	0.195	-0.349 - 1.739	0.9895

Table 5. One-way ANOVA Tukey's multiple comparisons for Figure 8.

DISCUSSION

Robust expression of both nicotinic and muscarinic receptors on cNTS GLP-1 neurons demonstrates a strong functional cholinergic GLP-1 interaction

Acetylcholine has a well-documented role in modulating the development and maintenance of drug-mediated behaviors, most notably cocaine, through its profound effects on reward, associative learning, explicit memory, attention, and arousal. The internal homeostatic state of the organism is a critically understudied component to these processes that all converge to regulate reward saliency and motivated behavior. The cNTS serves as the primary hub for integrating interoceptive input with higher order cognitive information and relays this throughout the brain, including direct connections with key sites of cholinergic modulation of reward[77,79]. Putative cholinergic terminals[80,81], AChE expression[82,83], and AChR expression in the cNTS[84–90] strongly indicate acetylcholine modulates cNTS activity. As such, cholinergic modulation of reward may extend to the cNTS. GLP-1 neurons are a particularly promising cellular population within the cNTS as they are the primary cell type within the cNTS with which the interceptive afferents synapse, have a well-documented role in modulating reward[96,97,101,113], and have been shown to mildly express the α5 nAChR subunit[91].

As such, we sought to elucidate the full profile of cholinergic receptors on cNTS GLP-1 neurons and investigate the role they may play in cocaine reward. This work provides the first comprehensive characterization of cholinergic receptor expression on cNTS GLP-1 neurons. Using high-resolution multiplexed FISH with MERFISH spatial transcriptomic validation, we demonstrated robust subtype-specific expression of both nAChR subunit and mAChR mRNA in cNTS GLP-1 neurons. These findings support the existence of a functional cholinergic input onto cNTS GLP-1 neurons, modulated by both nicotinic and muscarinic cholinergic systems.

Approach to the quantification of FISH-based mRNA expression

Interpreting these profiles of mRNA expression to definite which nAChRs and mAChRs are expressed on GLP-1 neurons poses an ongoing challenge. mRNA expression studies heavily rely on establishing a meaningful threshold to define when a cell is considered positive for a given transcript. Number of mRNA florescent puncta, percent of cell covered by florescence, or percent of cell with signal some level above background are common thresholding criteria. Regardless of thresholding method, determining the true point at which mRNA expression becomes biologically relevant is often arbitrary and likely not consistent across all genes. Furthermore, image analysis techniques for identifying nuclei, estimating corresponding cell boundaries, and quantifying florescence within those boundaries brings additional limitations. Slight misestimations of cell boundaries could direct a given cell to the wrong binary positive or negative classification. Subthreshold mRNA expression is likely a combination of nonspecific expression and true signal that is likely too low to result in biologically meaningful protein transcription and translation. Above threshold expression, while importantly defining the proportion of cells likely expressing biologically relevant mRNA levels, obscures the density of expression within positive cells. Alternatively, exclusively relying on overall abundance of mRNA in a given cell population of interest, can also mask potentially meaningful expression when that expression is confined to a small subset of cells. If a receptor is robustly expressed in only a limited population of neurons, its overall mean mRNA level may remain low and fail to reflect its functional significance in that cell type. Various studies often attempt to address these limitations by reporting a scale of mRNA expression such as low, moderate, and high expressors and the proportion of cells within each group. While this is a meaningful approach, it still relies on the need to determine a threshold for each level of expression without clarity on how various

levels relate to biologically relevant protein transcription and translation.

As such, we uniquely report the full distribution of receptor mRNA expression per GLP-1 neuron for every nAChR subunit and mAChR alongside mean receptor mRNA puncta per GLP-1 neuron and threshold-based receptor expression as a proportion of GLP-1 neurons. When observing the distributions of the number of receptor mRNA puncta per GP-1 neuron, we observed (1) unimodal distributions centered at zero indicative of few to no GLP-1 neurons likely expressing the receptor, (2) strongly right shifted unimodal distributions with a non-zero peak and little to no cells lacking any expression indicative of nearly all GLP-1 neurons likely expressing the receptor, and (3) bimodal distributions with a peak at zero and a second right shifted non-zero peak indicative of receptor expression in a subset of GLP-1 neurons. These distributions revealed a relatively consistent inflection point of approximately 4 mRNA puncta at which point (1) mRNA levels of low expressors largely dropped off, (2) mRNA levels of high expressors primarily began, and (3) mRNA levels of moderate expressors shifted bimodal peaks. As such, we used 4 mRNA puncta as our cutoff for threshold-based assessment of the proportion of GLP-1 neurons expressing each nAChR subunit and mAChR.

nAChR expression patterns suggest co-assembly of α7 and β2 on cNTS GLP-1 neurons

The β2 nAChR subunit was found to be densely expressed with nearly all cNTS GLP-1 neurons meeting the positivity threshold of 4 puncta and no GLP-1 neurons lacking any β2 mRNA expression. α4β2 ± α5 are one of the most prevalent nAChR subtypes in the mammalian brain and are critically involved in modulating synaptic transmission, attention, cognitive function, and the reinforcing effects of addictive substances[55]. The present study, however, found that only a small population of GLP-1 neurons reached positivity threshold for α4. The overall

abundance of α4 mRNA was significantly lower than β2, consistent with both the small proportion of GLP-1 neurons expressing α4 and relatively low mRNA levels within positive cells as compared to β2 expression. While the strength of mRNA expression is significantly lower compared to β2, the presence of non-zero α4 expression suggests β2 likely forms typical α4β2 heteromeric receptors on a small subset of cNTS GLP-1 neurons. α4β2-containing nAChRs exist in receptor complex both with and without the α5 subunit[55]. It has been reported that cNTS GLP-1 neurons express the α5 subunit[91], however, our data demonstrate that less than 1% of GLP-1 neurons reached the positivity threshold for α5 with near-zero overall abundance of α5 mRNA on GLP-1 neurons. Given the lack of significant expression of α5 on cNTS GLP-1 neurons, putative α4β2 nAChR heteromers are likely not in complex with the α5 subunit.

The β2 nAChR subunit cannot form homomeric receptor complexes[55] and, therefore, must exist in receptor complex with other nAChR subunits. The α7 subunit was found to be as densely expressed as β2 with most GLP-1 neurons also reaching the positivity threshold for α7. No other nAChRs were found to be significantly expressed on GLP-1 neurons. Furthermore, triple labeling confirmed that majority of GLP-1 neurons reach positivity threshold and co-express both β2 and α7, strongly suggestive of α7β2 heteromer expression. Triple labeling also revealed dense typical co-expression of α4 and β2 on non-GLP-1 neurons in the cNTS, however, GLP-1 neurons primarily expressed β2 without α4. Interestingly, triple labeling revealed that the small population of α4-expressing GLP-1 neurons also co-expressed α7, suggesting that putative α4β2 and α7β2 nAChRs are expressed on overlapping populations of GLP-1 neurons. While percent overlap data may suggest that a small percentage of non-α7, non-α4 expressing GLP-1 neurons express only the β2 nAChR, nearly all GLP-1 neurons had non-zero α7 expression and the mean overall number of β2 and α7 puncta per GLP-1 neuron were not significantly different

from each other. These findings underscore the importance of assessing overall transcript abundance in addition to threshold-based percent overlap.

Highly concordant expression patterns of nAChR subunit mRNA have previously been used to identify to presence of α7β2 heteromers on basal forebrain cholinergic neurons[59,114]. Tissue protein extraction, immunoprecipitation, and immunoblotting studies in the rat forebrain further confirmed these mRNA expression patterns corresponded with α7 and β2 co-assembly, and patch-clamp electrophysiology confirmed α7β2 functionality[114]. Our nAChR mRNA expression data strongly suggest that α7 nAChRs are co-assembling with β2 on cNTS GLP-1 neurons to form α7β2 heteromers.

It is important to note that the α7 containing nAChRs are generally considered homomers and one of the most prevalent nAChRs in the mammalian brain[115]. Even with dense co-expression of α7 with β2 and minimal expression of other subunits, these data do not rule out the possibility that the α7 subunit is also forming homomeric nAChRs on GLP-1 neurons in addition to co-assembling with β2.

Expression and function of α7β2 heteromers in the mammalian brain

While α7β2 remains a novel nAChR complex, there are growing reports of functional α7β2 nAChR complexes in both the rodent and human brain[116]. Most notably, α7β2 nAChRs have been shown to be expressed in the basal forebrain[114,116], hippocampus[117], and cortex[118]. The present study provides the first known report of α7β2 receptors in the cNTS on GLP-1 neurons.

Structurally, the α7β2 nAChR is a pentameric ligand-gated ion channel, predominantly composed of α7 subunits with one or more β2 subunits incorporated. This assembly results in a receptor that retains α7-like properties but exhibits unique functional attributes due to the

presence of β2 subunits. Similar to α7 homomers, α7β2 receptors are permeable to cations, including calcium. However, concatenated subunit expression in oocytes has demonstrated that the inclusion of β2 subunits modifies the receptor's kinetics, leading to slower activation and desensitization rates compared to α7 homomers. As the number of β2 subunits in the pentameric arrangement increases, the durations of channel openings and activation episodes increases progressively[118].

The available studies on the pharmacological properties of α7β2 are limited but suggest that α7β2 exhibit an altered pharmacological profile as compared to α7 homomers that largely maintain responsiveness to α7 drugs. The inclusion of the β2 does not appear to affect type II positive allosteric modulator (PAM) potentiation or activation by allosteric agonists, but results in impaired type I PAM potentiation[58]. α7β2 receptors display similar antagonism by α7-selective antagonists like MLA and α-bungarotoxin[118]. Activation by acetylcholine and non-selective nAChR agonist epibatidine appear unaffected[119]. While α7β2 receptors are still activated by α7-selective agonists, potentiated potency has been observed suggesting they may function as partial agonists. Most notably, multiple selective α4β2 agonists fail to activate α7β2 receptors. These data indicate that the inclusion of the β2 subunit does not shift the α7 receptor to a "β2-like" pharmacological profile[119].

cNTS α7-mediated attenuation of cocaine reward

The robust expression of α7-containing nAChRs as the primary nAChR expressed on cNTS GLP-1 neurons is particularly intriguing. While a functional role of nAChRs in cocaine reward has been well documented[11], the role of the specific nAChR subunits is limited. There is growing evidence, however, demonstrating α7 as one of the most implicated nAChR subunits in

cocaine reward. VTA MLA injections attenuate the reinforcing effects of cocaine as measured by cocaine-induced potentiation of ICSS[18]. Global α7 KO mice are hyporeactive to cocaine displaying attenuated acute cocaine-induced locomotion[65], which is likely not mediated by α7 expression in the NAc or VTA as MLA injections to these regions do not effect affect cocaine-induced locomotion and locomotor sensitization[19]. α7 expression on striatal cholinergic neurons mediate cocaine reinforcement as measured by cocaine-induced CPP and operant responding to cocaine[37]. As such, we sought to investigate the functional significance of α7 expression in the cNTS on cocaine-induced conditioned place preference.

We show that pharmacological activation of α7-containing receptors in the cNTS via local infusion of selective α7 agonist PHA-543613 significantly reduces the acquisition of cocaine CPP. These results demonstrate that local activation of cNTS α7-containing receptors is sufficient to attenuate cocaine reward learning. Notably, α7 mRNA was detected on non-GLP-1 neurons within the cNTS, indicating that the behavioral effect we observed is not exclusive to GLP-1 neurons. However, the robust expression of α7 on nearly all cNTS GLP-1 neurons and well-documented role of cNTS GLP-1 neurons in suppressing cocaine reward, strongly suggest that the observed behavioral effect is likely largely mediated by α7-expressing GLP-1 neurons. GLP-1 is thought to modulate cocaine reward by regulating cocaine-induced increases in dopamine[103–105]. As such, we hypothesize that the locally infused selective α7 agonist increased activity in GLP-1 neurons thus potentiating GLP-1-mediated attenuation of cocaine-induced dopamine release. As demonstrated, α7 is likely co-assembling with β2 on cNTS GLP-1 neurons and α7β2 receptors exhibit "α7-like" pharmacological profiles. As such, while action of PHA-543613 on α7β2 heteromers has not been tested, investigations of the potency of other α7 agonists suggests it would function as at least a partial agonist on α7β2 heteromers. α7

modulation has been shown to modulate other key functions of GLP-1 including the regulation of weight gain and glucose levels in mouse models of diabetes[120], promoting satiety and attenuating food intake[121], and modulating food-induced dopamine release[122]. Future studies employing cell type-specific α7 manipulations in the cNTS and their role in downstream effects of GLP-1 signaling are needed.

Cocaine may disinhibit cNTS GLP-1 neurons via M_2 mAChR inhibition, revealing a potential negative feedback loop further attenuating cocaine reward

In addition to robust nAChR expression, all cNTS GLP-1 neurons were found to densely express M_2 mRNA. Interestingly, cocaine has been shown to act directly on M_2 mAChRs as a competitive antagonist[71–73]. M_2 receptors are inhibitory Gi-coupled receptors[123]. Furthermore, voluntary intake of biologically relevant doses of cocaine has been shown to increase activity in cNTS GLP-1 neurons in rodents[108]. As such, our M_2 mRNA expression data suggests a novel mechanism whereby cocaine may disinhibit cNTS GLP-1 neurons via M_2 antagonism. This cocaine-induced disinhibition may contribute to enhanced GLP-1 signaling, thereby initiating a negative feedback loop that attenuates further drug seeking.

Cocaine also functions as a competitive antagonist at M_1 mAChRs, which the present study found to be expressed on a subset of cNTS GLP-1 neurons. While M_1 receptors are excitatory Gq-coupled receptors[123], and, therefore, may counteract some cocaine mediated disinhibition of M_2 expressing GLP-1 neurons, the overwhelming abundance of M_2 expression and clear ability of systemic cocaine to activate GLP-1 neurons suggests a net effect of disinhibition.

The rapidly expanding therapeutic potential of GLP-1 drugs and their limitations

The present finding that α7 activation in the cNTS attenuates cocaine reward learning and may be mediated by α7-expressing GLP-1 neurons, contributes to the profound body of pre-clinical and limited clinical studies demonstrating GLP-1's ability to powerfully modulate reward and drug-mediated behaviors. There are 4 newly completed and 9 ongoing clinical trials seeking to take FDA-approved GLP-1 receptor agonists widely used to treat Type 2 Diabetes (T2D) and Obesity and re-purpose them as a treatment for a variety of substance use disorders (SUD)[124]. These efforts are part of a dramatic and ongoing surge in scientific and public interest in GLP-1 drugs.

The success of GLP-1therapeutics as a treatment for T2D has proven successful for over two decades. Their efficacy at promoting weight-loss in individuals with obesity, however, remained only moderately successful until the recent development of Semaglutide, a GLP-1R agonists with unprecedented levels of weight loss (~20% of body weight), as compared to other GLP-1R agonists and weight-loss medications. FDA approval of Semaglutide for chronic weight management marked a pivotal turning point in the role of GLP-1 in modern therapeutics. Medicaid data indicates that from 2019 to 2023, GLP-1 prescriptions increased by over 400%, and gross spending on these medications rose by more than 500%. The global GLP-1 market is projected to be over $100 billion industry by 2029, with an estimated 40 million individuals using these medications. The prevalence and widespread use of GLP-1 agonists is likely to surpass these projections given that the potential applications of GLP-1 therapeutics extend beyond metabolic diseases, to include drug misuse as well as neurodegenerative disorders and inflammatory and immune disorders[124]. As such, an intimate understanding of the mechanisms and circuitry underlying the endogenous GLP-1 system that these drugs act on is critically

important now more than ever. The present study identifies and fully characterizes GLP-1 expression of cholinergic receptors, an entire major neurotransmitter system that was largely unknown to directly communicate with GLP-1 neurons. This work will critically contribute to potential future development of pharmacological agents targeted toward GLP-1 neurons.

While GLP-1 drugs are highly effective at treating T2D and Obesity, they have limitations that may impact their ability to translate to the SUD market. Most notably, currently FDA-approved GLP-1 drugs are administered systemically, and growing evidence has indicated that some may have worse blood brain barrier penetration than previously thought[94]. GLP-1 mediated modulation of insulin homeostasis is largely mediated by peripheral GLP-1 receptor activation, and there are numerous mechanisms by which peripheral GLP-1 receptor activation can regulate food intake. GLP-1 modulation of reward, however, has been shown to require central GLP-1 receptor activation. As such, GLP-1 drugs with limited blood brain barrier penetration would likely not be maximally effective at treating SUDs. While activation of peripheral GLP-1 receptors may induce the release of central GLP-1 via GLP-1 expressing vagal afferents projecting from the gut to the cNTS, this may not be sufficient to induce GLP-1 mediated attenuation of reward. This may contribute to the mixed efficacy seen in preliminary clinical trials investigating GLP-1 drugs for the treatment of SUD.

There are three clinical studies with data for alcohol use disorder (AUD). A 26-week randomized control trial (RCT) in 127 people with alcohol use disorder reported exenatide treatment reduced the number of alcohol drinking days but only in individuals with obesity[125]. Retrospective observational studies of patients with T2D and obesity report positive associations with GLP-1 drug use and reduced risk for incidence and recurrence of AUD[126] and reduced risk of alcohol-related events[127]. There are two clinical studies with data for nicotine use disorder. A

RCT of 84 prediabetic and overweight smokers reported exenatide over placebo promoted nicotine abstinence at 6 weeks[128], however, a more recent RCT of 255 nicotine dependent individuals did not show any effect of dulaglutide as compared to standard of care on promoting cigarette abstinence over 12 weeks of treatment[129]. Cocaine use disorder has one small proof-of-concept trial in 13 individuals across 2 days that did not show any effect of exenatide as compared to placebo on reducing the number of self-administered cocaine infusions. Finally, a retrospective observational study reported an association between semaglutide and a reduction in cannabis-use disorder in patients with T2D and obesity[130]. While the pre-clinical evidence for central GLP-1 signaling as a powerful modulator of reward and drug-mediated behaviors across all major drug classes is unequivocally clear, the mixed results of early clinical investigations suggests currently available GLP-1 drugs may not be sufficient to treat SUD. As such, advancements in GLP-1 therapeutics and alternative methods for pharmacological targeting the central GLP-1 system are be needed.

α7β2 nAChR expression on cNTS GLP-1 neurons as a putative novel therapeutic target for cocaine use disorder

In addition to suboptimal blood brain barrier penetration, systemic GLP-1 drugs, while effective for metabolic disorders, engage receptors throughout the body, including the pancreas, gut, and cardiovascular system, thus leading to peripheral side effects such as nausea, slowed gastric emptying, and changes in heart rate. GLP-1 modulation of drug-mediated behaviors, including cocaine, has been extensively shown to be mediated through central GLP-1 receptor activation and not relay on the peripheral GLP-1 systems. Targeting GLP-1 neurons directly, rather than relying on systemic GLP-1 receptor agonists, offers a promising strategy for

enhancing central GLP-1 signaling in the treatment of SUD with improved specificity. Our discovery of putative α7β2 nAChRs on cNTS GLP-1 neurons identifies a previously unexplored pharmacological target to selectively activate GLP-1 neurons without engagement of the peripheral GLP-1 system.

Pharmacological efforts to target the cholinergic system have long been hampered by the system's widespread anatomical distribution and receptor diversity, often resulting in off-target effects and poor therapeutic specificity. Both nicotinic and muscarinic acetylcholine receptors are broadly expressed throughout the central and peripheral nervous systems, playing roles in cognition, autonomic function, motor control, and reward. As a result, drugs targeting cholinergic signaling frequently engage unintended systems, leading to side effects that limit clinical utility. For example, muscarinic receptor agonist xanomeline initially showed promise in schizophrenia due to selective M_1/M_4 receptor engagement, but their clinical development was limited by peripheral cholinergic side effects such as gastrointestinal distress and bradycardia[131]. Similarly, acetylcholinesterase inhibitors like donepezil and rivastigmine, approved for Alzheimer's disease, enhance cholinergic tone non-specifically and often cause nausea, dizziness, and muscle cramps[132,133], reflecting a lack of spatial and receptor subtype selectivity.

Attempts to develop subtype-selective nicotinic drugs have faced similar challenges. ABT-418, a partial α4β2 nAChR agonist, was investigated for cognitive enhancement but failed in part due to narrow therapeutic windows and limited efficacy in larger trials[134,135]. Varenicline, a partial agonist at α4β2 and full agonist at α7 nAChRs, is effective as a smoking cessation aid but has been associated with neuropsychiatric side effects[136]. These examples illustrate the difficulty of cholinergic drug development. Even when targeting specific receptor subtypes, achieving cell-type or circuit-specific effects remains a major obstacle. Advances in genetic

targeting and localized delivery may overcome some of these limitations, but to date, clinical translation remains constrained by the inherent complexity of the cholinergic system.

Given the highly restricted expression of α7β2 receptors in the mammalian brain, novel modulators of this receptor subtype could allow for unprecedented selective control of cholinergic signaling and enable precise tuning of GLP-1 neuron activity with minimal off target effects. This selective engagement of central GLP-1 signaling could potentially offer a novel approach to reduce cocaine reward while avoiding the widespread activation of cholinergic systems that underlie significant side effects.

Conclusions

This study provides the first comprehensive characterization of cholinergic receptor expression on cNTS GLP-1 neurons, identifying robust expression of α7β2-containing nAChRs and M_2 mAChR expression. Functional studies revealed α7 activation in the cNTS modulates cocaine reward learning. We hypothesize that α7β2 expression GLP-1 neurons are the primary mediators of this behavioral effect given their well-documented effects on modulating cocaine reward. We further hypothesize that the dense M_2 expression of cNTS GLP-1 neurons identified in the present study provide a mechanism by which cocaine, a competitive antagonist of M_2, increases activity in GLP-1 neurons. This cocaine-induced disinhibition may contribute to enhanced GLP-1 signaling, thereby initiating a negative feedback loop that attenuates further drug seeking. These results support the existence of functional cholinergic input onto cNTS GLP-1 neurons, modulated by both nicotinic and muscarinic cholinergic systems. The discovery of putative α7β2 nAChRs on cNTS GLP-1 neurons elucidates a novel pharmacological target to selectively activate GLP-1 neurons in the treatment of cocaine and other substance use disorders.

Future Directions

Several important future directions emerge from the present findings. First, while our high-resolution mRNA expression studies strongly suggest co-assembly of α7 and β2 subunits into heteromeric receptors on cNTS GLP-1 neurons, direct confirmation at the protein level is needed. Future studies employing co-immunoprecipitation assays and immunohistochemical labeling for α7β2 complexes will be critical to validate the presence and assembly of these subunits. Second, functional studies are necessary to determine how activation or inhibition of α7β2 nAChRs and M_2 mAChRs modulate GLP-1 neuron activity. Electrophysiological recordings combined with pharmacological manipulations could elucidate how these receptors influence excitability and downstream GLP-1 signaling. Third, cell type-specific approaches, such as GLP-1 neuron-targeted optogenetic stimulation or chemogenetic activation of α7-containing receptors are needed to directly assess the causal role of GLP-1 neurons in mediating the observed attenuation of cocaine reward behavior.

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