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An acetylcholine-dopamine interaction in the nucleus accumbens and its involvement in ethanol's dopamine-releasing effect

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Abstract

Alcohol use disorder is a chronic, relapsing brain disorder causing substantial morbidity and mortality. Cholinergic interneurons (CIN) within the nucleus accumbens (nAc) have been suggested to exert a regulatory impact on dopamine (DA) neurotransmission locally, and defects in CIN have been implied in several psychiatric disorders. The aim of this study was to investigate the role of CIN in regulation of basal extracellular levels of DA and in modulation of nAc DA release following ethanol administration locally within the nAc of male Wistar rats. Using reversed in vivo microdialysis, the acetylcholinesterase inhibitor physostigmine was administered locally in the nAc followed by addition of either the muscarinic acetylcholine (ACh) receptor antagonist scopolamine or the nicotinic ACh receptor antagonist mecamylamine. Further, ethanol was locally perfused in the nAc following pretreatment with scopolamine and/or mecamylamine. Lastly, ethanol was administered locally into the nAc of animals with accumbal CIN-ablation induced by anticholine acetyl transferase-saporin. Physostigmine increased accumbal DA levels via activation of muscarinic ACh receptors. Neither scopolamine and/or mecamylamine nor CIN-ablation altered basal DA levels, suggesting that extracellular DA levels are not tonically controlled by ACh in the nAc. In contrast, ethanol-induced DA elevation was prevented following coadministration of scopolamine and mecamylamine and blunted in CIN-ablated animals, suggesting involvement of CIN-ACh in ethanol-mediated DA signaling. The data presented in this study suggest that basal extracellular levels of DA within the nAc are not sustained by ACh, whereas accumbal CIN-ACh is involved in mediating ethanol-induced DA release.

KEYWORDS

alcohol, cholinergic interneurons, in vivo microdialysis, muscarinic acetylcholine receptors, physostigmine, rat

1 | INTRODUCTION

Alcohol use disorder is a chronic and relapsing brain disorder associated with numerous serious medical consequences leading to preterm death, and alcohol remains one of the main avoidable risk factors highly contributing to the global burden of disease.[1] Alcohol activates the mesolimbic dopamine (DA) system leading to increased extracellular levels of DA in the main projection area, the nucleus accumbens (nAc),[2] an event implicated in reward-related behavior and reinforcing effects of drugs of abuse.[3,4]

Our research group has for more than two decades studied the mechanisms underlying ethanol's ability to release DA within the reward system. Cholinergic neurotransmission has been implicated, where activation of nicotinic acetylcholine (ACh) receptors (nAChRs) within the ventral tegmental area (VTA) in the midbrain seems crucial for ethanol-induced DA release in the nAc.[5–9] Data regarding the role of accumbal nAChRs in ethanol-induced DA release, however, are inconclusive. Some studies suggest that accumbal nAChRs are not critical as infusion of a nAChR antagonist locally into the nAc leaves the ethanol-induced DA release unaltered,[6,10] while others suggest involvement of specific subtypes of nAChRs in ethanol-induced DA release within the nAc.[11] Systemic administration of varenicline, a partial nAChR agonist, attenuates ethanol intake in both rodents[12] and humans[13–15] and increases extracellular levels of DA in rat nAc. The mechanism of action was originally thought to be due to activation of nAChRs in the VTA[16,17]; however, modulation of DA release via nAChRs located on dopaminergic terminals within the nAc has also been suggested.[18]

The main endogenous source of ACh in the nAc is cholinergic interneurons (CIN), and to a lesser extent, cholinergic projections from the midbrain.[19] The CIN are large and tonically active cells that exert regulatory impact through extensive axonal arborization.[20] Although scarce in numbers, constituting only 1% to 2% of the neuronal cell population, CIN have arisen as an important cell population within the nAc.[21,22] Interestingly, selective optogenetic activation of accumbal CIN induces DA release, independent of mesolimbic activation of the DA neurons.[23,24] On the contrary, selective activation of cholinergic neurons projecting from the midbrain does not induce striatal DA release,[24] suggesting that cholinergic regulation of DA release in the dorsal striatum is mediated by CIN.

The CIN-mediated augmentation of DA release has been proposed to be mediated via ACh acting on nAChRs located on DA terminals,[23,25] whereas others suggest the involvement of muscarinic ACh-receptors (mAChRs), as muscarinic agonists facilitate DA release.[26,27] Concurrently, DA regulates CIN via both D1 and D2 receptors.[28] Thus, a reciprocal presynaptic regulation of neurotransmitter release occurs locally in an intricate manner. Defects in CIN, and consequently a DA-ACh imbalance, have been suggested to be involved in disorders traditionally associated with deficient dopaminergic signaling, such as movement disorders,[29] schizophrenia,[30] and addiction.[31,32] The precise mechanisms through which endogenous cholinergic activity modulates DA release in response to ethanol administration in vivo and its role in the development of alcohol addiction are not known. We hypothesize that ethanol-induced DA release involves activation of CIN. In this study, we aimed to investigate the role of ACh and CIN in the regulation of basal accumbal DA tone and in the modulation of nAc DA release following ethanol administration. To this end, we used the in vivo microdialysis technique with local pharmacological manipulations combined with a toxin-based approach to selectively ablate accumbal CIN.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Wistar Han rats (n = 157, Envigo, Venray, The Netherlands), weighing 150 to 250 g at arrival to the facility, were group-housed under controlled environmental conditions with a 12-h light/dark cycle (lights on at 7:00 AM and lights off at 7:00 PM). The animals had free access to standard rat chow and water and were allowed to adapt to the novel environment for 1 week prior to the initiation of any experiments. All experiments were conducted in accordance with protocols approved by the Ethics committee for Animal Experiments, Gothenburg, Sweden (213/14, 214/14).

2.2 | Drugs and chemicals

Physostigmine salicylate, scopolamine hydrobromide trihydrate, and mecamylamine hydrochloride were all dissolved in Ringer's solution (140 mM NaCl, 1.2 mM $CaCl_2$, 3.0 mM KCl, and 1.0 mM $MgCl_2$) to a concentration of 50 μM and administered alone or in combination with 300 mM ethanol (95%, Kemetyl AB, Haninge, Sweden) locally into the nAc using reversed microdialysis in naïve animals. Pefusion with 300 mM ethanol is estimated to yield approximately 50 to 70 mM ethanol immediately outside the probe (unpublished results). The immunotoxin anticholine acetyl transferase (ChAT)–saporin and a sham-solution, rabbit-IgG-saporin (Advanced Targeting Systems Inc, San Diego, CA; Cat No.: AB-N34ap and Cat No.: IT-35, respectively) were diluted in Ringer's solution to a concentration of 0.5 μg/μl. All reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.3 | Surgery

Rats were anesthetized with 4% isoflurane (Baxter, Kista, Sweden), mounted onto a stereotactic instrument (David Kopf Instruments, Tujunga, CA, USA) and placed on a heating pad to prevent hypothermia. For microdialysis experiments, the skull was exposed and one hole was drilled above the nAc unilaterally and two additional holes were drilled for attachment of anchoring screws. A custom-made I-shaped probe, with a molecular cut-off of 20 kDa and an active space of 2 mm, was gently lowered into the nAc core-shell borderline region (A/P: +1.85, M/L: −1.4 relative to bregma; D/V: −7.8 relative to dura mater; Paxinos and Watson 6th ed. 2007). The dialysis probe together

with the two anchoring screws were fixed to the skull using Harvard cement (DAB Dental AB, Gothenburg, Sweden). Rats were housed individually and allowed to recover for 48 h prior to the in vivo microdialysis experiment. For lesioning of CIN, one hole was drilled unilaterally above the target area, nAc, after exposing the skull. A 10-µl Hamilton syringe attached to a 31-gauge microinjection canula (AMI-5T, AgnTho's AB, Lidingö, Sweden) was used to administer the active anti-ChAT-saporin- or sham rabbit-IgG-saporin-solution. The cannula was lowered to coordinates approximating nAc (A/P: +1.5, M/L: −1.4 relative to bregma, V/D: −7.8 relative to the skull; adjusted for age from Paxinos and Watson 6th ed. 2007), and the solutions were infused using a microperfusion pump (Univentor-864 Syringe Pump; AgnTho's AB, Lidingö, Sweden), at a flow rate of 0.05 µl/min for 10 min giving a total volume of 0.5 µl. The cannula was withdrawn 5 min after completion of the infusion, in order to allow diffusion. The wound was closed using staples, and the rats were given postoperative analgesia (Norocarp vet 1 mg/ml, 5 mg/kg, s.c; Apoteket AB, Sweden). The rats returned to their home cage and were housed for 4 weeks prior to insertion of microdialysis probe or being sacrificed for immunohistochemistry.

2.4 | In vivo microdialysis

Rats were awake and freely moving throughout the in vivo microdialysis experiment. The microdialysis probe was connected to a microperfusion pump and perfused with Ringer's solution at a rate of 2 µl/min, initially for a period of 2 h in order to equilibrate the fluid exchange before starting sampling (every 20 min). After obtaining four baseline samples, pharmacological substances diluted in Ringer's solution or ethanol solution were continuously perfused using reversed in vivo microdialysis. Microdialysate DA content was analyzed using high-performance liquid chromatography with electrochemical detection, as previously described.[33] Animals were sacrificed immediately after the experiment, and the brains were fixed in Accustain Formaline-free fixative for 3 to 7 days prior to verification of probe placement by gross examination of manually sliced sections. Animals with misplaced probes or visual defects were excluded from the statistical analysis. In the first set of in vivo microdialysis experiments, the ACh esterase inhibitor physostigmine was locally perfused into the nAc in order to study the local effect of ACh (Experiment 1). In the following set of experiments, ACh-antagonists were coperfused with physostigmine to determine the role of mAChRs and nAChRs, respectively, in ACh-mediated modulation of DA release (Experiment 2). In the third set of microdialysis experiments, the role of the different AChRs in ethanol-mediated DA elevation was studied (Experiment 3). In the last set of experiments, the role of cholinergic neurotransmission in ethanol-induced DA release was investigated using a CIN-lesioned in vivo model (Experiment 4) (schematic overview of experimental design in Figure 1A).

2.5 | Immunohistochemistry

Animals were deeply anesthetized with Allfatal (100 mg/ml Apoteket AB, Sweden, 350 mg/kg i.p.) and perfused transcardially with buffer (116 mM NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2$, 0.4 mM $MgSO_4$, 1.3 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5.5 mM glucose), followed by 4% ice-cold paraformaldehyde solution. The brains were postfixed in paraformaldehyde solution for 90 min followed by incubation in sucrose solutions of increasing concentration, 10% and 20%, before being snap frozen by isopentane on dry ice. Using a Leica CM1950 cryostat, the brains were cut in 40-µm sections throughout the nAc, placed in cryoprotective solution (30% glycerol, 30% ethylene glycol, 40% 1 × Tris-buffered saline (TBS; 0.15 M NaCl, 0.05 M Tris-HCL; pH 7.6)) and kept at −20°C until further processing. Brain sections were washed in 1 × TBS 3 × 10 min, pretreated with 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA), 0.1% triton-X-100 in 1 × TBS for 1 h, following incubation of primary antibodies, polyclonal goat anti-ChAT (Ab144p, Merck Millipore) 1:200, and monoclonal rabbit anti-NeuN (MABN140, Merck Millipore) 1:400 in 1 × TBS with 5% normal donkey serum and 0.1% triton X-100 overnight at 4°C. 1 × TBS was used to wash brain sections 3 × 10 min prior to incubation with secondary antibodies, donkey-antigoat Alexa 488 and donkey-antirabbit Alexa 555 (1:1000, Invitrogen, Thermo scientific) diluted in 1 × TBS with 5% normal donkey serum and 0.1% triton X-100 for 1 h. A last washing

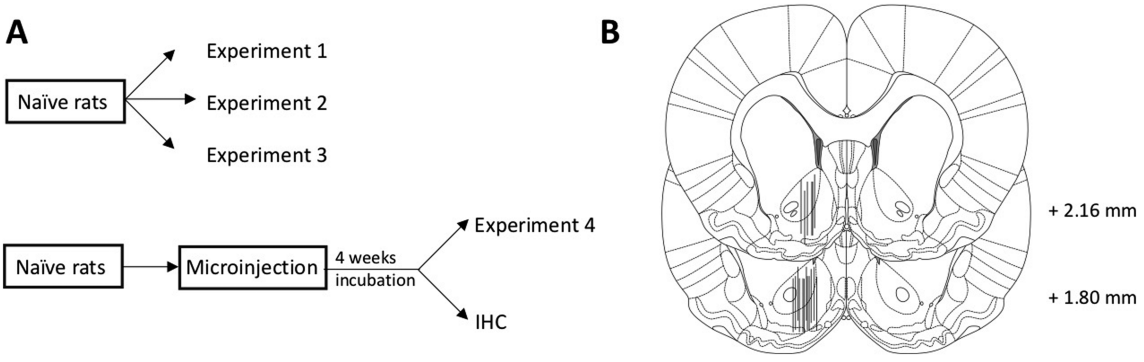

FIGURE 1 Overview and histology. (A) Schematic overview of the experimental design. IHC, immunohistochemistry. (B) Histology. Black lines show representation of a subset of the dialysis probe placements of the four separate microdialysis experiments. Approximately, every forth animal is represented. Numbers indicate distance from bregma

session was performed before the brain sections were mounted onto microscope slides using Fluoroshield, and images were obtained using a Zeiss LSM 700 Inverted confocal microscope (Zeiss, Jena, Germany). Quantification of ChAT-positive neurons within the nAc was performed using images representing nAc (in accordance with Paxinos and Watson 6th ed. 2007) obtained with a Nikon eclipse 80i microscope with a 10× objective, connected to a Nikon DS-Qi1Mc camera and an NIS-Element Image software (Nikon Instruments Inc.). Image J software (National Institute of Health, Bethesda, MD) was used for cell counting. Number of ChAT-positive neurons present in the microinjected hemisphere (either anti-ChAT-saporin or rabbit-IgG-saporin) were compared with the corresponding nontreated hemisphere in order to estimate the treatment effect as percent ChAT-positive cells. The treatment effect between toxin-treated and sham-treated animals were then compared in order to assess statistical significance. Cell counts from 15 to 21 sections from each brain (anti-Chat-sap $n = 3$, rabbit-IgG-Sap $n = 3$) were used for comparison.

2.6 | Statistics

GraphPad Prism 8 Software (San Diego, CA, USA) was used for all statistical evaluations. For all microdialysis experiments, area under curve (AUC) was analyzed for each individual animal, and *t*-test or one-way ANOVA with Dunnet's post hoc test was used for comparative analysis of the AUC between treatment groups. For quantification of CIN-depletion, percent ChAT-positive cells in toxin-treated animals were compared with sham-treated animals using unpaired *t*-test. All values are presented as mean ± standard error of the mean (SEM), and the significance level was set to $P < 0.05$.

3 | RESULTS

3.1 | ACh esterase inhibition elevates DA levels, an effect blocked by a muscarinic antagonist

Local administration of the ACh esterase inhibitor physostigmine (50 μM) into the nAc increased extracellular levels of accumbal DA as compared with vehicle (two-tailed *t*-test, $t^{19} = 5.37$, $P < 0.001$) (Figure 2B). In order to define if this effect was mediated via mAChRs or nAChRs, specific antagonists were used. When applying physostigmine combined with the mAChR antagonist scopolamine (50 μM), a significant attenuation of the DA-elevating effect was seen as DA levels returned back to baseline level (one-way ANOVA of AUC values, treatment effect: $F_{2, 31} = 3.89$; $P = 0.031$; post hoc test: $P = 0.034$) (Figure 2C,D). However, when combining physostigmine with the nAChR antagonist mecamylamine (50 μM), the physostigmine-mediated DA elevation was not altered (post hoc test: $P = 0.975$) (Figure 2C,D).

3.2 | Ethanol-induced DA elevation is blocked using a combination of muscarinic and nicotinic ACh receptor antagonists

In order to study if ethanol-induced DA elevation involves ACh, pretreatment with specific antagonists was given prior to local ethanol administration. Scopolamine and mecamylamine themselves did not alter extracellular levels of DA at the concentrations used (one-way ANOVA of AUC values, treatment effect: $F_{2, 22} = 0.29$, $P = 0.750$) (Figure 3A,B). Local perfusion of 300 mM ethanol into the nAc

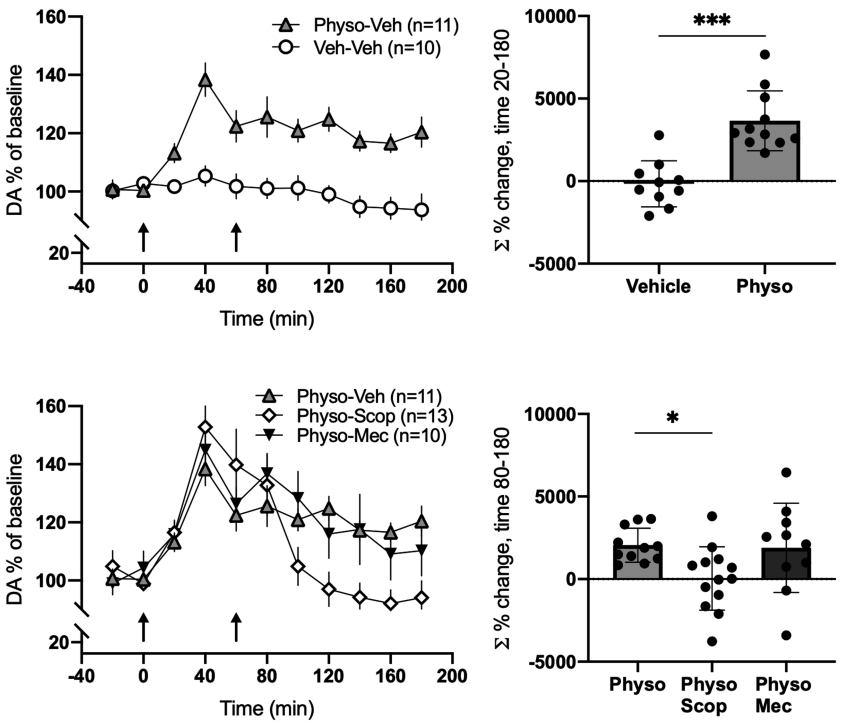

FIGURE 2 Local perfusion of an acetylcholine esterase inhibitor into the nucleus accumbens (nAc) increases extracellular levels of dopamine. in vivo microdialysis performed in the rat nucleus accumbens presented as dopamine (DA) percent of baseline mean ± standard error of the mean (SEM) (A and C) and area under curve (AUC) mean ± SEM (B and D). Arrows indicate start of local drug perfusion at timepoint 0 (A and C) and addition of a second drug to the perfusion (either scopolamine or mecamylamine) at timepoint 60 (C). Increased extracellular levels of DA was observed after local perfusion of physostigmine (50 μM) (A and B). Physostigmine-induced DA release was blocked by addition of scopolamine (50 μM) but not by addition of mecamylamine (50 μM) (C and D). *T*-test or one-way ANOVA with multiple comparison and Dunnet's post hoc test were performed for timepoints of interest. $^{*}P < 0.05$, $^{***}P < 0.001$

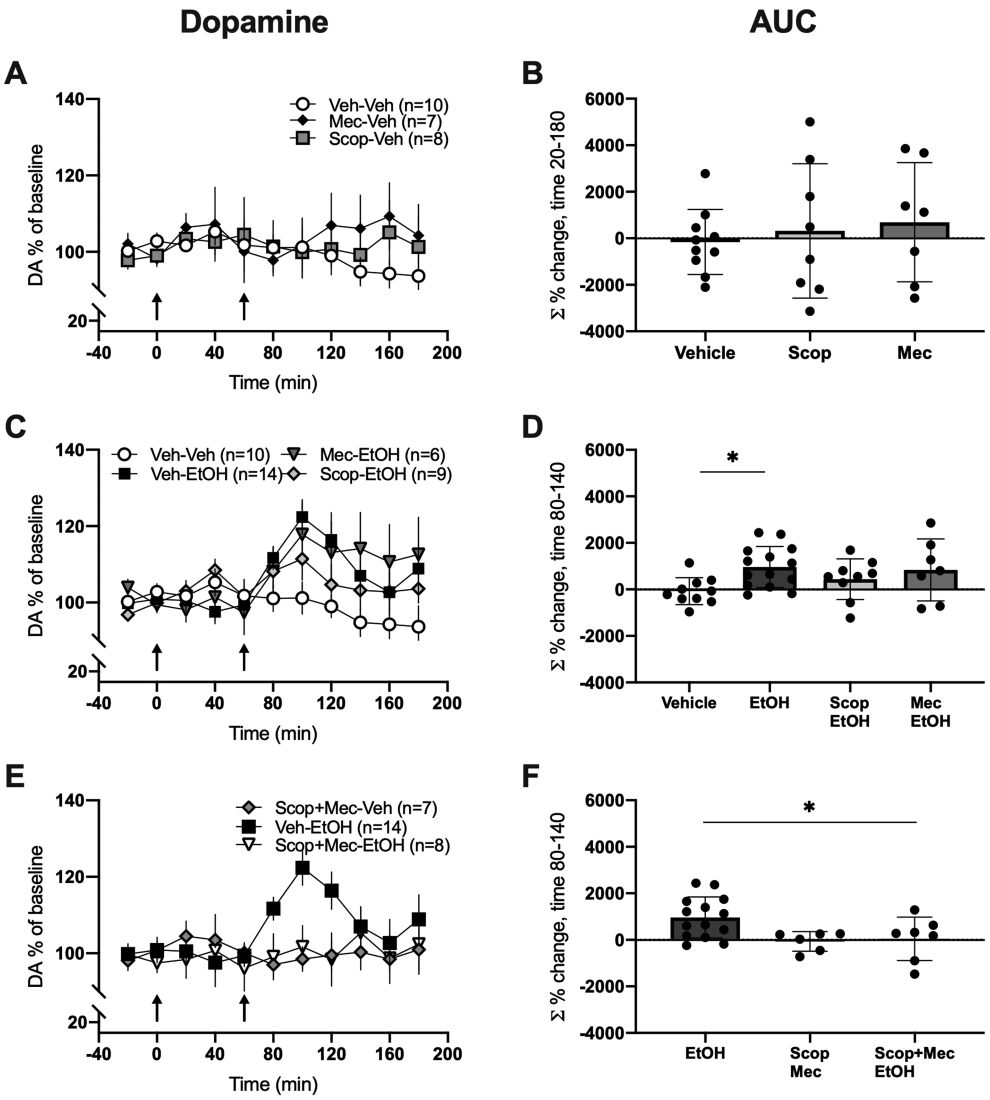

FIGURE 3 Ethanol-induced dopamine elevation is hindered when both muscarinic and nicotinic acetylcholine receptors are antagonized. in vivo microdialysis performed in the rat nucleus accumbens presented as dopamine (DA) % of baseline mean ± standard error of the mean (SEM) (A, C, and E) and area under curve (AUC) mean ± SEM (B, D, and F). Arrows indicate start of drug perfusion at timepoint 0 and addition of a second drug at timepoint 60 (A, C, and E). (B) Scopolamine (50 μM) and mecamylamine (50 μM) did not alter basal DA levels. (D) Local perfusion of ethanol (300 mM) increased extracellular levels of DA, an effect not significantly altered by neither scopolamine nor mecamylamine alone. (F) Pretreatment with a combination of scopolamine and mecamylamine blocked the ethanol-induced DA elevation. One-way ANOVA of AUC with Dunnet's post hoc test between treatment groups (B, D, and F). *$P < 0.05$

significantly increased the level of extracellular DA as compared with vehicle-treated controls (one-way ANOVA of AUC-values, treatment effect: $F_{3, 35} = 2.93$, $P = 0.047$; post hoc test: $P = 0.016$) (Figure 3C,D). Neither scopolamine (50 μM) nor mecamylamine (50 μM) altered the ethanol-induced DA elevation (post hoc test: $P = 0.420$ and $P = 0.985$, respectively) (Figure 3C,D). However, pretreatment with scopolamine (50 μM) combined with mecamylamine (50 μM) prevented the DA response to ethanol (one-way ANOVA of AUC values, treatment effect: $F_{2, 24} = 4.72$, $P = 0.019$; post hoc test: $P = 0.043$) (Figure 3E,F).

3.3 | Microinfusion of anti-ChAT-saporin into the nAc reduces the number of CIN

In order to further study the cholinergic modulation of accumbal DA release, a hypocholinergic in vivo model of the nAc was produced using local microinfusion of anti-ChAT-saporin. Following 4 weeks of incubation, immunohistochemical staining showed a significant reduction in ChAT-positive neurons in anti-ChAT-saporin-injected (toxin-treated) animals as compared with rabbit-IgG-saporin-injected (sham-treated) controls (two-tailed t-test, $t^4 = 17.59$, $P < 0.001$) (Figure 4C). Overall, an 81% ± 1 ($n = 3$) reduction of CIN was obtained in the toxin-treated animals, while no reduction of CIN was detected in sham-treated controls (−1.8% ± 5, $n = 3$). The effect was observed in both shell and core and restricted to this area as no reduction of cholinergic neurons was noted in adjacent brain areas such as the dorsal striatum or the vertical limb of the diagonal band (VDB) (Figure 4J). To verify the specificity of the immunotoxin, coimmunostaining with an antibody targeting NeuN, found exclusively in the nuclei of neuronal cells, was performed together with the antibody targeting ChAT. The anti-ChAT-saporin seems to be specific as no general neuronal degeneration was detected except for in a minor area directly adjacent to the injection site. When using a higher dose (0.75 μg/ul, 0.5 μl), CIN were completely ablated but at the expense of an extensive general neuronal degeneration (data not shown).

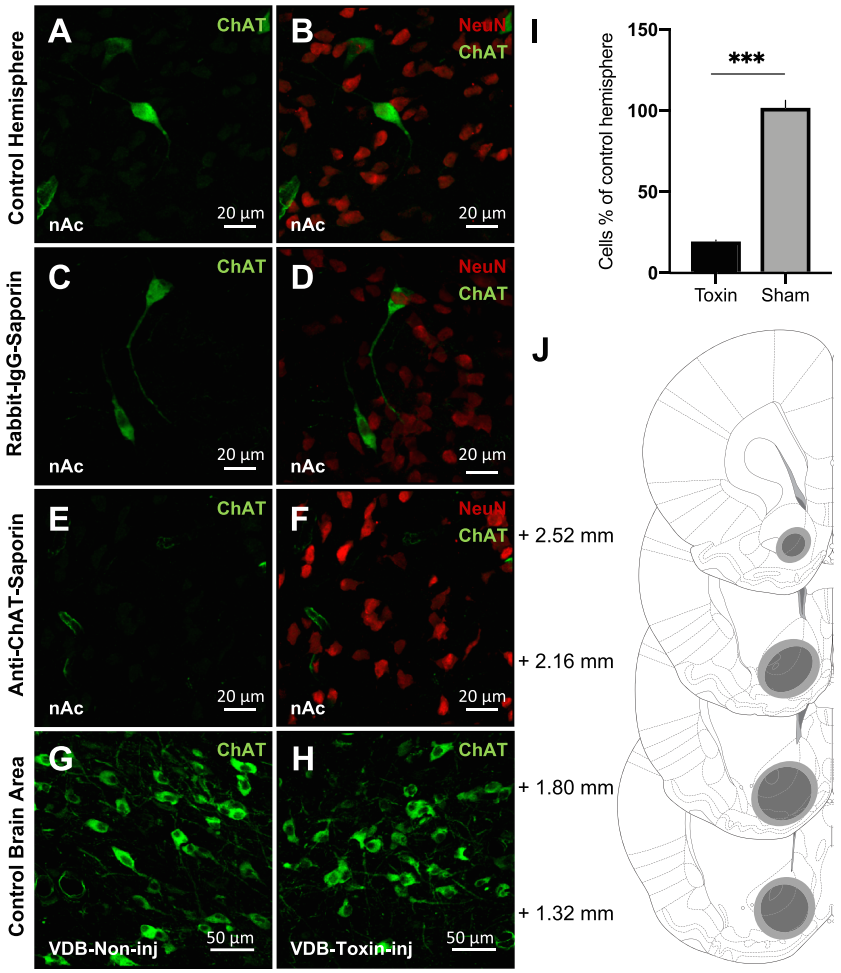

FIGURE 4 Local microinjection of anti-ChAT-saporin results in a significant reduction in number of cholinergic interneurons locally. (A, B, D, E, and G-I) Confocal images of ChAT (green) and NeuN (red) immunohistochemistry on sections representing nucleus accumbens (nAc) (A-F) and the vertical limb of the diagonal band (VDB) (G-H). Intact ChAT-positive neurons were present in control hemisphere (A and B) and sham-treated (rabbit-IgG-sap) hemisphere (C and D) but not in toxin-treated (anti-ChAT-sap) hemisphere (E and F). Intact ChAT-positive neurons were present in VDB, an area located adjacently to the nAc, in both noninjected hemisphere (G) and toxin-treated hemisphere (H). (I) In toxin-treated rats (n = 3), 19% of cholinergic interneurons (CIN) were remaining, as compared with corresponding control hemisphere, while no reduction of CIN was seen in sham-treated rats (n = 3) (t-test, mean CIN % of control hemisphere ± standard error of the mean [SEM]). (J) Schematic drawings on representative coronal sections of the rat nAc showing the smallest (dark gray) and largest (light gray) CIN-ablation. Numbers indicate distance from bregma. The CIN-ablation was spread in both nAc shell and core. $^{***}P < 0.001$

3.4 | Depletion of cholinergic signaling within the nAc results in decreased DA elevation in response to ethanol administration

To assess the effect produced by CIN depletion on baseline DA levels and ethanol-mediated DA-release, in vivo microdialysis experiments were performed. Baseline DA levels were not significantly altered in the nAc of toxin-treated animals as compared with sham-treated controls (2.81 nM ± 0.94 nM and 2.66 nM ± 0.94 nM, respectively; two-tailed t-test, $t^{16} = 0.32$, $P = 0.751$) (Figure 5A). However, toxin-treated animals exhibited a blunted increase in extracellular DA in response to local administration of ethanol as compared with vehicle-treated controls. Sham-treated controls conversely displayed a significant ethanol-induced DA elevation compared with vehicle-treated rats (one-way ANOVA of AUC-values, treatment effect: $F_{2,\ 24} = 9.11$, $P = 0.001$; post hoc test: $P = 0.097$ and 0.001, respectively) (Figure 5C).

4 | DISCUSSION

By means of in vivo microdialysis, we showed in this study that local administration of an ACh esterase inhibitor significantly increases accumbal DA levels via activation of mAChRs, implicating that activation of CIN may increase DA release. Conversely, baseline DA levels were unaffected by local administration of antagonists targeting nAChRs and/or mAChRs, suggesting that these receptors do not tonically control extracellular DA levels in the nAc. This result was further supported by the finding that basal DA levels were unaltered in animals with a targeted ablation of CIN, as compared with sham-treated controls. Furthermore, the DA-elevating property of ethanol was prevented following coadministration of scopolamine and mecamylamine as well as blunted in CIN-lesioned animals, suggesting involvement of CIN in ethanol-induced DA release.

Physostigmine inhibits ACh degradation[34] and therefore increased extracellular ACh levels can here be hypothesized, although not directly confirmed. The physostigmine-induced DA elevation presented in this study thus suggests that ACh augments DA release. In order to study if this DA release was mediated via mAChRs, nAChRs, or both, specific antagonists were used. Scopolamine completely reversed the physostigmine-induced DA release while mecamylamine did not, suggesting that the ACh-induced elevation of extrasynaptic DA in vivo was mediated via mAChRs within the rat nAc. Others demonstrate similar results in an in vitro setting where physostigmine facilitated DA signaling within the nAc, an event reversed by scopolamine, thus supporting our theory.[35] Whether the observed effect of

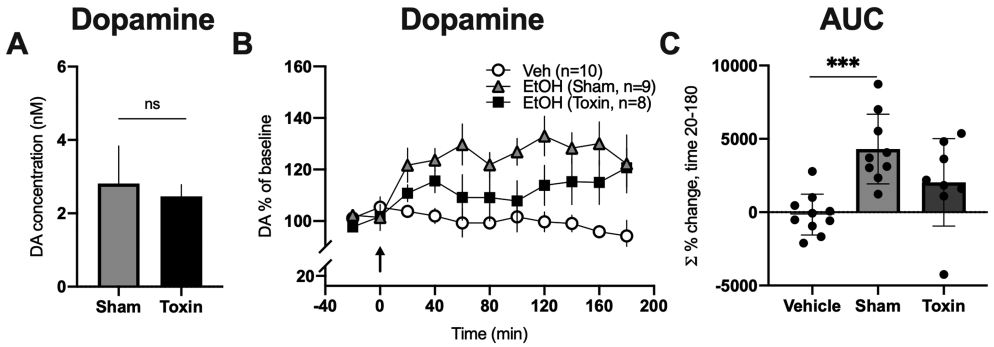

FIGURE 5 Rats with ablation of accumbal cholinergic interneurons (CIN) show an attenuated dopamine response to local perfusion of ethanol. Absolute dopamine (DA) concentrations at baseline timepoint 0 (A) were not significantly different between sham-treated (n = 9) and toxin-treated (n = 8) rats (two-tailed t-test, P = 0.751). (B) Graph representing the in vivo microdialysis experiment. Shown are means ± standard error of the mean (SEM) of DA levels in percent of baseline. The arrow indicates start of perfusion with 300 mM ethanol at timepoint 0 in toxin-treated and sham-treated rats. Sham-treated animals displayed an increase of extracellular levels of DA after ethanol perfusion, significantly different from vehicle-treated rats, presented as area under curve (AUC) for timepoints 20 to 180 min in C. (C) Toxin-treated rats showed an increase of extracellular DA in response to ethanol administration but not to the same extent and not significantly different from vehicle-treated rats. One-way ANOVA with Dunnet's post hoc test. $^{***}P < 0.001$

physostigmine is mediated via ACh-induced activation of mAChRs directly located on DA terminals or indirectly via adjacent neurons cannot be determined from the present studies. The muscarinic modulation within the nAc is complex. M_5 receptors, expressed on midbrain DA neurons,[36] facilitate DA release when activated,[35,37] and systemic administration of a negative allosteric modulator of M_5 decreased self-administration of ethanol but not natural rewards in rats,[38] suggesting an involvement of M_5 in the reinforcing effects of ethanol. Furthermore, striatal M_4 receptors may facilitate DA release via inhibition of GABAergic-projecting neurons resulting in decreased tonic $GABA_A$ receptor-mediated inhibition of DA terminals,[37] and/or DA cell bodies in the VTA. The DA elevation seen here could hence possibly be mediated via activation of M_5 receptors located on DA terminals and/or via activation of M_4 receptors located on medium spiny neurons (Figure 6). M_2 and M_4 receptors are also known to be expressed on CIN acting as autoreceptors decreasing ACh release and possibly decreasing DA release.[35,39] Many players are involved in accumbal signaling, and the exact mechanisms involved here are not possible to delineate with the methods used in this study. Interestingly, local accumbal injection of scopolamine, but not mecamylamine, has been shown to hamper reward-related behavior in mice,[40] thereby indirectly supporting a role for mAChRs in accumbal DA release in vivo.

Other studies suggest that nAChRs are responsible for ACh-mediated DA release.[23,25] This hypothesis was not supported by the present data. However, the abovementioned studies have investigated phasic DA release as opposed to this study where canges in DA release within the range of 20 min were studied. It should further be noted that physostigmine in addition to its ACh esterase inhibitory effect may directly inhibit nAChRs.[41] Thus, nAChRs might here already have been blocked by physostigmine, preventing ACh to produce its full effect and leaving little room for further blockade by mecamylamine. Moreover, excess ACh following physostigmine infusion may desensitize nAChRs, concealing the effect of mecamylamine when applied after choline esterase inhibition.[42] However, neostigmine, another choline esterase inhibitor, probably not inhibiting nAChRs in doses used,[43] also increased DA levels and this effect was not blocked by pre-perfusion with mecamylamine (Figure 1, Supporting Information) arguing against nAChR involvement in the DA elevation observed after ACh esterase inhibition. Firm conclusions regarding the tentative involvement of nAChRs in physostigmine-induced DA release remain, however, difficult to draw from these experiments.

Even though there are no studies confirming an increase in accumbal ACh in response to ethanol, voluntary ethanol intake has previously been shown to increase extracellular ACh levels in the VTA.[44] It is thus possible that ethanol has a general ability to activate cholinergic neurons. Furthermore, ethanol may also influence cholinergic neurotransmission by affecting cholinergic receptor activation, as studies have shown that ethanol may potentiate nAChR-mediated currents.[45] In order to study if ethanol-induced DA elevation in the nAc involves ACh, animals were locally pretreated with scopolamine, mecamylamine, or a combination of the two antagonists prior to accumbal perfusion of EtOH. Pretreatment with mecamylamine did not alter the ethanol-mediated DA response, which is in line with previous studies conducted by our research group.[6] Pretreatment with scopolamine slightly but nonsignificantly hampered the ethanol-induced DA elevation. However, when combining scopolamine with mecamylamine, the ethanol-induced DA release was totally blocked. Considering that the rewarding effect by ethanol has been linked to the DA releasing effect,[46,47] this finding suggests that both mAChRs and nAChRs are involved in mediating the rewarding effect by ethanol. One could hence speculate that ethanol activates CIN, inducing an ACh release locally within the nAc, causing activation of various different AChRs resulting in an increase of extracellular DA.

To further determine the role of ACh, most likely originating from accumbal CIN, in ethanol-mediated DA release, a toxin-based method to selectively ablate CIN locally within the nAc was used. An

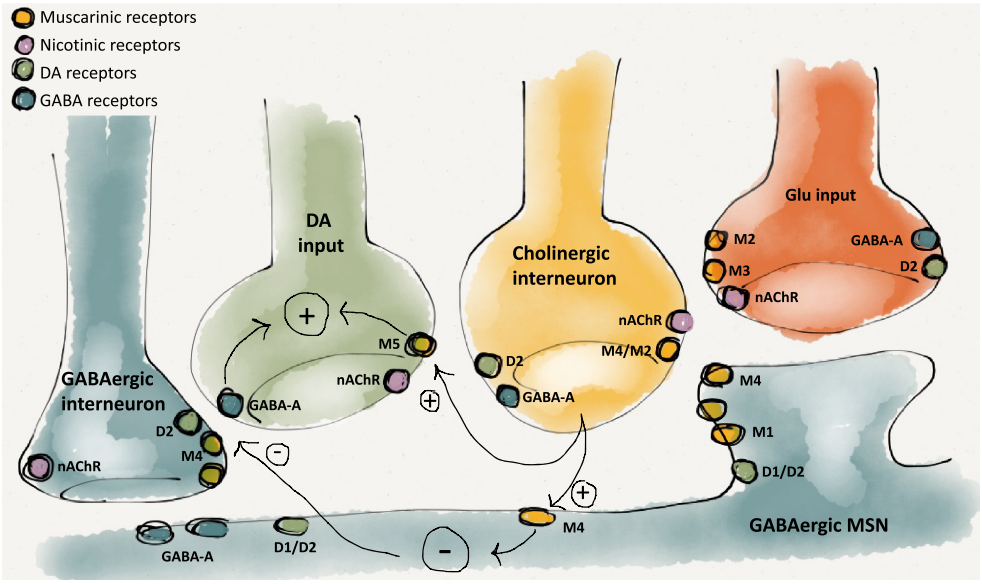

FIGURE 6 Simplified schematic illustration representing a plausible route of action for acetylcholine (ACh)–mediated increase in extracellular levels of dopamine (DA) within the nucleus accumbens (nAc). Increase in extracellular levels of accumbal ACh binds to M_5 receptors located on DA terminals facilitating DA release. ACh also binds to M_4 receptors located on GABAergic medium spiny neurons (MSN) inhibiting the neuron resulting in decreased inhibitory impact on DA terminals and/or on DA cell-bodies (via backward projecting MSNs), further facilitating DA release

irreversible ribosomal inhibitor saporin, conjugated to an antibody targeting ChAT,[48] enabled selective lesion of cholinergic neurons within the nAc. As a part of the optimization during the development of this in vivo model, different doses of the toxin were evaluated. A dose of 0.75 μg/ul, 0.5 μl of the anti-ChAT-saporin produced a 100% depletion of CIN; however, a widespread general neuronal degeneration was also observed. When using the dose of 0.5 μg/ul, 0.5 μl, also used in previous publications,[48–50] an 81% ablation of CIN was obtained, whereas no ablation was seen in sham-treated controls. The lower dose was therefore chosen for the following experiments. The fact that not all CIN were deleted may be an advantage, as a decrease in CIN might in fact better mimic a state of disease than a complete destruction. A human postmortem study has, for example, shown that schizophrenic patients have a decreased number of CIN within the ventral striatum (nAc) as compared with healthy controls.[30] No widespread neuronal degeneration was detected with the dose used, except for in a small area directly adjacent to the injection site, likely due to inevitable traumatic injury caused by the injection cannula. However, the affected area was considerably smaller than the area with CIN-ablation and therefore considered to be negligible. When administering ethanol locally into the nAc of toxin-treated rats, an attenuated DA response was observed. That the DA response was not completely abolished might be explained by the remaining CIN activity. The attenuation was hypothesized to be due to decreased ACh-transmission as a consequence of CIN ablation. Although not proven in this study, treatment with anti-ChAT-saporin has previously been shown to decrease the tissue content of ACh.[51] It should be noted that CIN seem to corelease glutamate.[52] Ablation of CIN may thus indirectly affect EtOH-induced DA release also by altering glutamatergic neurotransmission. The attenuated ethanol response is in line with the finding that scopolamine together with mecamylamine blocked the ethanol-mediated DA elevation.

The present results indicate that CIN ACh does not tonically control extracellular DA levels within the nAc. This conclusion is based on the findings that neither the AChR antagonists in combination nor the CIN ablation affected baseline DA levels. The possibilities remain, however, that the doses of the antagonists used were not sufficient to produce full antagonistic effects and that residual, minor CIN activity sustained extracellular DA levels. On the other hand, higher doses may convey unspecific effects and were therefore avoided. Our conclusion gains support from a previous study showing no difference in tissue levels of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) between control and CIN-ablated rats, using the same ablation-methodology.[49]

It has previously been demonstrated that accumbal glycine receptors regulate basal DA tone within the nAc[53] and are involved in the DA elevation following both systemic and local ethanol administration.[54,55] Interestingly, glycine receptors are expressed on striatal CIN, as shown by immunohistochemistry[56] and single-cell quantitative-PCR.[57] Ethanol has been suggested to interact with these receptors, inducing striatal ACh release originating from CIN.[58] The role of this interaction in ethanol-induced DA release is not clear, but the present data are in line with such a hypothesis. In contrast, glycine receptors controlling basal DA levels are probably not located on CIN since CIN ablation as well as scopolamine plus mecamylamine failed to alter baseline DA levels. Thus, different glycine receptors may be involved in controlling basal DA levels and the DA-activating effect of ethanol, respectively. It would apparently be interesting to further investigate the role of glycine receptors located on CIN in ACh-DA interactions.

A reciprocal homeostatic modulation between ACh and DA within the nAc appears to be of importance, where CIN-dysfunction in the striatum and the nAc are implied in neurodegenerative and neuropsychiatric disorders, respectively. How ethanol interferes with this reciprocal modulation is not known. Our findings indicate that ethanol produces part of its DA-releasing effect, when applied locally in nAc, through release of ACh from CIN, as shown by a combination of in vivo microdialysis and a toxin-based ablation of accumbal CIN.

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AUTHOR CONTRIBUTIONS

BS and ME were responsible for the study concept. BS, ME, and AL were responsible for the study design. AL performed the experiments, performed the statistical analyses, and drafted the manuscript. LA, ME, and BS provided critical revision of the manuscript for intellectual contents. All authors critically reviewed content and approved the final version of the paper.

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