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Dysfunktionale Lernprozesse und ihre neuronalen, behavioralen und (epi)genetischen Korrelate bei Patient*innen mit Alkoholabhängigkeit im Vergleich zu Kontrollen

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Abkürzungen

AUD	Alcohol Use Disorder/Alkoholgebrauchsstörung
BOLD	Blood-Oxygen-Level Dependent
BF	Bonferroni-korrigiert
CON	Kontrollen
CPG	5'-C-Phosphat-G-3'
CS	Konditionierter Stimulus
CR	Konditionierte Reaktion
DNA	Desoxyribonukleinsäure
DNAm	DNA-Methylierungs-Microarray-Daten
DSM	Diagnostic and Statistical Manual of Mental Disorders
EWAS	Epigenomweite Studien
EPIC	MethylationEPIC BeadChip Kit
FDOPA	[^{18}F]-Fluoro-Dihydroxyphenylalanin
fMRT	funktionelle Magnetresonanztomographie
FDR	False Discovery Rate
FSCV	Fast-Scan Cyclic Voltammetry
ICD	International Classification of Disease
mPFC	medialer Präfrontalkortex
MRS	DNA Methylation Risc Score
NAC	Nucleus accumbens
OFC	Orbitofrontalkortex
PET	Positronen-Emissions-Tomographie
PIT	Pavlovian-to-Intrumental Transfer
PRS	Polygenetischer Risikoscore
SNP	Single Nucleotide Polymorphismus
US	Unkonditionierter Stimulus

1. Einleitung

Die Alkoholabhängigkeit (Alcohol Use Disorder, AUD) ist mit einer Lebenszeitprävalenz von bis zu 30 % eine der häufigsten psychiatrischen Erkrankung weltweit (1). Der regelmäßige und anhaltende Alkoholkonsum ist assoziiert mit schwerwiegenden negativen Konsequenzen, sowohl für das Individuum als auch für die Sozialgemeinschaft. Trotz umfangreicher Behandlungsmöglichkeiten bestehend aus stationären Entgiftungen, Entwöhnungsprogrammen über mehrere Wochen und der Möglichkeit medikamentöser Behandlung sind die Rückfallraten im ersten Jahr hoch (2, 3). Fast zwei Drittel der Patient*innen erleiden bereits innerhalb der ersten drei Monate nach einer Entgiftung einen Rückfall (4). Die Sichtweise auf die Erkrankung und auch das diagnostische Regelwerk unterliegen einem Prozess, der zu einer stetigen Anpassung und Veränderung der Kriterien und der gesellschaftlichen Sicht auf die Erkrankung geführt hat. Dieser Prozess dauert bis heute an und hatte eine parallele und z. T. auseinandergehende Entwicklung der beiden Standard-Diagnosesysteme zur Klassifikation psychiatrischer Krankheitsbilder (das *International Classification of Disease*, ICD (5) und das *Diagnostic and Statistical Manual of Mental Disorders*, DSM (6)) zur Folge: Im diagnostischen Regelwerk der neuen Auflage des ICD 11 (7) wird voraussichtlich der Begriff der Alkoholabhängigkeit (*Alcohol Dependence*) verwendet. Die aktuelle Auflage des DSM V (8) hingegen benutzt das dimensionale Konzept einer Alkoholgebrauchsstörung (*Alcohol Use Disorder*), die von leicht und moderat bis schwer reicht. Diese Veränderung hat zur Folge, dass im DSM V nicht länger zwischen dem schädlichen Gebrauch und der Abhängigkeitserkrankung unterschieden wird (9, 10), sondern diese als Spektrum derselben Erkrankung definiert werden. Beiden gemeinsam sind als wesentliche definierende Kriterien der Alkoholabhängigkeit das Verlangen nach der Substanz, die Vernachlässigung anderer sozialer Aktivitäten, Toleranzentwicklung mit Entzugssymptomen sowie körperliche und psychische Probleme durch den Gebrauch. Bereits in den definierenden Kriterien dieser Erkrankung wird deutlich, dass ein komplexes Zusammenspiel körperlicher Reaktionen einerseits auf den Alkohol, andererseits auf die Erwartung, das Verlangen und die psychische Fixierung auf die Substanz eine Rolle spielen. Ähnlich umfangreich gestalten sich Erklärungsmodelle zur Entstehung der Erkrankung mit komplexen und teilweise widersprüchlichen Angaben im Hinblick auf die Bedeutung von sozialen, kulturellen, genetischen und lerntheoretischen Genesefaktoren (10–12). Obwohl die diagnostischen Kriterien in beiden Regelwerken nahezu identisch sind, liegt ein wesentlicher Unterschied in deren Klassifi-

kation als kategoriales vs. dimensionales Konzept. Das dimensionale Konzept der Alkoholabhängigkeit hat den Vorteil, dass Personen schon bei moderater Ausprägung der Erkrankung den Zugang zum Hilfesystem aus z. B. Beratungsstellen, psychiatrischer und psychotherapeutischer Behandlung in Anspruch nehmen können. Der Nachteil resultiert aus einer ggf. zu frühen Pathologisierung, einer fehlenden Trennschärfe zwischen dem riskanten, dem schädlichen und dem krankhaften Alkoholkonsum und damit einhergehend auch limitierten Ressourcen für schwerer erkrankte Patient*innen. Die Kontroverse zwischen einer dimensionalen vs. kategorialen Klassifikation hat zahlreiche wissenschaftliche Arbeiten angestoßen, darunter auch die im Folgenden aufgeführten (13–23). Die Arbeiten beschäftigen sich mit der Frage, welche Mechanismen eine Rolle bei der Entstehung der Alkoholabhängigkeit spielen, ihren weiteren Verlauf bestimmen und wie (oder ob) eine Transition von der moderaten in die schwere Alkoholgebrauchsstörung stattfinden wird. Eine besondere Rolle spielen hier lerntheoretische Überlegungen zu Entscheidungsprozessen und dem belohnungsabhängigen Lernen. Mit dem Ansatz, Lernprozesse verstehen zu wollen, die in die Abhängigkeit führen, sollen therapeutische Maßnahmen abgeleitet werden, die Wege aus der Abhängigkeit aufzeigen können.

Schon 1976, in den Anfängen der diagnostischen syndromalen Definition der Alkoholabhängigkeit, beschreiben die Autoren: *„His drinking is patterned by varying internal cues and external circumstances. At first, a person becoming caught up in heavy drinking may often widen his repertoire and also the range of cues that signal drinking“* (24). Edward und Gross (24) sprechen bereits von *„internal cues“* und *„cues that signal drinking“*, also der wichtigen Bedeutung von Reiz-Reaktions-Prozessen. Das Erlernen von initial neutralen Reizen, die im Verlauf der Erkrankung einen Signalcharakter für die Patient*innen bekommen, kann zur Aufrechterhaltung der Erkrankung beitragen.

Aus lerntheoretischer Perspektive finden verschiedene Prozesse statt, die dazu führen, dass ein primär positiver, belohnender Reiz in der Folge zu einem Signalreiz für maladaptives, schädliches Verhalten wird. Dazu gehören klassische Konditionierungsprozesse, instrumentelles Lernen und die Kombination beider Lernmodelle, konkret wie Pawlows gelernte Hinweisreize auf instrumentelles Verhalten Einfluss ausüben. Über den klassischen (oder Pawlowschen) Konditionierungsprozess wird ein unkonditionierter neutraler Stimulus (Unconditioned Stimulus, UCS, bei Pawlow die Glocke, in unserem Fall z. B. die Bar) mit einem valenten (positiven/negativen) Reiz gekoppelt (bei Pawlow die Futterpräsentation, bei uns

z. B. der Anblick des Bierglases), der üblicherweise eine körperliche Reaktion hervorruft (Unconditioned Response, UCR, bei Pawlow Speichelfluss, in unserem Fall z. B. soziale Enthemmung oder Entspannung). Durch diese Assoziation während der Konditionierung wird in der Folge der UCS zu einem konditionierten Reiz (Conditioned Stimulus, CS). Dabei überträgt sich also der Wert des valenten Reizes auf den vorher neutralen UCS und der so entstandene CS wird per se als valent erlebt. Werbeplakate, Gerüche, Erinnerungen, Situationen, örtliche Gegebenheiten, die Liste an so entstandenen potenziellen „cues" für Patient*innen mit AUD (also durch die Pawlowsche Konditionierung entstandene CSs im Verlauf der individuellen Trinkhistorie) ist lang und eine mögliche Erklärung für das hohe Risiko eines Rückfalls, „*reinstatement of the syndrome after abstinence*", das schon 1976 von den Autoren Edward und Gross als diagnostisches Element der Erkrankung vorgeschlagen wurde (24).

Neben klassischen Konditionierungsprozessen spielt das instrumentelle Lernen eine ebenso wichtige Rolle. Instrumentelles Lernen, auch als operante oder instrumentelle Konditionierung bezeichnet, beschreibt das Lernen von Verhalten über Belohnung und Bestrafung, indem der Organismus darin geschult wird, Belohnung zu maximieren und Bestrafung zu minimieren (25). Das Belohnungslernen geht zurück auf das 1911 von Thorndike etablierte „Law of Effect" (26). Thorndike konnte nachweisen, dass positive und negative Konsequenzen von Verhalten einen unterschiedlichen Effekt haben: Während sich die Wahrscheinlichkeit, ein Verhalten (Unconditioned Reaction, UCR) in der Zukunft erneut auszuüben, nach Belohnung erhöht (Annäherungslernen, Conditioned Reaction, CR), führt die Bestrafung desselben mit hoher Wahrscheinlichkeit zu dessen Minimierung (Vermeidungslernen, Conditioned Reaction, CR) in der Zukunft. Beide Arten der Verhaltensmodifikation spielen eine wesentliche Rolle beim instrumentellen Lernen.

Im Fall der Alkoholabhängigkeit findet über die Zeit eine Veränderung im instrumentellen Lernen statt, die sich in einer Veränderung des Annährungsverhaltens zeigt. Der initial belohnende Stimulus Alkohol löst zunächst eine Annäherungsantwort auf, die bewusst und zielgerichtet vom Individuum initiiert und beendet werden kann und an eine positive Erwartungshaltung gegenüber dem Stimulus gekoppelt ist. Bei fortgeschrittener AUD, wobei Alkohol hier eher negative Konsequenzen wie gesundheitliche Probleme und soziale Isolation auslöst, ist die Annährungsantwort losgelöst vom Wert des Stimulus. Alkohol wird in der Folge als Reaktion auf gelernte Umgebungsreize automatisiert aufgesucht, obwohl dessen negative Valenz

eigentlich eine Vermeidungsreaktion auslösen sollte. Unterschiedliche Faktoren, die im Individuum selbst und seiner Umwelt begründet sind, können den Verlauf dieser Transition, also das Erlernen von süchtigem Verhalten, begünstigen und so das Risiko, an einer AUD zu erkranken, erhöhen. Die in dieser Habilitationsschrift untersuchten Faktoren sind die Neigung, sich durch Umgebungsreize in seinem Verhalten beeinflussen zu lassen sowie der Einfluss, den genetische Varianten, die Ausprägung kognitiver Fähigkeiten und chronisches Stresserleben auf diese Neigung ausüben können.

Im Tiermodell konnte nachgewiesen werden, dass unterschiedliche Faktoren das Erlernen einer Suchterkrankung begünstigen können, die z. T. bereits genetisch determiniert sind (27, 28). Flagel und Kollegen trainierten und züchteten Ratten darin, eine Reiz-Reaktions-Antwort nach den Prinzipien des klassischen Konditionierens zu erlernen. Das Drücken eines Hebels (der CS) führte zur Ausgabe von Futter (UCS) an einer vom CS entfernten Futterstelle (29), woraufhin bei allen Tieren eine konditionierte Reaktion (CR) mit aufsuchendem Verhalten an der Futterstelle erfolgte. Ein wesentlicher Unterschied im Verhalten der Tiere war ihre Reaktion auf den CS und den UCS. Ein Teil der Tiere, sog. *sign tracker,* beschäftigte sich überdurchschnittlich lang durch Lecken und Zuwendung mit dem CS (Hebel), während die Futterstelle deutlich weniger Beachtung fand. Ein anderer Teil hingegen reagierte nach Präsentation des CS mit einer sofortigen Annäherung an die Futterstelle und wurde entsprechend als *goal tracker* bezeichnet. Über die direkte Ableitung der zellulären Antwort mittels Voltametrie (*fast-scan cyclic voltammetry*, FSCV) an der Synapse während der Darbietung von CS und US wurde nachgewiesen, dass diese eine direkte Korrelation mit der Dopaminausschüttung im Kern des Nucleus accumbens (NAC) hatte. Eine erhöhte phasische Dopaminausschüttung an der Synapse im NAC während der Darbietung des CS war prädiktiv für eine Zunahme des *sign tracking*-Verhaltens, also dafür, wie viel Bedeutung die Tiere dem CS entgegenbringen (in Form der Zuwendung). Es gelang Flagel und Kollegen die Ratten nach diesem Merkmal in 20 Generationen zu züchten. Es konnte des Weiteren gezeigt werden (27, 29–32), dass die Tendenz der *sign tracker*-Tiere, ihre Aufmerksamkeit eher dem konditionierten „ankündigenden" Reiz zuzuwenden, ebenfalls prädiktiv für deren Rezeptivität ist, auf weitere belohnungsassoziierte Reize und Suchtreize zu reagieren. Das heißt, die Tendenz des mesolimbischen Belohnungssystems vermehrt auf belohnungsassoziierte Reize zu reagieren, beeinträchtigt allgemeine Lernprozesse und ist z. T. genetisch determiniert.

In der Translation dieser Experimente auf den Menschen konnten ebenfalls Veränderungen im belohnungsabhängigen Lernen als moderierender Faktor für süchtiges Verhalten nachgewiesen werden. Dabei wurden unterschiedliche lerntheoretische Konzepte und Begrifflichkeiten genutzt, um Veränderungen im operanten und klassischen Konditionieren abzubilden. Da ihr Verständnis für die in der Habilitationsschrift verwendeten Paradigmen wesentlich ist, wird auf deren historische und konzeptuelle Entwicklung im Folgenden kurz eingegangen.

Im Hinblick auf das operante, instrumentelle Lernen wird auf der Begriffsebene zwischen dem sog. *modellfreien*, *habituellen* Verhalten auf der einen und dem *zielgerichteten*, *modellbasierten* Verhalten auf der anderen Seite unterscheiden. Während das modellfreie, habituelle Lernen schnell und mit geringem kognitivem Aufwand umgesetzt werden kann, ist es unflexibel und fehlerbehaftet, wenn es um die Adaptation an neue, komplexere Umgebungsbedingungen geht. In Situationen, in denen wir schnelle und in der Vergangenheit häufig wiederholte Entscheidungen treffen müssen, ist es jedoch von großem Vorteil gegenüber dem modellbasierten Lernen. Letzteres erfordert eine hohe kognitive Kapazität, da bestenfalls alle möglichen Alternativen und ihre Wertigkeit gegeneinander abgewogen werden. Dies ist ein aufwändiger, aber sehr verlässlicher Prozess, wenn wir neue, anspruchsvolle Aufgaben bewältigen müssen. Experimentell haben sich parallele Schulen zur Erforschung der Entscheidungsfindungsprozesse mit ihren eigenen Begrifflichkeiten und Operationalisierungen entwickelt. Aus der theoretischen, „klassischen“ Psychologie u. a. um Thorndike (26) und Skinner (33) entwickelten sich über klassische Konditionierungsparadigmen Modelle zum *habituellen* vs. *zielgerichteten* Verhalten. Aus den komputationalen Neurowissenschaften und deren Ziel, mathematische Vorhersagen über das Verhalten zu treffen, entwickelte sich das *modellfreie* vs. *modellbasierte* Verhalten. In ihrer Bedeutung für die Entstehung und Aufrechterhaltung der Alkoholabhängigkeit werden das habituelle und modellfreie Verhalten gleichgesetzt, ebenso wie andererseits das zielgerichtete und modellbasierte Verhalten (34, 35). Im Folgenden soll zum Verständnis der in dieser Habilitationsschrift verwendeten Paradigmen kurz auf die Gemeinsamkeiten und Unterschiede der konzeptuellen Umsetzung eingegangen werden.

Die **Operationalisierung von habituellem und zielgerichtetem Lernen** erfolgt vor allem über sogenannte Devaluationsparadigmen (36, 37). Ein belohnungsassoziierter Reiz wird über Sättigungs- und Intoxikationseffekte entwertet, also devaluiert. Die Entscheidung wird als habituell gewertet, wenn ein ehemals belohnter Stimulus trotz Devaluation weiter ausgewählt

wird, während der zielgerichtete Lerner den entwerteten Stimulus in der Folge meidet. Tricomi und Kollegen (36) konnten zeigen, dass das ständige Wiederholen und „Überlernen" eines zielgerichteten Verhaltens zu einer reduzierten Sensitivität gegenüber der Devaluation des überlernten Reizes führt. Das heißt, dass, obwohl der Wert des Reizes deutlich abnimmt, keine entsprechende Anpassung im Verhalten stattfindet. In der Folge kommt es zu einem *shift* weg vom zielgerichteten und hin zum habituellen Verhalten, der sich neuronal durch einen Zunahme der Aktivierung im dorsalen Putamen abbilden ließ. Die Zunahme eines zielgerichteten Verhaltens hingegen fördert die Aktivierung im orbitofrontalen Kortex (37). Wesentlich ist hier die Annahme, dass ein sogenannter *shift* von einem primär belohnungsassoziierten zielgerichteten Verhalten hin zu einem habituellen Verhalten neuronal durch einen entsprechenden *shift* von Aktivierungsmustern in frontalen zu striatalen Regionen stattfindet. Das dorsale Striatum und seine Konnektivität zu kortikalen Strukturen hat sich als zentrale Region innerhalb der Basalganglien zur Kodierung von automatisierten Entscheidungsfindungsprozessen herausgestellt, und entsprechend sind Veränderungen in der Aktivität verschiedener neuronaler Zelltypen in dieser Region an die Bildung automatischer Verhaltensweisen gekoppelt (38).

Die Operationalisierung von modellfreiem und modellbasierten Lernen erfolgt über mathematische Modelle (25) und dynamische Programmierungstechniken (39, 40), die auf sequenzielle Aufgabenstrukturen wie den *decision tree* (41) und die *two-step*-Aufgabe (42) angewendet werden. Über diese Modelle und Aufgaben kann eine relativ genaue Quantifizierung der Anteile von modellfreiem und modellbasiertem Verhalten bei einem Entscheidungsfindungsprozess getroffen werden. Der modellbasierte Lerner trifft Entscheidungen nach Evaluation aller Sequenzen im Entscheidungsbaum, während der modellfreie Lerner nur die zuletzt belohnte Stufe wertet. Sebold und Kollegen (43) führten die *two step*-Aufgabe bei Patient*innen mit Alkoholabhängigkeit durch und berichteten über einen größeren Anteil an modellfreiem Lernen bei Patient*innen im Vergleich zu Kontrollen.

Wendet man diese mathematischen Modelle auf die eingangs beschriebenen tierexperimentellen Befunde von Flagel et al. (29) zu *sign* und *goal trackern* an, zeigt sich eine Kopplung der phasischen Dopaminausschüttung an das modellfreie Lernen insbesondere während der Salienzzuschreibung eines konditionierten Stimulus. Das bedeutet, der modellfreie Lerner weist dem konditionierten Stimulus eine höhere Bedeutung (Salienz) zu und reagiert entsprechend auch stärker auf suchtassoziierte Reize. Dieser Befund ist

vergleichbar mit der Zunahme der neuronalen Aktivität im dorsalen Striatum von Tricomi et al. (36) beim habituellen Lernen. Die Entkopplung der dopaminergen Modulation von einem initial modellbasierten Verhalten kann in der Folge dysfunktionale Lernprozesse begünstigen und zur Entstehung und Aufrechterhaltung einer Alkoholabhängigkeit beitragen (44).

Eine Möglichkeit der **Kombination klassischer Konditionierungsparadigmen mit dynamischen Entscheidungsfindungsprozessen in der Operationalisierung** am Menschen gelang über das *Pavlovian-to-instrumental-transfer*-Paradigma (PIT) (20-22). PIT erfasst das Ausmaß, in dem ein belohnungsassoziierter Reiz das instrumentelle Verhalten beeinflussen kann. Es gibt zahlreiche Varianten im Design der Transferphase (für eine Übersicht siehe (45)), aber die Abfolge gliedert sich immer in die folgenden drei Abschnitte: 1) instrumentelles Lernen, 2) Pawlowsches Lernen und 3) Transfer.

Das in dieser Habilitationsschrift verwendete Design verwendet Fraktale und Muscheln als primär neutrale Reize (UCS), die dann in der Folge über instrumentelle und klassische Lernprozesse zu konditionierten Reizen (CS) werden. In der ersten Phase lernen die Probanden Muscheln unterschiedlicher Form und Farbe entsprechend einem damit verbundenen monetären Gewinn (0 bis 2 Euro) als gut vs. schlecht zu identifizieren (instrumentelles Konditionieren). In der zweiten Phase werden neutrale Stimuli (Fraktale unterschiedlicher Form und Farbe) durch Kopplung mit Geldgewinnen (0 bis 2 Euro) und Tönen positiv (Gewinn) und negativ (Ausbleiben von Gewinn) klassisch konditioniert. In der dritten Phase findet die Kopplung von klassische konditioniertem und instrumentell konditioniertem Verhalten statt, indem die Probanden die Auswahl der (guten und schlechten) Muscheln vor dem Hintergrund der (positiv und negativ) konditionierten Fraktale treffen müssen. Der PIT-Effekt ist dann besonders stark ausgeprägt, wenn der Wert des im Hintergrund eingeblendeten konditionierten Stimulus einen starken Einfluss auf die Auswahl der Muscheln im Vordergrund hat, d. h. also, dass z. B. besonders schnell und viele gute Muscheln eingesammelt werden, wenn im Hintergrund ein positives Fraktal erscheint (sog. *Go-Trial*), während das Liegenlassen einer schlechten Muschel vor dem Hintergrund eines negativen Fraktals erleichtert wird (sog. *No-Go-Trial*). Das Ausmaß des PIT lässt sich über die Anzahl der Knopfdrücke beim Einsammeln einer Muschel in Abhängigkeit von der Wertigkeit der Muschel und des Fraktals im Hintergrund berechnen. In der Entstehung und Aufrechterhaltung der Alkoholabhängigkeit steht der PIT-Effekt für das Ausmaß, in dem ein Reiz, der mit Belohnung oder Bestrafung in Verbindung gebracht wurde, die

Motivation, ein Verhalten auszuüben, verändern kann, und dient als paradigmatisches Modell für den reizinduzierten Rückfall (21).

Den dargestellten Lern- und Verhaltensprozessen ist gemeinsam, dass sie auf neuronaler Ebene z. B. über fMRT-Untersuchungen am Menschen mit Aktivierungsmustern im mesolimbischen System, insbesondere der Amygdala und dem NAC, in Verbindung gebracht werden (21). Hier konnte gezeigt werden, dass eine Verhaltensänderung im Sinne eines Wechsels von modellbasiertem zu mehr modellfreiem Lernen, z. B. im Rahmen von Automatisierungsprozessen, aber auch bei der Entstehung einer Abhängigkeitserkrankung, mit einem *shift* in der neuronalen Aktivierung von ventralen zu dorsalen Aktivierungsmustern im Kern des NAC (36, 37, 46–48) einhergeht.

Bereits Flagel und Kollegen konnten am Tiermodell nachweisen, dass die Tendenz, auf belohnungsassoziierte Reize mit vermehrter Aufmerksamkeit zu reagieren, genetisch determiniert ist und diese Disposition das Entstehen von Suchtverhalten begünstigt (49, 50). Untersuchungen am Menschen zeigen im Hinblick auf die Alkoholabhängigkeit eine hohe genetische Disposition (51). Angaben zur Erblichkeit schwanken zwischen 33 % für einen Zusammenhang mit der Diagnose der Alkoholabhängigkeit (52) und 13–18 % für einen Zusammenhang mit der Trinkmenge (53). Ähnliche Schätzwerte liefern genomweite Analysen mit einer Varianzaufklärung in Verhaltensmaßen bis zu 12 % (54). Die veröffentlichten Studien zeigen z. T. widersprüchliche Ergebnisse zum Einfluss einzelner Gene auf klinische Aspekte der Erkrankung wie den Krankheitsverlauf, die Trinkmenge und das Rückfallgeschehen. Gleichzeitig werden berichtete Genbefunde vor allem dann, wenn sie sich auf ein spezifisches Verhalten wie Menge des Alkoholkonsums oder Rückfall beziehen, nur sehr selten repliziert (55). Neben dem dopaminergen System spielen das opioide System und seine genetischen Determinanten eine wichtige Rolle bei der Wertigkeit, die einem belohnungsassoziierten Reiz gegeben wird. Über die Modulation von Opioidrezeptoren lässt sich im Tiermodell sowohl die hedone als auch die motivationale Komponente von Reizen beeinflussen, z. B. über die Stimulation des µ-Opioid-Systems (MOP) im NAC (Pecina und Berridge, 2013). Eine entsprechende Modulation von Motivation und Valenzzuschreibung lässt sich über die pharmakologische Modulation des MOP auch am Menschen replizieren (56–58).

Neben der Auswertung einzelner Gene soll die mathematische Berechnung genetischer Risikoscores die Interaktionen und Abhängigkeiten einzelner Gene untereinander berücksichtigen

und so den Phänotypen des abhängigen Verhaltens besser abbilden können. Im Rahmen dieser Analysen ergeben sich Varianzaufklärungen durch genetische Risikoscores von ca. 1 % in Verhaltensmaßen. Im Vergleich zeigen Risikoscores für andere psychiatrische Krankheitsbilder wie die paranoide Schizophrenie eine Varianzaufklärung von bis zu 20 % (59, 60). Mit der Erforschung von Methylierungsprozessen an der DNA, sog. epigenetischen Prozessen, wuchs die Hoffnung, Gen-Umwelt-Interaktionen besser abbilden zu können. Epigenetik bezieht sich auf molekulare Prozesse, die die Genexpression verändern, ohne die Desoxyribonukleinsäure (DNA)-Sequenz selbst zu modifizieren (61). Hierzu zählen DNA-Methylierung, Histonmodifikationen und nicht-kodierende RNAs. Die DNA-Methylierung stellt den am häufigsten untersuchten epigenetischen Mechanismus bei Sucht dar. Ethanol selbst führt zu Veränderungen der Methylierung, die im Tiermodell noch in der nächsten Generation nachgewiesen werden konnten (62). Veränderungen waren hier erneut vor allem im limbischen System nachweisbar (63). Es wurde gezeigt, dass Unterschiede in den durch Alkoholkonsum induzierten Methylierungsmustern über Generationen bestehen bleiben (64), ebenso aber auch durch spezifische chemische Interventionen rückgängig gemacht werden können (65). Veränderungen im Methylom können neben dem Alkohol selbst durch zahlreiche weitere Einflüsse induziert werden, dazu gehören Umweltstressoren wie mangelnde mütterliche Fürsorge und nachteilige Lebenserfahrungen (66, 67). Bei der Entstehung eines riskanten und schädlichen Alkoholkonsums wurde die chronische Stressexposition als ein wesentlicher Mechanismus in der Vermittlung von Gen-Umwelt-Interaktion identifiziert (68). Weaver und Kollegen konnten an Ratten zeigen, dass die mütterliche Fürsorge in den ersten Wochen nach der Geburt das Epigenom der Nachkommen durch Methylierungsprozesse am Glukokortikoidrezeptor (GR)-Genpromotor im Hippocampus verändert (67). Der Hippocampus ist Teil des mesolimbischen Belohnungssystems und hat insbesondere bei der Vermittlung von Gedächtnis und Lernprozessen eine hohe Relevanz (69). Eine weitere Möglichkeit, sich mit epigenetischen Veränderungen bei AUD auseinanderzusetzen, sind Alterungsprozesse im Epigenom, die als Modelle einer epigenetischen Uhr in der Lage sind, das chronologische vom biologischen Alter über Methylierungsprozesse an spezifischen CPG Stellen zu trennen (70–72), um so den differenziellen Einfluss von Alkohol auf Alterungsprozesse auf Ebene der DNA zu untersuchen (73, 74).

Eine zentrale Herausforderung für die zukünftige Forschung auf dem Gebiet der Genetik und Epigenetik wird es sein zu entschlüsseln, wie genau Veränderungen in der Gentranskription

beim Menschen zur Entwicklung und Aufrechterhaltung von Suchtverhalten beitragen können.

Basierend auf den eingangs beschriebenen Forschungsarbeiten setzen sich die folgenden Publikationen mit drei wesentlichen ätiologischen Faktoren der Entstehung und Aufrechterhaltung der Alkoholabhängigkeit auseinander: 1) Veränderungen im belohnungsabhängigen Lernen, hier im Besonderen Veränderungen im PIT-Effekt, und deren neuronale Korrelate, 2) (epi)genetische Korrelate eines anhaltenden Alkoholkonsums und deren methodische Besonderheiten und 3) die Rolle von chronischem Stresserleben auf belohnungsabhängiges Lernen in Abhängigkeit von kognitiven Funktionen.

2. Eigene Arbeiten

2.1 Pawlowsch-instrumenteller Transfer im Nucleus accumbens ist mit dem Rückfallgeschehen bei Patient*innen mit Alkoholabhängigkeit assoziiert.

Garbusow M, Schad DJ, Sebold M, Friedel E, Bernhardt N, Koch SP, et al. Pavlovian-to-instrumental transfer effects in the nucleus accumbens relate to relapse in alcohol dependence. Addict Biol. 2016;21(3):719–31.

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2.2 Zusammenhang zwischen dem OPRM1-Polymorphismus und Konditionierungslernen bei Patient*innen mit Alkoholabhängigkeit

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Association of the *OPRM1* A118G polymorphism and Pavlovian-to-instrumental transfer: Clinical relevance for alcohol dependence

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Abstract

Background: Pavlovian-to-instrumental transfer (PIT) quantifies the extent to which a stimulus that has been associated with reward or punishment alters operant behaviour. In alcohol dependence (AD), the PIT effect serves as a paradigmatic model of cue-induced relapse. Preclinical studies have suggested a critical role of the opioid system in modulating Pavlovian–instrumental interactions. The A118G polymorphism of the *OPRM1* gene affects opioid receptor availability and function. Furthermore, this polymorphism interacts with cue-induced approach behaviour and is a potential biomarker for pharmacological treatment response in AD. In this study, we tested whether the *OPRM1* polymorphism is associated with the PIT effect and relapse in AD.
Methods: Using a PIT task, we examined three independent samples: young healthy subjects (N=161), detoxified alcohol-dependent patients (N=186) and age-matched healthy controls (N=105). We used data from a larger study designed to assess the role of learning mechanisms in the development and maintenance of AD. Subjects were genotyped for the A118G (rs1799971) polymorphism of the *OPRM1* gene. Relapse was assessed after three months.
Results: In all three samples, participants with the minor *OPRM1* G-Allele (G+ carriers) showed increased expression of the PIT effect in the absence of learning differences. Relapse was not associated with the *OPRM1* polymorphism. Instead, G+ carriers displaying increased PIT effects were particularly prone to relapse.
Conclusion: These results support a role for the opioid system in incentive salience motivation. Furthermore, they inform a mechanistic model of aberrant salience processing and are in line with the pharmacological potential of opioid receptor targets in the treatment of AD.

Keywords
Alcohol dependence, learning, decision making, *OPRM1* A118G, opioid system

Introduction

Contextual stimuli are important modulators in the way we learn and can promote specific behaviours. One mechanism underlying contextual learning is the so-called Pavlovian-to-instrumental transfer (PIT). The PIT effect capture the influence of Pavlovian conditioned stimuli (CSs) on instrumental behaviour, with appetitive Pavlovian stimuli specifically promoting approach and reducing withdrawal, and aversive Pavlovian stimuli promoting withdrawal and reducing approach (Huys et al., 2011), thus reflecting a powerful mechanism affecting behavioural choices across humans (Talmi et al., 2008) and animals (Dickinson et al., 2000; O'Connor et al., 2010). Moreover, the PIT effect has been used as a quantification of incentive salience attribution, that is, the extent to which formerly neutral cues become attractive, themselves desired, and therefore 'wanted' (Huys et al., 2014; Meyer et al., 2012).

Crucially, incentive salience attribution is one prominent mechanism underlying several disorders of compulsivity, such as alcohol dependence (AD; Corbit and Janak, 2007) and other addictive disorders (LeBlanc et al., 2012). Also, interindividual differences in PIT have been associated with addiction vulnerability and maintenance. For instance, preclinical work suggests an association between the magnitude of PIT and addictive behaviour, such as compulsive alcohol drinking (Barker et al., 2012; Corbit and Janak, 2007). Preclinical studies have also consistently reported that non-drug-related (e.g. food or sucrose reward) CSs lead to increased

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responding during PIT in addicted animals (LeBlanc et al., 2013; Ostlund et al., 2014; Saddoris et al., 2011). Moreover, we have recently shown that the PIT effect in humans serves as a vulnerability marker for the development and maintenance of AD (Garbusow et al., 2014, 2019; Schad et al., 2019a; but see van Timmeren et al., 2020). The behavioural and neural correlates of PIT have been associated with relapse in AD (Garbusow et al., 2016; Sekutowicz et al., 2019; Sommer et al., 2020) and were predictive of future drinking behaviour in adolescents (Sekutowicz et al., 2019).

Although contemporary theories emphasise the involvement of the dopaminergic system in incentive salience, recent findings suggest the opioid system as another important player (Pecina and Berridge, 2013; van Steenbergen et al., 2019). The opioid system has been primarily linked to hedonic features of a reward, also termed 'liking' as opposed to 'wanting', which reflects the motivational properties to promote a certain behaviour rather than its hedonic value. However, preclinical studies have shown that stimulation of the μ-opioid (MOP) system in the nucleus accumbens directly enhances incentive motivation (or 'wanting') for reward (Pecina and Berridge, 2013). In animals, experimental manipulation of the opioid system can mediate the influence of reward-guided and stimulus-guided decisions on choice (Laurent et al., 2012), increase motivation for different reward types (Mahler and Berridge, 2012) and mediate the motivating influence of cue-triggered reward expectations (Lichtenberg and Wassum, 2017). In humans, evidence for a functional role of the opioid system in mediating 'wanting' mainly stems from pharmacological challenges. For instance, MOP agonists and antagonists selectively enhance and decrease processing efficiency in a reward task (Eikemo et al., 2017) and increase and decrease the motivation to view positive valenced stimuli, respectively (Chelnokova et al., 2014). Likewise, opioid receptor antagonists reduced physical effort produced to obtain reward and increased negative facial reactions during reward anticipation (Korb et al., 2019).

In humans, the role of the opioid system in mediating the PIT effect as one further quantification of incentive salience (or 'wanting') is less clear. The opioid receptor antagonist naltrexone could decrease alcohol cue-induced activation of the ventral striatum (Myrick et al., 2008) and cue-induced impulsive responding (Mitchell et al., 2007). However, to date, there are only two studies investigating the role of the opioid system in mediating human PIT-like effects (Weber et al., 2016; Wiers et al., 2009), reporting reduced PIT after blockade of the MOP receptor (naltrexone) in healthy humans (Weber et al., 2016) and increased automatic approach tendencies in G+ carriers of the OPRM1 polymorphism to alcohol-associated stimuli (Wiers et al., 2009). The overarching aim of our study was to further elucidate the role of the human opioid system in mediating the PIT effect in both healthy subjects and those with AD.

A common mechanism of quantifying interindividual differences in the human opioid system is the determination of the MOP receptor single nucleotide polymorphism (*OPRM1*). The *OPRM1* gene codes for the MOP receptor, an inhibitory G-protein coupled receptor that binds endogenous opioid peptides such as β-endorphin and enkephalins as well as exogenous opioids such as morphine and heroin (Burns et al., 2019; Kieffer and Gaveriaux-Ruff, 2002). Opioid receptors are distributed widely in the human brain and modulate brain function at all levels of neural integration, including the mesolimbic system as part of the brain's reward pathway.

Human studies investigating the *OPRM1* polymorphism have suggested a crucial role of this single nucleotide polymorphism (SNP) in AD, treatment response and automatic approach biases to conditioned cues (Chamorro et al., 2012; Filbey et al., 2008; Ray and Hutchison, 2004; Wiers et al., 2009). The A118G (rs1799971) polymorphism of the *OPRM1* gene alters the function of MOP receptors, such that the G variant binds beta-endorphin three times more strongly than the A variant, potentially also affecting receptor availability (Heinz et al., 2005). We henceforth refer to the minor *OPRM1* G-allele carriers as G+ carriers. G+ carriers were shown to report higher subjective alcohol-associated feelings of intoxication (Ray and Hutchison, 2004) and craving (Van Den Wildenberg et al., 2007) and have a higher risk for positive family history (Ray and Hutchison, 2004). However, conflicting results stem from large genome-wide association studies (GWAS) and candidate gene studies (Kong et al., 2017), which could not replicate an association between AD and *OPRM1* genotype, corresponding with a recent report on converging evidence against an association between the *OPRM1* A118G polymorphism and alcohol consumption and sedation (Sloan et al., 2018).

The analyses presented here aimed to answer three questions. (1) Is the *OPRM1* polymorphism associated with the PIT effect across three independent cohorts? (2) Is the association between the PIT effect and the *OPRM1* polymorphism different in patients with AD compared to healthy controls (HCs)? (3) Is the association between the PIT effect and the *OPRM1* polymorphism relevant for treatment outcome in the way that it is different in prospectively relapsing and abstinent patients with AD?

Methods

Subjects

All subjects were recruited between 2012 and 2018 as part of a larger study (LeAD study, ClinicalTrials.gov identifiers: NCT01744834, NCT01679145 and NCT02615977) investigating behavioural, genetic and neuroimaging alterations associated with reward-based learning as (a) predictors for the development of AD in a sample of young 18-year-old male subjects recruited from the national registry and (b) the maintenance of AD with respect to relapse and drinking behaviour in a sample of patients suffering from AD and an age, education and sex-matched HC sample (for previously published results of our sample, see, amongst others, Garbusow et al., 2014, 2016, 2019). Thus, this study comprised two independent HC samples that significantly differed with regards to several sociodemographic variables (see Supplemental Table S2 for between-group differences). As previous analyses (Sebold et al., 2016) indicated substantial differences in PIT effects between these cohorts, we did not merge both control samples but instead analysed the influence of the *OPRM1* polymorphism on the PIT effect separately in these two control cohorts (analysis 1).

The assessed samples were a subsample of the three cohorts mentioned above for which genetic data were available: 18-year-old male subjects ($N = 161$, henceforth referred to as young controls), recently detoxified patients with AD ($N = 186$) and age-matched HCs ($N = 105$, henceforth referred to as middle-aged controls). For a precise overview of the selection procedures, see Supplemental Information 1 and Supplemental Figure S1.

For a complete description of exclusion criteria, see Garbusow et al. (2016). Briefly, all subjects were free from psychotropic medication, had no history of substance dependence (DSM-IV, except from AD in the AD group) or current substance use (DSM-IV, except for nicotine use), no other current DSM-IV axis 1 psychiatric or neurological disorders and no borderline personality disorder as assessed by the computer-based Composite International Diagnostic Interview (Jacobi et al., 2013; Wittchen, 1997). Participants' demographic and clinical characteristics are outlined in Table 1. Participants gave written informed consent before study inclusion. The study was approved by the local ethics committees of the Technical University of Dresden and Charité Universitätsmedizin Berlin.

To define relapse across patients with AD, a three-month follow-up was performed using the Time Line Follow Back procedure (Sobell and Sobell, 1992). Relapse was defined as at least five standard drinks (e.g. one standard drink = 0.33 L beer) on one occasion for male participants and at least four standard drinks for female participants according to the World Health Organization (WHO; 2014) definition of high-risk consumption. A total of 51 patients were classified as relapsers (of whom 37 were G– and 14 were G+ carriers), whereas 94 patients were classified as abstainers (of whom 78 were G– and 16 were G+ carriers). The remaining 41 patients could not be contacted during the follow-up period.

Task

We used a PIT task as previously described (Garbusow et al., 2014, 2016; Sommer et al., 2017). The task consisted of four phases (of which the first three phases are depicted in Figure 1): (a) instrumental learning, (b) Pavlovian learning, (c) PIT and (d) forced choice task followed by a rating scale of the stimuli.

Instrumental learning. Subjects had to learn to collect 'Go' shells and leave 'No-Go' shells by repeatedly pressing a button while receiving probabilistic feedback. In order to collect a shell, subjects had to move a red dot onto the selected shell by repeated button presses within two seconds. We instructed the subjects to maximise their profit. For this, they should use the probabilistic feedback to find out via trial and error what is a 'good shell', which in 'most cases' lead to wins when collected, and leave 'bad shells', which in 'most cases' lead to wins when not collected. Each button press moved the red dot a fraction of the way towards the shell. To collect a 'Go' shell correctly, subjects had to press the button five or more times, and to leave a 'No-Go' shell, subjects had to perform between zero and four button presses. The subjects did not know about the number of button presses, but we instructed them to press the button as often as possible to collect a shell to maximise instrumental performance. Correct responses were rewarded with 20 cents in 80% of trials and punished with a loss of 20 cents in 20% of trials, and for wrong responses it was vice versa (see Figure 1.1 for 'Go' and 'No-Go' trials). The shell set consisted of six different shells (three 'Go' shells and three 'No-Go' shells).

Participants performed 60–120 trials, depending on their performance. In order to ensure that all subjects were at comparable performance levels before advancing to the PIT part, a learning criterion was enforced (80% correct choices over 16 trials after a minimum of 60 trials).

Pavlovian learning. Pavlovian learning consisted of 80 trials in which compound visual and auditory stimuli (CS) were predictive of distinct monetary rewards or punishments (unconditioned stimulus (US); Figure 1.2). Each trial began with a three-second presentation of a compound CS (fractal picture and tone) which was then followed by a three-second presentation of two fixation crosses (on the left and right side of the screen). Then, the US (monetary reward or punishment) was presented for three seconds on the side where the CS had not been presented. Subjects were instructed to view the CS–US pairings passively and to memorise these associations. The set of CS consisted of six stimuli of which each was paired with positive (+2€/+1€), neutral (0€) or negative (−1€/−2€) outcomes, henceforth referred to as 'money CS'.

PIT phase. Subjects performed 162 trials of the instrumental task again, this time without outcome feedback. Subjects were instructed that their choices still counted towards the final monetary outcome (so-called nominal extinction). The instrumental stimuli superimposed one of the money CSs presented during Pavlovian training (Figure 1.3), or one of four beverage stimuli (results not presented here, but see (Schad et al., 2019a; Sekutowicz et al., 2019; Sommer et al., 2017, 2020). Each instrumental stimulus (three 'Go' shells and three 'No-Go' shells) was combined with each money CS (fractal stimulus previously associated with either of −2€, −1€, 0€, +1€, +2€) for three times, resulting in 90 trials, which were of primary interest for this study. Each trial lasted 3.6 seconds.

Forced-choice task. This part of the task aimed to verify the acquisition of Pavlovian learning. In each trial, subjects had to choose between two sequentially presented compound money CSs from the Pavlovian training, each presented for two seconds. All possible compound CS pairings were presented three times in an interleaved randomised order.

Pleasantness ratings. After the task, subjects were asked to rate the pleasantness of the CSs (fractals and shells) from the Pavlovian learning phase and the instrumental learning phase on a Likert scale from 1 to 7 on the screen.

Genotyping

To genotype our sample, DNA was extracted semi-automatically with a Chemagen Magnetic Separation Module (PerkinElmer, Waltham, MA) from whole blood. All samples were genotyped with the Illumina Infinium PsychArray Bead Chip (Illumina, San Diego, CA). We assessed rs1799971, a SNP that is an A-to-G substitution (A118G), resulting in a functional amino acid substitution (Asn40Asp; Hartwell et al., 2020).

Because of the limited sample size, G-allele carriers (AG and GG) were grouped together. This approach is in keeping with precedent in the field (Persson et al., 2019; Way et al., 2009).

Behavioural analyses

Data were analysed using the R programming language (R Foundation for Statistical Computing, Vienna, Austria). Demographic, clinical and neuropsychological comparisons between G+ and G– *OPRM1* carriers were examined using chi-square and *t*-tests (Table 1).

Table 1. Demographic, clinical and neuropsychological characteristics for all cohorts; young controls, middle-aged controls and patients with AD, split by *OPRM1* polymorphism.

Cohort	Alcohol-dependent patients (N = 186)			Middle-aged controls (N=105)			Young controls (N=161)		
OPRM1	G– (N=154)	G+ (N=32)	Test statistics	G– (N=79)	G+ (N=26)	Test statistics	G– (N=120)	G+ (N=41)	Test statistics
	M (SD)	M (SD)		M (SD)	M (SD)		M (SD)	M (SD)	
Demographic variables									
Age	46.17 (10.49)	47.09 (11.03)	t=–0.42, p=0.67	43.64 (11.1)	46.04 (10.5)	t=–0.99, p=0.33	18.36 (0.2)	18.37 (0.2)	t=–0.33, p=0.74
Sex (% male)	84%	81%	χ^2=0.2, p=0.67	83%	81%	χ^2=0.11, p=0.75	100%	100%	NA
Years of education	14.97 (4.07)	14.29 (2.52)	t=1.3, p=0.22	15.98 (3.22)	15.37 (3.32)	t=0.79, p=0.43	11.7 (0.75)	11.51 (1.34)	t=0.85, p=0.4
Clinical characteristics									
Anxiety[a]	4.37 (3.41)	4.8 (3.37)	t=–0.63, p=0.53	2.32 (2.04)	1.88 (2.21)	t=0.89, p=0.38	2.31 (2.19)	2.92 (2.89)	t=–1.2, p=0.23
Depression[b]	3.5 (3.7)	4.33 (3.33)	t=–1.23, p=0.23	1.48 (1.98)	1.85 (2.62)	t=–0.65, p=0.52	1.67 (1.75)	1.8 (2)	t=–0.39, p=0.7
Craving[c]	12.76 (7.94)	12.52 (8.57)	t=0.14, p=0.88	2.4 (2.41)	3.68 (4.03)	t=–1.31, p=0.2	3.47 (3.01)	4.65 (3.48)	t=–1.91, p=0.7
Impulsivity[d]	31.63 (6.67)	31.84 (5.57)	t=–0.19, p=0.85	29.32 (5.4)	28.84 (5.3)	t=0.39, p=0.69	29.99 (5.15)	31.82 (4.56)	t=–2.13, p=**0.04**
Neuropsychological testing									
Cognitive speed[e]	9.27 (2.76)	9.48 (2.78)	t=–0.39, p=0.7	10.58 (2.82)	10.92 (3.78)	t=–0.42, p=0.68	11.5 (2.2)	11 (2.59)	t=1.11, p=0.27
Working memory[f]	6.5 (1.93)	6.77 (1.61)	t=–0.82, p=0.41	7.41 (1.95)	7.62 (2.43)	t=–0.40, p=0.69	8.04 (1.95)	8.02 (2.21)	t=0.04, p=0.96

The variables were assessed by means of [a]the anxiety and [b]depression subscale of the Hospital Depression and Anxiety Questionnaire; [c]the Obsessive Compulsive Drinking Scale, [d]the Barratt Impulsiveness Scale and the following subtest of the Wechsler Intelligence Test: [e]the Digit Symbol Substitution Test and [f]the Digit Span Backwards Test.
AD: alcohol dependence.

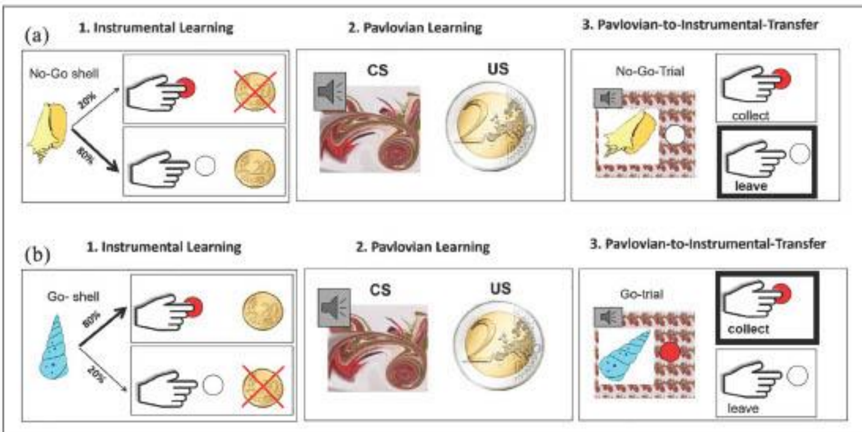

Figure 1. Phases 1–3 of the paradigm for (a) the 'No-Go' trial and (b) the 'Go' trial. 1. Instrumental learning: The subject's task was to move a dot towards the stimulus by repeated button presses in order to collect it or to do nothing within two seconds. These two instrumental choices resulted in monetary wins or losses, presented immediately after each trial via a picture of a 20€ coin for 1.5 seconds. Feedback was probabilistic. A 'Go' shell was rewarded in 80% and punished in 20% of trials if collected and vice versa if not collected. A 'No-Go' shell was rewarded in 80% and punished in 20% of the trials if not collected and vice versa if collected. 2. Pavlovian learning: Neutral fractal and audio stimulus compounds (CS) are repeatedly paired with monetary outcomes (US: e.g. here a 2€ coin). 3. Pavlovian-to-instrumental transfer (PIT) phase: Subjects performed the instrumental task in nominal extinction, that is, no explicit monetary outcomes were presented (A. leave button to not collect a 'No-Go' shell and B. press button to collect 'Go' shell superimposed on the audiovisual Pavlovian stimulus; here: the Pavlovian stimulus previously paired with 2€ and the respective tone pitch).

Analysis of the PIT phase was of primary interest, but we analysed all other phases as well (see Supplemental Information 6, Supplemental Information 7, Supplemental Information 8 and Supplemental Information 10). In the PIT phase, the PIT effect reflects the interaction between the valence of the background stimulus and the accuracy of the foreground instrumental action. We were specifically interested whether the *OPRM1* genotype covaried with PIT effect, that is, the way that positive and negative stimuli influence 'Go' and 'No-Go' actions. More precisely, we asked whether the genetic phenotype would interact with the extent to which a positive stimulus facilitates 'Go' responses but impairs 'No-Go' responses and, vice versa, a negative stimulus facilitates 'No-Go' responses but impairs 'Go' responses.

As outlined in the introduction, the analyses presented here aimed to elucidate: (a) the association between the *OPRM1* polymorphism and the PIT effect, (b) the clinical relevance of this association for AD and (c) the relevance of this association for treatment outcome. Across these different analyses, we coded a participant's accuracy of the PIT phase as correct (1) if a 'Go' shell was collected or a 'No-Go' shell was left, and as false (0) if a 'No-Go' shell was collected or a 'Go' shell was left. We used a binomial mixed effect regression as implemented in the lme4 package (Bates et al., 2015). We regressed the participant's accuracy (correct or incorrect) on Pavlovian valence (negative, neutral or positive, dummy coded with neutral as the reference), instrumental action ('Go' or 'No-Go', coded as 0.5 and −0.5) and *OPRM1* polymorphism (G− or G+, coded as −0.5 and +0.5) and tested for interaction between these factors. Subjects were added as random effects (random intercept model). We performed model comparisons to ensure that this model was the best-fitting model across subjects (see Supplmenetal Information 2).

Analysis 1: Association between the PIT effect and the OPRM1 *polymorphism across cohorts.* To test whether the *OPRM1* polymorphism was associated with the PIT effect in all three cohorts, we performed the above-described analysis for all three cohorts separately (Supplemental Figure S1).

Analysis 2: Alcohol-related group differences for the association between the PIT effect and the OPRM1 *polymorphism.* To test whether the interaction between the PIT effect and the *OPRM1* polymorphism was significantly different between HCs and patients with AD, we performed the above-described regression model (see analysis 1) but additionally added group (HC or AD, coded as 0.5 and −0.5) as an additional fixed effect and allowed interaction with all predictors (Supplemental Figure S1). For this analysis, we only included patients with AD and middle-aged control subjects (who were initially sampled as a comparison group of patients with AD). Both groups profoundly differed across several socio-economic and clinical variables (Supplemental Table S2). Increased depression, anxiety, craving and impulsivity as well as reduced cognitive speed and working memory are features instead of confounders of AD. Thus, as suggested by Miller and Chapman (2001), we did not control for these variables. Years of education was the only variable we added as a covariate because groups significantly differed in these variables despite our efforts of matching.

Table 2. Results of analysis 1. Effects of the regression analysis from the PIT part for all three cohorts.

Group	Alcohol-dependent patients (N=186)		Middle-aged controls (N=105)		Young controls (N=161)	
	χ^2	p-Value	χ^2	p-Value	χ^2	p-Value
Pavlovian CS valence	11.723	**0.003**	5.599	0.061	15.105	**0.001**
Instrumental behavior	7.057	**0.008**	13.108	**0.0003**	0.159	0.690
OPRM1 polymorphism	0.002	0.963	0.046	0.831	0	0.994
Pavlovian valence × instrumental behavior	2074.63	**<0.0001**	912.67	**<0.0001**	365.68	**<0.0001**
Pavlovian valence × *OPRM1* polymorphism	0.224	0.894	0.074	0.964	0.629	0.730
Instrumental behavior × *OPRM1* polymorphism	13.917	**0.0002**	18.930	**<0.0001**	7.757	**0.005**
Pavlovian valence × instrumental behavior × *OPRM1* polymorphism	12.723	**0.002**	9.027	**0.011**	20.691	**<0.0001**

All interaction effects with the *OPRM1* polymorphism in the young control cohort remained significant after controlling for self-reports of impulsivity, which was significantly different between G+ and G– carriers in this cohort (see Table 1). Statistically significant values are shown in bold.
PIT: Pavlovian-to-instrumental transfer; CS: conditioned stimulus.

Analysis 3: Relapse-related group differences for the association between the PIT effect and the OPRM1 *polymorphism.* To test whether the interaction between the *OPRM1* polymorphism and the PIT effect was significantly different between patients with AD who relapsed and those who remained abstinent, we performed the above described regression analysis (see analysis 1) but added relapse (relapsers or abstainers, coded as 0.5 and −0.5) as an additional fixed factor and allowed interaction with all predictors. For this analysis, we only included patients with AD for whom relapse data were available ($n=145$; Supplemental Figure S1). Relapsing patients did not differ from abstaining patients in any demographic or clinical variables, except for craving (where relapsing patients had significantly higher OCDS scores (Anton et al., 1995; Mann and Ackermann, 2000) than abstaining patients ($t=-2.66$, $p=0.01$). Thus, we added craving as a covariate of no interest in this analysis.

Post hoc analyses

For analyses 2 and 3, we were particularly interested in how the PIT effect was modulated by the *OPRM1* polymorphism and whether this depended on group, respectively. Thus, in our post hoc analyses, we focused on these contrasts (analysis 2: G+ vs. G– carriers/HCs vs. ADs; analysis 3: G+ vs. G– carriers/relapsers vs. abstainers) and considered effects as significant when they survived Bonferroni correction for four comparisons ($p<0.01$).

Results

Genotyping

Genotyping resulted in 353 participants homozygous for the major A allele, 89 participants with the AG combination and 10 participants homozygous for the G allele. *OPRM1* genotype distribution did not significantly differ from Hardy–Weinberg equilibrium ($\chi^2_{(df=1)}=2.31$, $p=0.13$).

Demographic, clinical and neuropsychological comparisons between G+ and G– carriers in all three cohorts indicated no group differences (Table 1), except from increased self-reports of impulsivity assessed via BIS-15 (Meule, 2011) in G+ carriers compared to G– carriers in young healthy adults. Moreover, we found no evidence for a functional association between the *OPRM1* polymorphism and AD. Descriptively, there were proportionally more G+ carriers among the HCs compared to the AD group – from the literature we would have expected the reverse results – although this difference was formally not statistical significant ($\chi^2_{(df=1)}=3.62$, $p=0.06$). Also, we found no evidence for a functional association between the *OPRM1* polymorphism and relapse ($\chi^2_{(df=1)}=1.60$, $p=0.21$).

Behavioural data

Analysis 1: Association between the PIT effect and the OPRM1 *polymorphism across cohorts.* The first aim of this study was to test whether the *OPRM1* polymorphism influences the PIT effect across three independent cohorts. In all three cohorts we found a significant PIT effect, that is, the interaction between Pavlovian valence (negative, neutral or positive) and instrumental action ('Go' or 'No-Go'; Table 2), indicating that positive stimuli facilitated 'Go' responses but impaired 'No-Go' responses, whereas negative stimuli facilitated 'No-Go' responses but impaired 'Go' responses.

In all groups, respectively, we found no interaction between Pavlovian valence and *OPRM1* polymorphism. However, the *OPRM1* polymorphism interacted with instrumental action (Table 2). Crucially, we found a three-way interaction between Pavlovian valence, instrumental action and *OPRM1* polymorphism in all cohorts. This result suggests that the *OPRM1* polymorphism strongly interacts with the PIT effect in all three independent cohorts. In fact the PIT effect was significantly higher in G+ carriers compared to G– carriers (Figure 2 and Table 2).

To rule out that our PIT-related *OPRM1* effect was simply due to the fact that G+ carriers showed stronger cue-induced modulation of liking, we further performed analyses of the rating data of the Pavlovian stimuli (pleasantness ratings; Supplemental Information 10). To this end, we first tested whether the *OPRM1* polymorphism was associated with ratings of the stimuli, depending on the Pavlovian valence. In all cohorts, the *OPRM1* polymorphism did not interact with Pavlovian valence (Supplemental Information 10). Moreover, adding the rating data as an additional covariate in our PIT analyses, all interaction between the

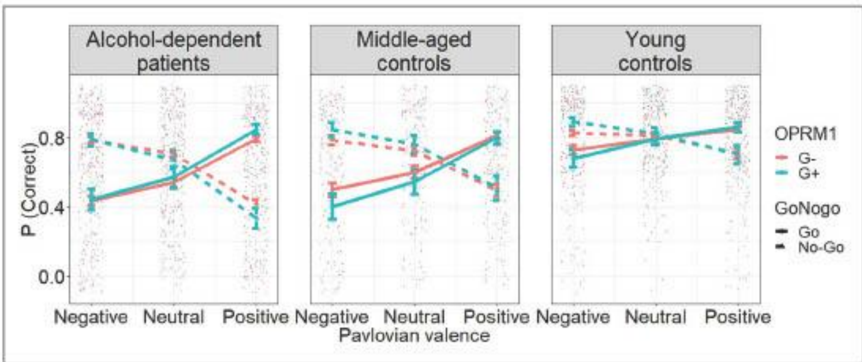

Figure 2. Results of the PIT phase as a function of group (patients with alcohol dependence (AD), middle-aged controls and young controls) and *OPRM1* polymorphism. Each panel shows the PIT effect in the respective group, that is, there was a significant influence of Pavlovian background valence on instrumental action (accuracy: percent correct choices), here visualised by the slope of the lines. Crucially, in each of the three cohorts, this was steeper in G+ carriers compared to G− carriers, as indicated by the three-way interaction between *OPRM1* polymorphism, Pavlovian valence and instrumental action (analysis 1), that is, in each of the three independent cohorts, the PIT effect was modulated by the *OPRM1* polymorphism. However, this was not different between alcohol-dependent patients and matched middle-aged controls (analysis 2).

Table 3. Results of analysis 2. Effects of the regression analysis from the PIT part where we tested whether the interaction between the PIT effect and the *OPRM1* polymorphism was significantly different between patients with AD and HCs.

	χ^2	*p*-Value
Pavlovian valence	13.183	0.001
Instrumental action	18.391	**<0.0001**
OPRM1 polymorphism	0.007	0.933
Group	2.316	0.128
Years of education	7.651	0.006
Pavlovian valence × instrumental action	2888.726	**<0.0001**
Pavlovian valence × *OPRM1* polymorphism	0.031	0.984
Instrumental action × *OPRM1* polymorphism	0.374	0.540
Pavlovian valence × group	3.661	0.160
Instrumental action × group	4.187	0.041
OPRM1 polymorphism × group	0.015	0.901
Pavlovian valence × instrumental action × *OPRM1* polymorphism	16.909	**<0.0001**
Pavlovian valence × instrumental action × group	22.695	**<0.0001**
Pavlovian valence × *OPRM1* polymorphism × group	0.257	0.880
Instrumental action × *OPRM1* polymorphism × group	30.727	**<0.0001**
Pavlovian valence × instrumental action × *OPRM1* polymorphism × group	0.318	0.853

HC: healthy control.

OPRM1 polymorphism, Pavlovian valence and instrumental action remained significant (patients with AD: $p=0.0004$; middle-aged controls: $p=0.006$; young controls: $p<0.0001$).

Analysis 2: Alcohol-related group differences for the association between the PIT effect and the OPRM1 *polymorphism.* The second aim of this study was to test whether the interaction between the PIT effect and *OPRM1* polymorphism was significantly different between patients with AD and HCs. This analysis indicated a three-way interaction between Pavlovian valence, instrumental action and group and also a three-way interaction between Pavlovian valence, instrumental action and *OPRM1* polymorphism. Thus, AD and the *OPRM1* polymorphism were significantly and independently associated with the strength of the PIT effect per se (see Figure 2). Moreover, we found a three-way interaction between instrumental action, group and *OPRM1* polymorphism. However, the four-way interaction between Pavlovian valence, instrumental action, group and *OPRM1* polymorphism was not statistically significant (Table 3). Thus, the interaction between the PIT effect and the *OPRM1* polymorphism was not statistically different between patients with AD and matched control subjects (Figure 2).

Table 4. Results of analysis 3. Effects of the regression analysis from the PIT part where we tested whether the interaction between the PIT effect and the *OPRM1* polymorphism was significantly different between relapsers and abstainers.

	χ^2	*p*-Value
Pavlovian valence	10.27	**0.006**
Instrumental action	0.002	0.965
OPRM1 polymorphism	0.324	0.569
Relapse	0.706	0.401
Craving	0.053	0.817
Pavlovian valence × instrumental action	1535.13	**<0.0001**
Pavlovian valence × *OPRM1* polymorphism	0.426	0.808
Instrumental action × *OPRM1* polymorphism	11.706	**0.001**
Pavlovian valence × relapse	0.513	0.774
Instrumental action × relapse	12.786	**<0.0001**
OPRM1 polymorphism × relapse	0.042	0.838
Pavlovian valence × instrumental action × *OPRM1* polymorphism	16.786	**0.001**
Pavlovian valence × instrumental action × relapse	13.647	**0.001**
Pavlovian valence × *OPRM1* polymorphism × relapse	0.571	0.752
Instrumental action × *OPRM1* polymorphism × relapse	1.988	0.159
Pavlovian valence × instrumental action × *OPRM1* polymorphism × relapse	30.347	**<0.0001**

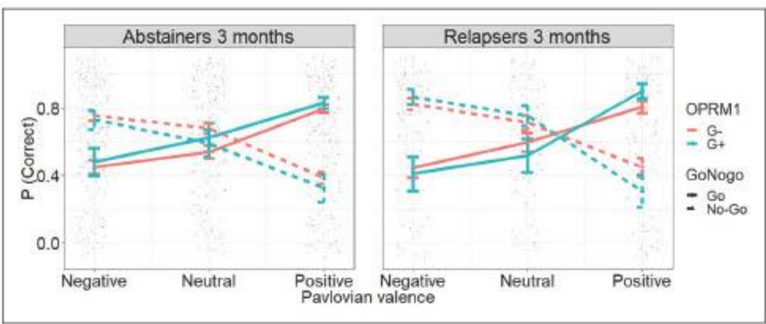

Figure 3. Results of the PIT phase as a function of treatment outcome (abstainers vs. relapsers) and *OPRM1* polymorphism (analysis 3). Patients with AD who relapsed showed a stronger interaction between the PIT effect and the *OPRM1* polymorphism compared to patients with AD who remained abstinent. Moreover, G+ carriers showed a strong and significant interaction between the PIT effect and treatment outcome, whereas G– carriers did not.

Analysis 3: Relapse-related group differences for the association between the PIT effect and the OPRM1 *polymorphism.* Last, we tested whether the observed interaction between the *OPRM1* polymorphism and the PIT effect was associated with relapse. Again, we found a three-way interaction between the *OPRM1* polymorphism, Pavlovian valence and instrumental action (Table 4). In addition, we observed an interaction between relapse status and instrumental action, and a three-way interaction between Pavlovian valence, instrumental action and relapse. This interaction was further modulated by the *OPRM1* polymorphism, resulting in the expected four-way interaction between Pavlovian valence, instrumental action, *OPRM1* polymorphism and relapse status (Figure 3 and Table 4). Thus, the interaction between the *OPRM1* polymorphism and the PIT effect was statistically different between patients with AD who prospectively relapsed and those who remained abstinent. Post hoc tests indicated that the interaction between Pavlovian valence, instrumental action and the *OPRM1* polymorphism was only significant for relapsers ($p<0.0001$) but not for abstainers ($p=0.328$). Moreover, the interaction between Pavlovian valence, instrumental action and relapse was significant for G+ carriers ($p<0.0001$) but not for G– carriers ($p=0.09$).

Discussion

To explore and further understand the behavioural and genetic underpinnings of 'wanting' as an expression of incentive salience attribution in humans and to bridge the gap to preclinical results, we investigated the association between the *OPRM1* polymorphism, PIT effect and relapse across a large cohort of patients with AD and two independent cohorts of HCs.

We demonstrate that (a) in all three independent cohorts, G+ carriers showed an increased PIT effect; (b) there is no difference between patients with AD and HCs in the interaction between *OPRM1* and PIT; but (c) when merely investigating AD, relapsing patients carrying the G+ allele showed an increased PIT effect as opposed to abstaining patients, who did not show an association between *OPRM1* genotype and PIT. We henceforth discuss these three main results.

Analysis 1: Association between the PIT effect and the OPRM1 *polymorphism across cohorts*

The first analysis demonstrated a clear association between the *OPRM1* genotype and PIT in three independent human cohorts. Two studies have previously investigated the role of the human opioid system in PIT-like effects in healthy human subjects. By using a pharmacological challenge, Weber et al. (2016) demonstrated that naltrexone reduces PIT effects for primary reinforcers (e.g. food rewards). We here demonstrate that the opioid system is also involved in modulating PIT effects for secondary reinforcers (e.g. monetary rewards). Beyond this, the experimental design from Weber et al. (2016) also differed in several other aspects from our study. Weber et al. (2016) focused on the positive 'limb' of the PIT effect (the extent to which positive stimuli affect responses), whereas our paradigm also enabled us to examine the negative 'limb' of the PIT effect (the extent to which negative stimuli affect responses). Moreover, our instrumental task included both 'Go' and 'No-Go' responses, whereas the instrumental task by Weber et al. (2016) merely included a 'Go' component. Thus, in line with previous investigations (Guitart-Masip et al., 2011, 2014; Swart et al., 2017), our experimental manipulation enabled us to test for more complex valence–action interactions. These previous tasks in line with our results have identified a potentially phylogenetically induced bias for congruent action–valence responses (e.g. better performance when a 'Go' response was acquired to win) compared to incongruent action valence (e.g. when a 'No-Go' response was acquired to win).

A second study published by Wiers et al. (2009) investigated automatic appetitive action tendencies in male heavy-drinking carriers of the *OPRM1* G allele. Heavy-drinking G+ carriers showed increased automatic approach tendencies not only to alcohol-associated stimuli but also to other appetitive stimuli (Wiers et al., 2009). This is in line with our finding of increased behavioural modulation in the presence of appetitive cues in AD G+ carriers. However, Wiers et al. did not include a control group in their study design and only included male sex, which limits generalisability and comparability to our results.

In summary, our data support the notion that the *OPRM1* polymorphism serves as one biological agent associated with human PIT effect in both AD patients and HCs.

Analysis 2: Alcohol-related group differences for the association between the PIT effect and the OPRM1 *polymorphism*

We did not find a significantly different association between the PIT effect and the *OPRM1* polymorphism between patients with AD and HCs, which partly reflects the ongoing debate and contradictory results published so far on the association between the *OPRM1* genotype and AD (Hendershot et al., 2016; Kong et al., 2017; Ray and Hutchison, 2004; Sloan et al., 2018). Instead, we found that AD and the *OPRM1* polymorphism are independent factors that both increase the PIT effect. Moreover, we found an interaction between instrumental action, *OPRM1* polymorphism and group, indicating that the opioid system differently affects instrumental responses in AD patients and HCs. Exploratory post hoc analyses (Supplementary Information 4) indicated that AD G+ carriers showed increased 'Go' responses compared to 'No-Go' responses, whereas HC G+ carriers showed increased 'No-Go' responses compared to 'Go' responses. Of note, a positive PIT effect is accompanied by an overall increase of 'Go' responses, while a negative PIT effect is accompanied by an overall increase in 'No-Go' responding. Thus, the *OPRM1* polymorphism may influence the positive PIT effect in patients with AD and the negative PIT effect in HC. A core feature of AD is the persistent substance consumption despite the negative consequences of consumption (Stacy and Wiers, 2010). We speculate that this paradox might partly be explained by an increased responsiveness of patients with AD to positively conditioned cues, which is stronger in G+ carriers. On the other hand, an increased responsiveness to negative stimuli might reveal a protective mechanism of healthy G+ carriers (S3 and S4). Clearly, future studies need to validate this speculation.

Analysis 3: Relapse-related group differences for the association between the PIT effect and the OPRM1 *polymorphism*

Only relapsers but not abstainers showed a significant interaction between the PIT effect and the *OPRM1* polymorphism. Moreover, only relapsing G+ carriers showed an increased PIT effect compared to abstainers, whereas there was no difference between the PIT effect in relapsers and abstainers in G– carriers. One speculative interpretation of these findings is that there may be two pathways to relapse, and that these fundamentally differ with regard to the *OPRM1* polymorphism and the PIT effect. On the one hand, in G+ carriers, the mechanisms driving PIT might also be related to relapse, whereas in G– carriers, these mechanisms could be less related to relapse. Our finding of an increased PIT effect in relapsing AD G+ carriers might also be relevant for precision medicine, particularly in the light of the ongoing discussion of the *OPRM1* polymorphism as a potential biomarker for the effectiveness of naltrexone treatment (Chamorro et al., 2012; Hartwell et al., 2020; Oslin et al., 2003; Setiawan et al., 2012; Ziauddeen et al., 2016). Strikingly, treatment response to naltrexone was also particularly high in patients with AD classified as reward drinkers (Mann et al., 2018; Witkiewitz et al., 2019) and reduced craving, most notably in social drinkers, who had high positive alcohol expectancies (Palfai et al., 1999).

Similar considerations might be relevant to nalmefene, the MOP antagonist and partial κ-agonist, recently approved for the treatment of AD (Gual et al., 2013), with similarly conflicting results. According to a meta-analysis, the drug is able to improve behavioural outcomes in patients with AD (Mann et al., 2016),

while others show that it has a limited efficacy in AD therapy (Palpacuer et al., 2015; Soyka and Muller, 2017). Nalmefene administered in a modified 'Go'/'No-Go' paradigm mildly reduced vigor to alcoholic cues in patients with AD (Gal et al., 2019). However, no major differences were observed between the treatment group and the placebo group with respect to behavioural and neural correlates of approach/avoidance tendencies. Given our data, future studies could investigate whether naltrexone and/or nalmefeme might be particularly effective in alcohol-dependent patients who are G+ carriers and additionally show large PIT effects.

Outlook: How does OPRM1 *influence neural reward processing?*

The neural correlates of PIT have been associated with relapse in AD within the mesolimbic reward system (Garbusow et al., 2016; Sekutowicz et al., 2019; Sommer et al., 2020) and could predict future drinking behaviour in adolescents (Sekutowicz et al., 2019). Recent studies have suggested a direct link between the *OPRM1* polymorphism and the mesolimbic dopaminergic system. For instance, by using a mouse model of the *OPRM1* A118G SNP, Popova et al. (2019) demonstrated that A- and G-allele carriers show significantly different regulation of mesolimbic dopaminergic firing. One potential underlying mechanism is that MOP receptors (which are affected by the *OPRM1* polymorphism) mediate opioid-induced disinhibition of midbrain dopaminergic neurons (Jalabert et al., 2011; Jhou et al., 2012; Matsui et al., 2014). Recent work in rodents has proven that optogenetic manipulations of those dopaminergic neurons can bidirectionally modulate online action selection (Howard et al., 2017). Thus, we speculate that the *OPRM1* polymorphism is associated with the extent to which Pavlovian stimuli functionally activate the mesolimbic dopaminergic system in AD. This speculation is in line with functional magnetic resonance imaging studies using cue reactivity paradigms in substance-dependent individuals. For instance, some studies suggest that AD G+ carriers display increased neural responses to alcohol-associated stimuli in mesocorticolimbic areas (Bach et al., 2015; Courtney et al., 2015; Filbey et al., 2008; but see Schacht et al., 2013). In line with this, humanised mice carrying the G+ allele of the *OPRM1* polymorphism displayed increased striatal dopamine release in response to an intravenously infused alcohol dose (Ramchandani et al., 2011). Clearly, future studies should further investigate how the *OPRM1* polymorphism affects the underlying neural mechanisms of the PIT effect in humans.

Limitations

The generalisability of our results is limited by the lack of preregistration, additional analyses designed after study protocol and the use of single gene analyses. The correlational nature of the analyses only allows speculation about causal relationships and needs to be further validated in a longitudinal design. Even though candidate genes as opposed to large-scale GWAS studies have come into disrepute, we believe that there is still a high relevance in connecting single genes and their respective pathways to specific neurocognitive processes and thus providing the opportunity for more specific interventions in precision medicine (Deb et al., 2010; Di Martino et al., 2020). Another limitation of our design is that the procedure used here to indicate Pavlovian learning (task phase 4) was not designed to detect between-group effects but instead served to identify subjects who did not learn the Pavlovian contingencies (Supplemental Information 8). Across all cohorts, subjects could almost perfectly identify the best Pavlovian stimuli, and these ceiling effects potentially lowered statistical power to detect differences in Pavlovian learning. Several studies across humans and animals have demonstrated that individuals who attribute incentive salience to reward predicting stimuli through Pavlovian conditioning (so called sign-trackers) will also show an increased PIT effect (Garofalo and di Pellegrino, 2015; Schad et al., 2019b). Future studies should therefore use more sensitive methods to identify sign-tracking humans (such as eye-tracking; Schad et al., 2019b) and test the role of the *OPRM1* polymorphism in this phenomenon. One further limitation is the relatively small sample size of relapsers versus abstainers in analysis 3. Importantly, the group of G+ carriers that relapsed versus abstained was 16 versus 14, respectively. Thus, future stratification studies need to replicate our findings in larger sampling sizes, for example by oversampling G+ carriers in AD.

Summary

This study presents strong evidence for an association between the *OPRM1* polymorphism and the PIT effect in both patients with AD and HCs. It is the first to show that the *OPRM1* polymorphism modulates the extent to which Pavlovian stimuli exert control over behaviour and suggests a functional difference of this gene–behaviour interaction between relapsers and abstainers.

Declaration of conflicting interests

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Supplemental material

Supplemental material for this article is available online.

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2.3 Der Einfluss von Alkoholkonsum, Abstinenz und Rückfall auf epigenetische Veränderungen bei Patient*innen mit Alkoholabhängigkeit

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Impact of Long-Term Alcohol Consumption and Relapse on Genome-wide DNA Methylation Changes in Alcohol-Dependent Subjects: A Longitudinal Study

Eva Friedel, Henrik Walter, Ilya M. Veer, Ulrich S. Zimmermann, Andreas Heinz, Helge Frieling, and Tristan Zindler

Background: Genetic factors play an important role in the development and maintenance of alcohol use disorder (AUD). Significant and widespread differences in methylation levels of multiple regions within the genome have been reported between AUD patients and healthy controls in large epigenome-wide association studies (EWASs). Also, within patient populations, methylation changes over time (both during and after withdrawal) have been identified as sensitive indicators for disease activity. The detection of changes in methylation levels is a powerful tool to further explore and understand the biological correlates and underpinnings of AUD. Although there is strong and convincing evidence for differences in methylation of various sites between AUD patients and controls, only few studies assessed changes within patients over longer periods of time while taking into account alcohol consumption, relapse, and abstinence. So far, the longest period assessed as a within-subject design using EWASs was 4 weeks.

Methods: Here, we investigated changes in whole-genome methylation levels within a sample of 69 detoxified AUD patients over a period as long as 12 months for the first time, comparing patients that relapsed within the follow-up period to those that remained abstinent.

Results: Whole-genome methylation patterns of individual CpG sites over time did not differ between abstinent and relapsing patients. However, there was a negative association between global mean methylation at the 12-month follow-up and alcohol consumption within our sample.

Conclusion: Although the present study represents the largest study of methylation levels in a sample of AUD patients with a follow-up period of 1 year and accounting for alcohol consumption and relapse to date, the sample size might still not be large enough to detect genome-wide significant effects. Therefore, large-scale, long-term studies with AUD subjects are needed to determine the utility of DNA methylation for the assessment and monitoring of persons with alcohol use disorders.

Key Words: DNA Methylation, Alcohol Use Disorder, Longitudinal Design, Relapse, Epigenetics, Alcohol Consumption.

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CHRONIC EXPOSURE TO alcohol has been associated with far-reaching consequences, including loss of quality of life years, negative effects on mental and physical health (Charlet and Heinz, 2017), and increased mortality (Lozano et al., 2012). Despite the considerable heritability of alcohol use disorder (AUD) proven by adoption and twin studies, there is still a limited number of genetic variants that have been identified in traditional genome-wide association studies (GWASs) as risk factors for AUD, with an estimated 40–60% of genetic factors contributing to the variance in susceptibility to AUD (Deak et al., 2019). In addition to genetic mechanisms, epigenetic regulation—a possible bridge between genetic and environmental factors—has been proposed as one mechanism of interest in neuroadaptation that contributes to the development and maintenance of AUD (Berkel and Pandey, 2017; Hagerty et al., 2016; Hamilton and Nestler, 2019). Epigenetics refer to molecular processes that alter gene expression without altering the deoxyribonucleic acid (DNA) sequence itself (Nestler, 2014). The most commonly accepted mechanisms coded as epigenetic

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mechanisms are DNA methylation, histone modifications, and noncoding RNAs. These mechanisms interact with chromatin, the protein complex that organizes DNA and thus can alter the extent to which genes are accessible to transcription factors. Here, we refer to epigenetic mechanisms after assessing DNA methylation as the most commonly studied epigenetic mechanism in addiction providing a possible bridge between genetic and environmental factors. However, when evaluating the current literature on AUD and methylation changes, we encounter a high inconsistency of published results and an extremely limited number of replications. This might be partly due to varying study designs, heterogeneous cohorts, and unstandardized methodology within this highly dynamic and developing research field (Harlaar and Hutchison, 2013; Zhang and Gelernter, 2017). Furthermore, differences in tissue types used to assess methylation levels might add up to the heterogeneity of results. Although there has been a recent effort in providing cross-tissue and cross-phenotypic approaches using postmortem brain cells, peripheral blood and buccal cells in AUD patients (Hagerty et al., 2016; Lohoff et al., 2018) the majority of studies stems from peripheral blood (Bruckmann et al., 2016; Koller et al., 2019; Liu et al., 2018; Philibert et al., 2018; Philibert et al., 2014a; Philibert et al., 2012) and saliva (Xu et al., 2019), which does not entirely reflect methylation changes in the brain (Rollins et al., 2010). Nevertheless, previous reports stress the value of analyzing peripheral tissue to explore molecular mechanisms and detect new genes, pathways and biomarkers for psychiatric diseases (Pajer et al., 2012; Pedroso et al., 2012).

A considerable number of studies have reported the cross-sectional difference in whole-genome methylation patterns between subjects consuming alcohol (heavy drinkers and AUD patients) and healthy controls (e.g., Bruckmann et al., 2016; Harlaar et al., 2014; Liu et al., 2018; Philibert et al., 2014a; Philibert et al., 2012; Xu et al., 2019; Zhao et al., 2013), with sample sizes ranging from 10/10 (patients/controls) (Zhao et al., 2013) up to n = 13,317 (2018)). The majority of studies revealed differential methylation in a number of genes that are involved in alcohol metabolism, stress immune response, and signal transduction with respect to alcohol consumption. However, replication of specific sites is scarce. One finding recently replicated is for CpG sites in the promoter region of *GDAP* (Bruckmann et al., 2016). Liu and colleagues (2018) assessed DNA methylation as a possible biomarker of alcohol consumption in 13 population-based cohorts (n_{total} = 13,317), identifying 144 CpGs associated with current heavy alcohol consumption. However, the latest study on whole-genome methylation patterns and alcohol consumption (Xu et al., 2019) reported only small effects of alcohol consumption on individual CpG sites, including 64 new CpG sites. Only 6 of the CpG sites previously reported to be associated with AUD, liver function, body mass index, and lipid metabolism could be replicated (n = 1,135).

Longitudinal studies of whole-genome methylation patterns with respect to alcohol consumption are extremely rare: So far, only one study of AUD patients (Bruckmann et al., 2016), 2 studies of healthy drinkers (Philibert et al., 2014a; Philibert et al., 2012), and one of healthy subjects developing AUD after 10 years (Weng et al., 2015) have been published with longitudinal data. Time between baseline and follow-up ranged from 21 days (Bruckmann et al., 2016) to 10 years (Weng et al., 2015), respectively. Philibert and colleagues (2012) assessed changes in methylation patterns in 165 healthy female subjects over a period of 6 months with respect to alcohol consumption. They reported severity-dependent changes in the degree of genome-wide methylation, with 2 regions reaching genome-wide significance. In 2 other studies from the same group (Philibert et al., 2018; Philibert et al., 2014a), changes in whole-genome methylation levels were reported for 66 subjects (33 cases with heavy alcohol consumption, 33 controls) that entered and exited a 30-day inpatient treatment program. At baseline, the case–control comparison revealed a total of 56 CpG sites reaching genome-wide significance. With respect to alcohol-dependent changes over time (max of 25 days), no single CpG site crossed the threshold of genome-wide significance. Interestingly, there was no significant overlap between the CpG sites reported for alcohol consumption and the CpG sites previously reported for smoking (Dogan et al., 2014; Philibert et al., 2014a) or for CpG sites associated with alcohol in healthy women (Philibert et al., 2012). The longest follow-up period was reported for a subgroup of 10 subjects (compared to 10 healthy controls) who developed AUD after 12 years (starting off as healthy participants) (Weng et al., 2015). The authors reported an association between changes in methylation levels of 6 genes and alcohol consumption for this group. However, taking into account that the authors did not correct for multiple testing, this finding should be interpreted with extreme caution until replicated. The most recent longitudinal design assessing whole-genome methylation changes at baseline and 3 weeks after completion of an inpatient alcohol treatment program was reported by Bruckmann and colleagues (2016). The authors performed an EWAS in 49 AUD patients and 47 healthy controls. They report significant differences between *GDAP1* DNA methylation levels in patients before and after alcohol treatment and there was a trend toward a negative association between the mean DNA methylation levels of 3 associated CpG sites and the years of alcohol dependency. *GDAP1* was previously reported as a significant finding in an EWAS of heavy drinkers by Philibert et al. (2014a).

With respect to AUD and alcohol consumption–associated cross-sectional global DNA methylation changes, Zhang and Gelernter (2017) provide an overview of 6 studies published until April 2016, again revealing the inconsistency of published results. Two studies reported an overall increase of methylation with alcohol consumption (Bonsch et al., 2004; Kim et al., 2016), one study an inverse relationship (Zhu et al., 2012), and 3 studies no association at all (Ono et al., 2012; Zhang et al., 2011). Since then, 5 more studies have been published (Bruckmann et al., 2016; Hagerty et al., 2016; Koller et al., 2019; Liu et al., 2018; Xu et al., 2019). Only one of these 4 studies (Koller et al., 2019) reported

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changes in global methylation status between cases and controls (with an increase of methylation for AUD patients after withdrawal). The other studies (Bruckmann et al., 2016; Hagerty et al., 2016; Liu et al., 2018; Xu et al., 2019) refer to methylation in specific CpG sites without accounting for global methylation status.

Evaluating the current literature on epigenetic mechanisms in AUD, the urgent need for more longitudinal within-subject designs comparing the effect of alcohol consumption, relapse and abstinence becomes evident. Long-term follow-ups allow investigators to address changes in methylation beyond effects that can be attributed to the epigenetic property of alcohol itself (Zakhari, 2013) and changes that are detectable only during the short period of detoxification and withdrawal (Bruckmann et al., 2016). When comparing DNA methylation differences between AUD cases and controls, it is unclear whether epigenetic differences are already present before alcohol exposure (and should be considered as risk factors for AUD) or whether they are the consequence of chronic alcohol use.

We therefore investigated whole-genome methylation patterns as well as mean methylation levels in 69 well-characterized detoxified alcohol-dependent patients over a period of 1 year and assessed the influence of alcohol consumption, relapse, and abstinence on intraindividual changes in methylation patterns.

MATERIAL AND METHODS

Sample

We assessed 69 subjects suffering from AUD at baseline and after a 1-year follow-up, including blood analysis and detailed information on alcohol consumption. Data were collected as part of the LeAD study (Learning and Alcohol Dependence). The protocol has previously been reported in detail elsewhere (Garbusow et al., 2016; Nebe et al., 2018; Sebold et al., 2019). All patients fulfilled diagnostic criteria for AUD according to ICD-10 and DSM-IV-TR ("Diagnostic and Statistical Manual of Mental Disorders," 2000) for a minimum of 3 years. Patients with history of current or past substance use disorder (except alcohol and nicotine dependence), other major psychiatric disorder (as assessed with the computer-based Composite International Diagnostic Interview, CIDI (Wittchen, 1997)), or neurological disease were excluded. All subjects were free of psychotropic medication known to interact with the central nervous system for at least 4 half-lives (including illegal drugs and detoxification treatment tested by a drug urine). Study participation of the patients took place shortly after detoxification (3–21 days). The Alcohol Dependence Scale (Skinner, 1984), Obsessive Compulsive Drinking Scale (German version; Mann, 2000), and estimated lifetime alcohol consumption (in kg) were assessed for severity of alcohol use (Sobell, 1992). There was no difference between abstaining and relapsing patients in alcohol use severity at inclusion. However, relapsing patients entered the study with a higher number of inpatient detoxification treatments before inclusion ($p < 0.05$) (for a detailed sample description, see Table 1). Smoking status was assessed with the Fagerström Test for Cigarette Dependence (FTCD) (Fagerström, 2012). Within the 12-month period, one abstaining patient and 2 relapsing patients changed smoking status (from smoker to nonsmoker). There was no significant difference in percentage of smoking patients at either baseline or at the 12-month follow-up between abstaining and relapsing patients (Table 2).

Alcohol consumption

After study participation, AUD patients were regularly contacted over a period of 12 months (every 2 weeks during the first 3 months, every 6 weeks from month 3 onward, and every 12 weeks from month 6 onward) to assess alcohol consumption using the alcohol timeline follow-back method (Sobell, 1992). Patients were contacted either via the telephone (on weeks 6, 10, 18, and 36 after baseline) or in a personal assessment (which took place at baseline and on weeks 4, 8, 12, 24, and 48 after baseline). Relapse was defined as consumption of 60 or 40 grams of alcohol on any occasion for males and females, respectively. This definition was used according the WHO (WHO, 2000) criteria of current high-risk versus current low-risk consumption. Personal assessment included alcohol breath tests to validate self-reports. Breathalyzers were used by trained instructors only, who guided patients through the procedure and documented the respective results.

During the follow-up period, 16 patients discontinued participation in the study (15%). In 2 cases, we only had relapse reports from close relatives, which we accepted for classification. Altogether, 38 patients relapsed during the follow-up period, whereas 31 remained abstinent. Within the group of patients classified as abstainers according to WHO criteria, 27 patients reported zero alcohol consumption over the period of 12 months. Four subjects consumed an average of 34.88 (80.55) g on 0.66 (1.56) occasions (see Table 1). Demographic and clinical characteristics of this sample are shown in Tables 1 and 2. As a reliable biological marker for alcohol consumption, gamma-glutamyltransferase (U/l) showed a significant decrease in abstaining patients only.

Analysis

DNA methylation quantification and quality control. Genomic DNA was extracted from peripheral whole-blood samples at baseline and after 12 months from the same subjects. Bisulfite-converted DNA samples were used in the array-based DNAm assay, the Illumina Infinium Human MethylationEPIC BeadChip (Illumina, San Diego, CA) which interrogates DNAm at roughly 850K CpG sites. DNAm profiling was conducted at Hannover Medical Center. For sample distribution on plates and chips with respect to relapse and time point, see Fig. S1.

Raw Illumina EPIC methylation data were preprocessed and converted into beta values using the ChAMP pipeline at baseline and the 12-month follow-up. Subsequent quality control of the sample data included removal of probes with a detection p-value above 0.01. Additionally, probes with a bead count less than 3 and probes with SNPs were removed (Zhou et al., 2017).

Only probes present in both datasets after quality control were included in further analyses, resulting in datasets for the baseline and 12-month follow-up with probes of 740,391 CpG sites of 69 samples. Quality control for batch effects performing singular value decomposition method (SVD) revealed a significant batch effect between the plates, chips, and sample well (see Fig. S2). Batch correction was performed using ComBat as implemented in the sva package (Leek et al., 2012) without the use of a moderating variable after applying probe type normalization with the BMIQ method. A new surrogate variable analysis (SVA) plot showed a considerable reduction of the batch effects after ComBat (see Fig. S2). All subsequent analyses were validated with the untransformed and uncorrected raw beta values to reduce the possibility of introducing false positive results with ComBat and a simulation of the given factor structure regarding the effects of ComBat was carried out in order to rule out a considerable distortion of the test statistics[1].

[1]To further ensure the validity of our sample, we were able to replicate the frequently reported effect of smoking on cg05575921 of the AHRR gene within our sample (see Supplementary Material A).

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Table 1. Sample Characteristics

	Abstainer (N = 31; 6 female)				Relapser (N = 38; 4 female)			
	Baseline (BL)		Follow-up (FU12)[a]		Baseline (BL)		Follow-up (FU12)[a]	
	n	$M(SD)$	n	M(SD)	n	M(SD)	n	M(SD)
Smoking (in %)	31	77% (n = 24)	31	81% (n = 25)	38	79% (n = 30)	38	74% (n = 28)
Age (in years)	31	48.51 (11.55)	31	49.46 (11.78)	38	47.24 (9.57)	34	48.21 (9.73)
ADS[b] score	31	13.45 (6.76)	26	10.27 (7.16)	38	14.71 (6.30)	37	11.06 (7.29)
Drinking per occasion past year (in g)	31	173.61 (97.05)	29	34.88 (80.55)	38	212.45 (94.29)	34	163.40 (123.83)
Drinking frequency past year	31	4.29 (1.30)	29	0.66 (1.56)	38	4.66 (0.75)	34	3.79 (1.63)
Drinking per day past year (in g)	31	135.24 (96.09)	29	15.77 (50.15)	38	186.45 (111.53)	34	113.44 (116.55)
Lifetime alcohol intake in kg (pure alcohol)	31	2,081.64 (1416.79)			38	1,973.02 (981.66)		
γ-GT[c] U/l	26	181.92 (273.22)	28	42.09 (33.61)	31	77.05 (68.23)	30	84.54 (115.26)
GOT[d] U/l	26	42.08 (20.13)	28	25.42 (7.66)	31	35.65 (29.94)	30	41.36 (39.91)
GPT[e] U/L	26	59.23 (44.53)	28	27.23 (18.30)	31	34.50 (22.65)	30	42.14 (45.23)
OCDS[f]	31	10.28 (8.60)	31	2.75 (3.31)	36	10.61 (7.21)	38	8.66 (6.76)
Outpatient detoxification	31	26% (n = 8)			38	26% (n = 10)		
Inpatient detoxification	31	87% (n = 27)			38	95% (n = 36)		
Time to relapse (in days)			31				38	111.76 (82.33)

[a]After 12 months.
[b]Alcohol Dependence Scale.
[c]Gamma-glutamyltransferase.
[d]Glutamate-oxaloacetate transaminase.
[e]Glutamate-pyruvate transaminase.
[f]Obsessive Compulsive Drinking Scale.

Table 2. Descriptive Statistics for Group Comparisons at Baseline (BL–BL), at Follow-Up (FU12–FU12), and for Changes Over Time Within Each Group Separately (BL–FU12)

	BL–BL (Abstainer–Relapser)		FU12–FU12 (Abstainer–Relapser)		BL–FU12 (Abstainer)		BL–FU12 (Relapser)	
	t (df)	p	t (df)	p	t (df)	p	t (df)	p
Smoking (in %)		1[a]		1[a]		1[a]		1[a]
Age (in years)	0.49 (58.20)	0.63	0.45 (54.40)	0.65				
ADS[b] score	−0.79 (62.24)	0.43	−0.42 (54.44)	0.68	1.71 (52.10)	0.09	2.26 (65.69)	0.03
Drinking per occasion past year (in g)	−1.67 (63.47)	0.10	−4.95 (57.26)	<0.0001	6.04 (57.21)	<0.0001	1.87 (61.39)	0.07
Drinking frequency past year	−1.40 (45.68)	0.17	−7.79 (60.11)	<0.0001	9.76 (54.52)	<0.s	2.84 (45.11)	
0.007			Drinking per day past year (in g)	−2.05 (66.78)	0.05	−4.43 (46.31)	<0.0001	
6.09 (45.85)	<0.0001	2.71	(68.32)	0.009				
Lifetime alcohol intake in kg (pure alcohol)	0.36 (51.67)	0.72						
γ-GT[c] U/l	1.91 (27.62)	0.07	−1.93 (34.22)	0.06	2.59 (25.70)	0.02	−0.31 (46.80)	0.76
GOT[d] U/l	0.96 (52.69)	0.34	−2.15 (31.28)	0.04	3.96 (31.64)	<0.001	−0.63 (53.77)	0.53
GPT[e] U/l	2.57 (35.63)	0.01	−1.67 (38.79)	0.10	3.41 (32.72)	0.002	−0.83 (42.37)	0.41
OCDS[f]	−0.17 (58.86)	0.87	−4.74 (56.00)	<0.0001	4.55 (38.71)	<0.0001	1.20 (70.99)	0.23
Outpatient detoxification	0.64 (11.30)	0.53						
Inpatient detoxification	−2.15 (53.03)	0.04						

[a]Fisher's exact test.
[b]Alcohol Dependence Scale.
[c]Gamma-glutamyltransferase.
[d]Glutamate-oxaloacetate transaminase.
[e]Glutamate-pyruvate transaminase.
[f]Obsessive Compulsive Drinking Scale.

Statistical analysis. The statistical analyses were conducted within the R (v3.6.1) environment and the Bioconductor (v3.9) framework. We performed exploratory genome-wide methylation analysis to identify relevant changes in methylation within our patients over a period of 12 months with respect to drinking behavior and relapse.

First, we checked for differences in overall methylation with a repeated-measures ANOVA from baseline to the 12-month follow-up for relapse versus nonrelapse (at the 12-month follow-up). Additionally, the Spearman rank correlation between alcohol consumption in grams (g) per past year and the mean methylation at the 12-month follow-up was conducted.

Second, we performed a series of t-tests for the differences in methylation with respect to relapse/nonrelapse at the 12-month follow-up for every probe (DMP) with a false discovery rate (FDR) of $< 5\%$. In addition to the DMP comparison, an analysis for the identification of differentially methylated regions (DMRs) was carried out with the Bumphunter algorithm implemented in the

ChAMP package (Tian et al., 2017) on the beta value differences in the 12-month follow-up period. An ingenuity pathway analysis was planned for genes with sites significant after considering the FDR. To rule out the effect of alcohol consumption within the sample of abstaining patients (4 patients continued the consumption of alcohol on a low level with an average of 34.88 (80.55) g on 0.66 (1.56) occasions; see Table 1), all t-tests were reanalyzed using a criterion of zero alcohol consumption (for a detailed description, see Supplementary material B).

In an additional approach, we tried to identify relevant CpGs and their corresponding genes in 2 steps: In the first step, we performed a partial correlation of lifetime kg alcohol consumption, age and whole-genome methylation at baseline. The top 1,000 CpG sites correlated with lifetime alcohol intake were then advanced to the second step of the analysis. In this second step, we calculated the difference in methylation between baseline and 1-year follow-up of the previously identified top 1,000 CpG sites and correlated this difference score with g alcohol intake for the 12-month time period between the methylation measurements. FDR correction for the second step was applied to 1,000 calculations to reduce the probability of false negative results. Third, we calculated a series of Spearman's rank correlation coefficients for all probes with the average daily total drinking volume and change in liver enzymes (GPT/GOT) over the observation period. Correlations with a false discovery rate (FDR) of < 5% were defined as significant.

RESULTS

The repeated-measures ANOVA did not show any significant effects on mean methylation level: Neither the effect of time, $F(1, 134) = 2.36$, $p = 0.13$, nor the group effect between the relapse and nonrelapse groups, $F(1, 134) = 0.01$, $p = 0.94$ was significant. There was a nonsignificant but trendwise interaction between time and group, $F(1, 134) = 3.24$, $p = 0.07$), pointing toward a decrease in mean methylation difference for relapsers compared to abstainers.

The Spearman rank correlation between mean methylation at the 12-month follow-up and alcohol consumption (over a period of 12 months) revealed a significant, moderate, and negative ($r_s = -0.30$, $p = 0.01$) association. This again provided evidence for decreasing methylation with increasing alcohol consumption.

A Wilcoxon signed-rank test indicated no difference in kg lifetime alcohol intake at baseline in relation between abstinent (median = 1,806.555) and relapsing (median = 2,017.337), W = 586, $p = 0.98$ patients. Correlation of the top 1,000 CpGs (with highest correlation between lifetime alcohol consumption and baseline methylation (T0)) between difference in methylation (T0–T1) and g alcohol intake (for the 12-month follow-up period) revealed no significant results. The highest negative Pearson product–moment correlation between the methylation-difference score T0–T1 and alcohol consumption during 12-month follow-up was not FDR significant and small, $r(67.00) = -0.21$, 95% CI [−0.43, 0.02], $p = 0.08$, FDR $p = 1.00$) for cg22544563 (SLC35F1). The highest positive Pearson product–moment correlation between the methylation-difference score T0–T1 and alcohol consumption during 12-month follow-up was not significant and small, $r(67.00) = 0.28$, 95% CI [0.05, 0.48], $p < 0.05$, FDR $p = 1.00$) for cg03452160 (RBFOX3). For all correlations of the 1,000 CpGs, see Table S1. The conducted series of t-tests to identify differentially methylated probes revealed that none of the 740,391 tested CpG probes exceeded the significance level of FDR < 5% (Fig. 1). The analysis for identification of DMRs also showed no significant result. The range of methylation changes within our cohort was relatively small ($M = -0.008997147$, median = −0.009088333, SD = 0.0389543; for a detailed description, see Table S2). Since no significant CpG probes could be identified, the planned pathway analysis was subsequently not carried out. Reanalyses of t-tests for the differences in methylation with respect to relapse/nonrelapse at the 12-month follow-up with a 0/>0 criterion of alcohol consumption did not lead to more significant results, but the p-values tend to increase (for a detailed description, see Supplementary material B and Table S3).

There were no correlations exceeding the significance level of FDR < 5% between the difference in methylation rate for every probe between T1 and T0 and the average daily alcohol consumption during the follow-up period and change in liver enzymes. Subsequent analyses with the untransformed and uncorrected raw beta values yielded validation of the reported results.

DISCUSSION

This is the first study to date investigating the long-term effects of abstinence and alcohol consumption on patients diagnosed with AUD according to DSM-IV-TR. We did not find an association of methylation patterns when comparing individuals with long-term alcohol consumption to those of abstinent individuals suffering from AUD. A trendwise interaction between relapse and time for mean whole-genome methylation and a negative correlation of mean whole-genome methylation with alcohol consumption at the 1-year follow-up could be detected.

To date, only 2 studies with a maximum follow-up period of 30 days systematically investigated the effects of an alcohol treatment program on the epigenome (Bruckmann et al., 2016; Philibert et al., 2014a). In the first study (Philibert et al., 2014a) comparing the methylation levels of heavy drinkers at the beginning of the alcohol treatment and after 4 weeks of treatment, no genes reached epigenome-wide significance. However, 56 CpGs reached epigenome-wide significance when comparing AUD patients to controls at inclusion. The second study (Bruckmann et al., 2016) was able to validate one of the findings (*GDAP1*) as an indicator for disease severity and treatment outcome after a 21-day alcohol treatment program. The authors report 48 differentially methylated CpG sites at $p < 0.1$ and with a DNA methylation difference of> 5%. Findings in *GDAP1* were validated by pyrosequencing. Interestingly, the exclusion of 8 patients who had been abstinent for more than 3 days before hospital admission enhanced the observed effect of differential *GDAP1* methylation between control individuals and

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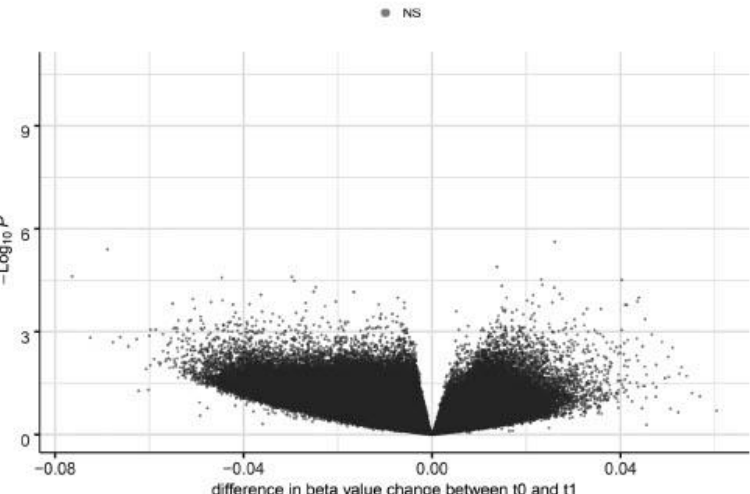

Fig. 1. Volcano plot for the group differences regarding relapse and abstain with -log10 *p*-value on the *y*-axis and the difference in beta value change between the 2 time points (t0 = baseline; t1 = 12-month follow-up) on the *x*-axis. The volcano plot enables visual identification of CpG sites with large changes between t0 and t1 that are also statistically significant. More specifically, it enables quick visual identification of sites with large beta value change and their respective significance. This subsequently can help to identify the most biologically significant sites. Here, no significant changes in beta value occur.

patients at baseline. Time to last drink and time to relapse until methylation analysis might have been too long to detect the previously described short-term effect of alcohol consumption in AUD patients in our sample. Relevant epigenetic mechanisms have been identified during the period of withdrawal (Biermann et al., 2009; Bruckmann et al., 2016; Hillemacher et al., 2009); however, within our sample, important changes might have already occurred before inclusion. All patients had been abstinent from alcohol for at least 30 days until first relapse (Tables 1 and 2), and all patients had a minimum of 3 years of AUD according to DSM-IV criteria before inclusion. Homogeneity in sense of disease severity within this sample might explain the overall relatively small range of methylation changes comparing abstaining and relapsing patients after the 1-year follow-up. More extreme group comparisons with respect to duration of abstinence versus duration and amount of alcohol consumed might be necessary to detect differences using within-sample designs.

We did find a trendwise increase of mean global methylation levels in abstinent compared to relapsing patients, which is in line with the most recently published results (Koller et al., 2019) but inconsistent with previously reported increase of methylation levels in AUD cases compared to controls (Bonsch et al., 2004; Kim et al., 2016). There was a negative correlation between mean methylation at the 1-year follow-up and alcohol consumption, accordingly. However, a stepwise correlation taking into account age and lifetime alcohol consumption between the methylation-difference score of both time points and 12-month alcohol intake did not yield significant results.

DNA methylation studies of AUD, especially those on whole-genome methylation patterns, are still at an early stage (Zhang and Gelernter, 2017). Most studies have been published in the past 5 years, with the vast majority reporting differences between healthy control individuals and AUD patients (Hagerty et al., 2016; Liu et al., 2018; Xu et al., 2019). There has been great enthusiasm with recent findings in large case–control cohorts (Hagerty et al., 2016; Liu et al., 2018; Xu et al., 2019); however, we need to develop a more detailed picture of the biological processes involved in the detected changes of methylation patterns. Most of what we currently know about epigenetic processes and addiction stems from animal studies, which enable the experimental manipulation of important factors such as the type, extent, and timing of substance exposure (Bekdash et al., 2013; Finegersh and Homanics, 2014a; Finegersh and Homanics, 2014b; Wieting et al., 2019). Besides the epigenetic impact of alcohol itself, (dys)functional learning processes (Garbusow et al., 2014; Sebold et al., 2017), social status, early (stressful) life experience (Sebold et al., 2019b), tolerance toward alcohol exposure, and the high comorbidity with other substance use disorders make this research in human subjects far more challenging. As in many other studies exploring AUD, nicotine dependence has not been excluded (Beck et al., 2012; Garbusow et al., 2016; Garbusow et al., 2018; Schad et al., 2019; Sebold et al., 2019a; Sebold et al., 2017; Sekutowicz et al., 2019; Weinberger et al., 2016). And even though

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nicotine dependence is among the most common comorbidities in AUD (Weinberger et al., 2016) results are hence limited in their generalizability to nonsmoking AUD patients. Another limitation of generalizability and replicability might be caused by the usage of a nonzero alcohol consumption as relapse criterion, which here was defined according to the WHO criterion of low- versus high-risk consumption (WHO, 2000). With respect to abstinence rate, in our sample this was relatively high with 45%. Using zero alcohol consumption as abstinence criterion, abstinence rates decrease to 39%. However, results of supplementary analysis using the zero criterion did not substantially differ from our results and a significant decrease in liver enzymes in abstaining patients only might serve as one indicator of the validity of the above reported relapse criterion. This is in line with previously reported designs on AUD using the same criterion (e.g., Beck et al., 2012; Charlet et al., 2014; Garbusow et al., 2016; Garbusow et al., 2018; Schad et al., 2019; Sebold et al., 2017; Sekutowicz et al., 2019; Witkiewitz et al., 2017; Witkiewitz et al., 2020).

When using cross-sectional designs, it remains unclear whether differences between AUD cases and controls were already present before alcohol exposure (and should be considered as risk factors for AUD) or whether they are the consequence of chronic alcohol use. Another well-known limitation of studies assessing epigenetic changes in humans is the tissue analyzed. Tissues commonly accessible and thus of potential benefit for daily clinical use in AUD patients are blood and saliva (as opposed to, e.g., tumor tissue in cancer research), whereas brain tissue (of various regions within the brain) would be the tissue clearly stronger associated with addictive behavior (Hagerty et al., 2016; Lohoff et al., 2018). Some studies have shown that brain cellular heterogeneity may bias DNA methylation patterns (Guintivano et al., 2013). We hence understand peripheral mechanisms of alcohol consumption rather than exploring its neural mechanisms, except to the extent that peripheral measures reflect central activity.

Methodological differences between studies make it extremely difficult to replicate findings and to disentangle false positives from valid differences between groups. Out of 3 studies that assessed methylome-wide changes using within-subject designs (Bruckmann et al., 2016; Philibert et al., 2014a; Weng et al., 2015), one study reported a correlation of methylation changes with alcohol consumption but without accounting for the effect of multiple comparisons (for approximately 28,000 CpG site comparisons in 20 subjects (Weng et al., 2015)). Therefore, validation of results with an alternative method and replication in larger samples is urgently needed. Also, the publication of negative findings must also be encouraged to get a better idea of the average effect sizes. Unfortunately, due to publication bias, small and nonsignificant effects are either often not submitted for publication or have a higher probability of being denied for publication by reviewers or editors (Bakker et al., 2012; John et al., 2012; Schafer and Schwarz, 2019), making it even more difficult to replicate findings.

Although the present study represents the largest study of methylation levels in a sample of AUD patients with a follow-up period of 1 year and accounting for alcohol consumption and relapse to date, the sample size might still not be large enough to detect genome-wide significant effects. Therefore, large-scale, long-term studies on AUD subjects are needed to determine the utility of DNA methylation for the assessment and monitoring of persons with AUDs. These future results might help to identify reliable biomarkers that remit as a function of abstinence and allow to explore the biological correlates and underpinnings of AUD.

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CONFLICT OF INTEREST

All authors have no competing interests of financial or other nature.

AUTHORS' CONTRIBUTIONS

AH, HW, UZ, EF, and IV were responsible for recruitment of alcohol-dependent patients. TZ and EF were responsible for further statistical analyses with support of HF, HW, IV, and UZ. EF and TZ drafted the manuscript. HF, HW, IV, UZ, and AH provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved the final version for publication.

ETHICS APPROVAL

Ethical approval for the study was obtained from the ethics committee of Charité-Universitätsmedizin Berlin (EA1/157/11) and Universitätsklinikum Dresden (EK228072012). Participants received a monetary compensation of 10 /hour for study participation.

DATA AVAILABILITY STATEMENT

This manuscript contains previously unpublished data. The name of the repository and accession number(s) are not available due to patient confidentiality and participant privacy. Methylation array data can simultaneously identify individuals and convey protected health information (2014b). Patients did not provide written informed consent on the publication of individual methylation profiles. Requests to access the datasets should be directed to Tristan Zindler (Zindler.Tristan@mh-hannover.de).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Upper panel: Single value decomposition analysis (SVD) using RAW values. Lower panel: Single value decomposition analysis (SVD) using COMBAT corrected values.

Fig. S2. Distribution of all samples (relapser and abstainer) on chips and plates for t0 = baseline and t1 = 12-month follow up.

Table S1. Correlation of the top 1,000 cpgs (with highest correlation between lifetime alcohol consumption and baseline methylation(T0)) between difference in methylation (T0-T1) and g alcohol intake (for the 12 month follow up period)

Table S2. *T*-tests for the differences in methylation with respect to relapse/non-relapse at the 12-month follow-up.

Table S3. Re-anaylses of *t*-tests for the differences in methylation with respect to relapse/non-relapse at the 12-month follow-up with a 0 / >0 criterion of alcohol consumption.

Data S1. Supplementary materials.

Data S2. Figure legends.

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2.4 Wie Alkohol unsere epigenetische Uhr schneller ticken lässt und Abstinenz diese Uhr zurückstellen kann

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ORIGINAL ARTICLE

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How alcohol makes the epigenetic clock tick faster and the clock reversing effect of abstinence

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Abstract

This study investigated the recently reported association between alcohol dependence and accelerated ageing and the potential effects of abstinence and relapse on DNA methylation status using Levine's epigenetic clock to estimate DNA methylation age in two independent cohorts. The first sample comprised 88 (15 female) detoxified patients with alcohol use disorder (AUD) and 32 (5 female) healthy control (CON) subjects (NCT02615977), and the second included 69 (10 female) AUD patients that were followed up for 12 months with respect to relapse ($n = 38$, 4 female) and abstinence ($n = 31$, 6 female) (NCT01679145). To account for the different aspects of ageing captured by various clocks, we performed additional analyses of the first-generation Horvath clock and next-generation Zhang clock. To account for the genetic liability of AUD and its potential influence on DNA methylation, we calculated a polygenic risk score for alcohol dependence. We found that ageing was accelerated by 3.64 years in AUD patients compared with the CON group according to Levine's DNAm PhenoAge. Furthermore, in a second longitudinal sample, we found that abstaining AUD patients displayed a decrease in DNAm PhenoAge by 3.1 years, but we found an over proportional increase by 2.7 years in those who relapsed. Polygenic risk did not affect epigenetic ageing within our sample. These results confirm the age acceleration associated with AUD and provide the first evidence for a recovery of this effect upon abstinence from alcohol.

KEYWORDS
ageing, alcohol use disorder, epigenetic clock, relapse

1 | INTRODUCTION

All multicellular organisms undergo an ageing process that is characterized by the gradual deterioration of biological functions, which is referred to as biological ageing in contrast to chronological ageing

Tristan Zindler and Helge Frieling contributed equally.

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(corresponding to the time since birth).[1] There are different options to assess biological ageing and its molecular and cellular correlates, for example, cell cycle arrest,[2] telomere length in leukocytes,[3] secretion of specific factors and cytokines,[4] all providing important insights into the cellular signs of ageing. However, biomarkers used to measure cellular senescence are often unspecific and might fail to address the multi-level aspects of ageing. Epigenetic clocks provide a further valuable tool for monitoring the ageing process, because they result from changes in the organism at different levels. Deoxyribonucleic acid methylation (DNAm) clocks are comprehensive statistical models that use methylation levels at several specific cytosine and guanine dinucleotides (CpG) sites to calculate epigenetic age. There are two fundamental approaches to the methodology of DNAm clocks: The first aims to predict the chronological age of an individual as accurately as possible from DNAm (chronological DNAm clocks), whereas the second calculates biological age using other known indicators of biological ageing in addition to chronological age (biological DNAm clocks).[5] The term 'biological aging' will subsequently be used with respect to DNA methylation estimates. Hence, aspects of ageing independent from methylation signals will not be covered by these estimates. While the molecular details of DNAm clocks are not well understood, they nonetheless enable investigations of the biological ageing process, which also reflects a person's health and wellbeing.[6]

Alcohol use disorder (AUD) is a chronic debilitating disorder associated with a reduced life expectancy due to an increased risk of mortality, limited treatment options and a poorly defined pathophysiology.[7] Chronic exposure to alcohol over the lifespan has undisputed detrimental effects on health and well-being.[8] It can therefore be assumed that AUD has an impact on the ageing process as measured with DNAm clocks. Research on the pathophysiology of AUD has revealed various effects of genes and their functional pathways on the development, maintenance and treatment of alcohol dependence.[9] Changes in methylation state (known as epigenetic marks) are an important mechanistic link between specific genes and the behaviours that drive the course of the disease.[7]

The first evidence of an association between epigenetic ageing and AUD came from Rosen and colleagues[10] who explored the effect of excessive alcohol consumption on age acceleration, including DNA methylation levels in whole blood and tissue samples from five independent cohorts of AUD subjects and controls (CON) using a chronological DNAm clock (Horvath clock[11]). Only two of the five datasets (blood and liver tissue) showed accelerated ageing, highlighting the need for further investigations on the mechanisms of the ageing process in AUD. A subsequent study from the same group demonstrated age acceleration by 2.2 years in AUD patients compared with CON using Levine's DNAm PhenoAge,[12] a biological DNAm clock; the effect was greater in individuals with more severe AUD-associated phenotypes such as elevated liver transaminases and a higher number of heavy drinking days.

To this end, the present study examined the relationship between AUD and biological ageing in two independent cohorts. We first used the same basic methodologic approach as Luo et al.[13] to analyse the discrepancy between true chronological age and biological age measured with Levine's DNAm PhenoAge, as well as chronological DNAm age measured with the Horvath clock and Zhang's age predictor as a next-generation statistical DNAm model. There have been no studies to date on the long-term effects of relapse and potential benefits of abstinence on biological ageing. The study by Luo et al. had a cross-sectional design,[13] and the results did not specify whether differences in DNA methylation levels and epigenetic age acceleration are predisposing factors for addiction or if they are consequences of long-term alcohol use. To examine this interplay of genetic and environmental factors, we used methylation data from two time points (baseline and 12-month follow-up) previously published by our group[9] to examine how alcohol exposure and epigenetic ageing interact and whether the absence of chronic, heavy alcohol consumption in abstinent AUD patients affects the deviation between true chronological age and biological age measured with Levine's DNAm PhenoAge.

The complex interplay between genetic and environmental factors poses an additional challenge to investigate epigenetic processes. Several genetic variants from recent Genome-Wide Association Studies (GWAS) have been associated with epigenetic age acceleration, for example TERT (the catalytic subunit of telomerase),[14] DHX57 (an ATP-dependent RNA helicase) or MLST8 (a subunit of both mTORC1 and mTORC2 complexes),[15] however, without reference to AUD. It is recognized that AUD is highly polygenic. We therefore aimed to investigate in an additional exploratory approach how a polygenic risk score (PRS) for alcohol dependence derived from a large genome-wide association study assessing alcohol dependence and problematic alcohol use (PAU)[16] affects epigenetic age acceleration.

2 | MATERIAL AND METHODS

2.1 | Subjects

All subjects were recruited between 2012 and 2020 as part of a larger study (ClinicalTrials.gov identifiers: NCT01679145 and NCT02615977) investigating behavioural, genetic and neuroimaging alterations associated with reward-based learning in AUD over two funding periods. Thus, this study comprised two independent AUD cohorts from funding period 1 ($DS_{Case@BaseVSLong}$) and funding period 2 ($DS_{CaseVSCon}$). In addition, a healthy CON group was recruited in which subjects were matched with the AUD patients of $DS_{CaseVSCon}$ with respect to smoking status and several socio-economic variables (Data S1). Whole epigenome data for $DS_{Case@BaseVSLong}$ (changes in methylation levels over time) have been previously analysed and published by our group[9] (Data S2A and S2B). Briefly, whole-genome methylation patterns of individual CpG sites over time did not differ between abstinent and relapsing patients. However, there was a negative association between global mean methylation at the 12-month follow-up and alcohol consumption within our sample. All patients fulfilled the diagnostic criteria for alcohol dependence according to International Classification of Disease-10 and Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) text revision[17] for a minimum of 3 years. Patients with history of current or past

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substance use disorder (except alcohol and nicotine dependence), other major psychiatric disorders (as assessed using the computer-based Composite International Diagnostic Interview)[18,19] or neurologic disease were excluded. All subjects were free of psychotropic medication known to interact with the central nervous system for at least four half-lives (including illegal drugs and detoxification treatments as determined by a urine test). Patients were enrolled in the study shortly after detoxification (3–21 days) in both funding periods. Patients in $DS_{Case@BaseVSLong}$ were followed up for 12 months with the Alcohol Timeline Followback method.[20] Relapse during this period was defined as consumption of 60 or 40 g of alcohol on any occasion for males and females, respectively, according to World Health Organization[21] criteria of current high-risk versus low-risk consumption. Individual assessment included alcohol breath tests to validate self-reports. For $DS_{CaseVSCon}$, methylation data were available from baseline and 2-week follow-up assessments only. Smoking status was assessed with the Fagerström Test for Cigarette Dependence.[22] AUD patients and CON subjects were matched according to smoking status.

2.2 | Genotype QC and imputation

The genotype QC and imputation was performed using Rapid Imputation for COnsortias PipeLIne (RICOPILI) GWAS pipeline.[23] The subjects and SNPs passed the QC if the following parameters were satisfied: SNP missingness < 0.05 (before sample removal); subject missingness < 0.02; autosomal heterozygosity deviation (|F het| < 0.2); SNP missingness < 0.02 (after sample removal); difference in SNP missingness between cases and controls < 0.02; and SNP Hardy–Weinberg equilibrium ($p > 10e^{-6}$ in controls or $p < 10e^{-10}$ in cases). Three population outliers were excluded by visually selecting a threshold from 2D plots of principal components 1 and 2 from a principal component analysis (PCA, see Figure S6). One subject failed the above missingsness filter, resulting in $n = 185$ subjects. For relatedness testing, 65 828 autosomal SNPs which were left after linkage disequilibrium (LD) pruning ($r^2 > 0.02$) with minor allele frequency (MAF) > 0.05 were used. No pairs of subjects with an estimated proportion of IBD (PIHAT) > 0.2 were identified.

The genotype imputation was conducted using the pre-phasing/imputation stepwise approach implemented in Eagle (https://alkesgroup.broadinstitute.org/Eagle/)/MINIMAC3 (https://genome.sph.umich.edu/wiki/Minimac) with variable chunk size of 132 genomic chunks and default parameters. The imputation reference set consisted of 54 330 phased haplotypes with 36 678 882 variants from the publicly available Haplotype Reference Consortium (HRC) reference (https://ega-archive.org/datasets/EGAD00001002729).

2.3 | Quantification of DNA methylation level and QC

Data preprocessing for both datasets was performed using the respective default functions of the The Chip Analysis Methylation (ChAMP) pipeline,[24] mainly applying the default settings in the ChAMP functions. This includes the conversion of raw iDat files into beta values as well as the following six preprocessing steps that were performed as recommended by the authors of ChAMP: (1) Filter for probes with detection p value (default > 0.01). (2) Filter out probes with <3 beads in at least 5% of samples per probe. In this process, sample's failed probes' ratio above a threshold of 0.1 is regarded as failed measurements; in the $DS_{CaseVSCon}$, one sample was excluded in this preprocessing step. (3) Filter out all non-CpG probes contained in the dataset. (4) Filter out all SNP-related probes. (4) Filter out all multi-hit probes. (5) Filter out all probes located in chromosome X and Y.

Based on our previous experience with batch effects that can lower data quality and yield false positives, we used 2-week follow-up measurements as systematic twofold data of all subjects in $DS_{CaseVSCon}$ with a stratified randomized distribution of the samples. All samples were analysed with the Illumina Infinum Human MethylationEPIC BeadChip (Illumina, San Diego, CA, USA).

As the 2-week age difference is negligible for the research question at hand and because there is no systematic difference in predicted age between the two measurements ($t_{Levine}[119] = 0.01$, $p = 0.99$; Cohen's $d = 0.00$), the measurements were used as measurement repetitions and subsequently averaged for each subject in order to reduce measurement errors and to exclude systematic batch effects. One sample was excluded during QC due to showing a high fraction of failed probes. As there was no repeat measurement, the subject was completely excluded.

In order to meet the requirements for DNAm clock calculations, averaged samples were normalized for type I and II probe differences using the beta mixture quantile normalization (BMIQ) method.[25] As previously reported,[9] data for the first funding period ($DS_{Case@BaseVSLong}$) had no measurement repetitions, and significant batch effects for sample plate, chip and row were corrected using ComBat from the sva package[26] without target variables after normalizing the data by BMIQ as for $DS_{CaseVSCon}$. Additionally, a significant batch effect between $DS_{Case@BaseVSLong}$ and $DS_{CaseVSCon}$ was adjusted with ComBat using only AUD patients from $DS_{CaseVSCon}$ as a reference group again without target variables.

2.4 | Calculating DNAm age

We evaluated DNAm age using three different statistical models: the chronological Horvath clock comprising 344 CpG sites[11]; the biological Levine's DNAm PhenoAge DNAm clock model comprising 513 CpGs in 505 genes[12]; and Zhang's age predictor comprising a best linear unbiased prediction model based on 319 607 CpGs.[27] While the Horvath clock was developed for Illumina 27K and 450K arrays and not for the EPIC arrays used in the present study, Levine's DNAm PhenoAge and Zhang's age predictor were developed for EPIC array data. CpG sites not measured due to the based array or missing due to exclusion were imputed for the Horvath clock and Levine's DNAm PhenoAge. While 27 (7.6%) CpG sites were missing from the

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TABLE 1 Pearsons product-moment correlations between biological age and DNAm clock predictions

DNAm clock	r $DS_{Case@BaseVSLong}$	$r_D DS_{CaseVSCon}$
Horvath clock	0.66**	0.84**
Levines DNAm PhenoAge	0.56**	0.81**
Zhangs age predictor	0.78**	0.95**

Abbreviation: DNAm, DNA methylation.
*$p < 0.05$.
**$p < 0.01$.

Horvath clock specific instructions from the Horvath work group were applied, 12 (2.3%) missing CpG sites for Levine's DNAm PhenoAge were imputed using the mean methylation. Zhang's age predictor does not need imputation due to its high number of predictors. For the calculation of the Horvath clock and Levine's DNAm PhenoAge, the implementation in the Bioconductor package Methylclock[28] was used; for Zhang's age, original code provided by the author was utilized. We then calculated the difference between actual age and age predicted with the DNAm clock models (Δ). Pearson's product-moment correlations between these values were positive, significant, and very large for all three DNAm clocks (Table 1), supporting the assumption of sufficient sample quality and correct sample preprocessing.

2.5 | Calculating the PRS

For calculating the PRS, we used the summary statistics of the phenotypes AUD (cases/controls: 57 564/256 395) and PAU (n = 435 563) from a recent genome-wide association study from Zhou et al.[16] Both the summary statistics were LD clumped (discarding variants within 500 kb of and in $r^2 \geq 0.1$ with another more significant marker), resulting in 120 446 (in AUD) and 235 621 (in PAU) SNPs, used for scoring. Specifically, we multiply the effect size from the training data set with the number of risk alleles on each SNP, summing up over each individual to have a whole-genome PRS in PLINK v1.90b4.1.[29] A p value threshold of $p = 1$ representing the composite additive effect of all SNPs was applied, historically showing the strongest scoring results in psychiatric disorders.[30] In Data S7, we additionally show results for more stringent p value thresholds ($p = 0.05$ and $p = 0.001$).

2.6 | Single SNP analyses

For exploratory analyses only, we intended to assess the impact of ADH1B (rs1229984) and ALDH2 (rs671) on our epigenetic clocks. Both genetic variants have so far shown the strongest and most replicated association with alcohol dependence. However, due to the small sample size, the low frequency of risk alleles (MAF = 0.01) did not allow for further analyses. For replication purpose, we analysed the candidate SNP rs916264 in APOL2, previously shown to be associated with epigenetic age acceleration by Luo and colleagues.[13]

2.7 | Statistical analysis

All statistical analyses were performed in the R (4.1.1) environment and Bioconductor (3.13) framework. To evaluate differences between AUD and CON groups in terms of the deviation between DNAm age determined with the Horvath clock, Levine's DNAm PhenoAge and Zhang's age predictor and true chronological age (Δ),[27] we carried out analysis of variance (ANOVA) for the $DS_{CaseVSCon}$ dataset with group and age as independent and dependent variables, respectively. As in the study of Luo et al.,[13] we added sex as a covariate and corrected for immune cell counts obtained using the 'champ.refbase' function of the ChAMP Package in Bioconductor.[24] In a second step due to the high collinearity of the cell distribution, the variance inflation factors (VIFs) of the independent variables were calculated, and highly correlated predictors were removed accordingly.

To explore genes associated with CpGs of Levine's DNAm PhenoAge Δ, we calculated Pearson's product-moment correlations between each of the CpG sites included in the model and Δ. For this step, a false discovery rate (FDR) < 0.05 was defined as significant. We then calculated correlations between predicted DNAm PhenoAge and liver function indices (gamma-glutamyltransferase [γ-GT], aspartate aminotransferase [AST], alanine aminotransferase [ALT]). We also revisited previously published data of our $DS_{Case@BaseVSLong}$ cohort[9] to investigate the possible development of/intergroup differences in Levine's DNAm PhenoAge over a 12-month period (which had not been previously examined) with a mixed linear model to predict Levine's clock Δ, with relapsers/abstainers, sex and immune cell counts as covariates over time. Again, VIF was checked for high collinearity and collinear predictors were removed accordingly.

As for the analyses of $DS_{CaseVSCon}$, we calculated Pearson's product-moment correlations between each of the CpG sites included in the Levine's DNAm PhenoAge model and Δ, with FDR < 0.05 defined as significant.

Association between the PRS (see methods above) and phenotypes (AUD and PAU) was tested using logistic regression and adjusted for population stratification (using PC's as covariates 1–5) in $DS_{CaseVSCon}$ ($n = 116$, 85 cases/31controls, $DS_{Case@BaseVSLong}$ was excluded due to missing controls). The explained variance was estimated with Nagelkerke's R^2 (NKr^2) by comparing scores generated from a full model (containing covariates and PRS) and a reduced model (covariates only).

Variance in Levine's DNAm PhenoAge Δ explained by PRS for AUD and PAU was then tested using linear regression analysis in $DS_{CaseVSCon}$ and $DS_{Case@BaseVSLong}$ ($n = 185$). Similar to previous analysis, the analysis was also corrected for population stratification using PCs (1 to 5). The beta coefficients and adjusted R^2 were estimated.

Association between the SNP rs916264 in APOL2 and Levine's DNAm PhenoAge Δ was tested using PLINK (v1.90b4.1) based linear regression analysis. The analysis was adjusted for population stratification using PCs (1 to 5) in $DS_{CaseVSCon}$ and $DS_{Case@BaseVSLong}$ ($n = 171$ subjects, 14 subjects had to be excluded due to missing genotype, MAF = 0.19).

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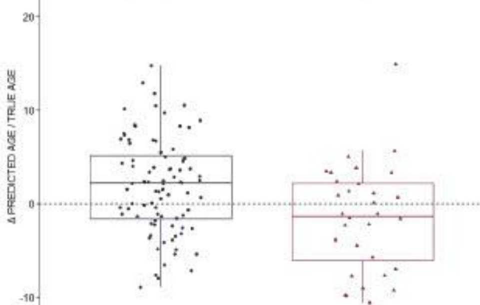

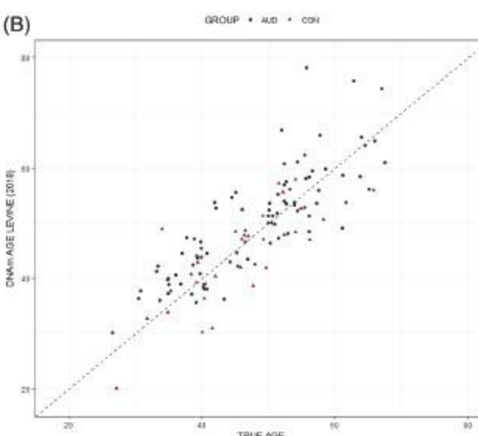

FIGURE 1 (A) Boxplot of Levines DNAm PhenoAge Δ in alcohol use disorder (AUD) patients and control (CON) subjects for $DS_{CaseVSCon}$. (B) Scatterplot of Levines DNAm PhenoAge versus true chronological age (Δ) for $DS_{CaseVSCon}$

3 | RESULTS

ANOVA for differences between AUD and CON groups in the $DS_{CaseVSCon}$ with regard to age Δ did not show a significant effect for Horvath's DNAm clock (F[1, 112] = 0.43, p = 0.51; η^2 [partial] = 0.00) (Data S3A). The analysis for Zhang's age predictor yielded comparable results (F[1, 112] = 0.40, p = 0.53; η^2 [partial] = 0.00) (Data S3B). However, Levine's DNAm PhenoAge showed a significant medium size effect (F[1, 112] = 11.10, p = 0.00; η^2 [partial] = 0.09) (Figure 1A, B and Table 2). AUD patients showed a mean (±SD) accelerated ageing of 2.94 (±5.69) years compared with their chronological age whereas in CON subjects, and the mean (±SD) Δ was −0.66 (5.7) years, yielding a mean difference of 3.6 years between the two groups.

Pearson's product-moment correlations in the $DS_{CaseVSCon}$ revealed four CpG sites with an FDR-corrected significant influence of Δ (Data S4): cg05851163 located in the 5′ untranslated region of the dermatan sulphate epimerase-like (*DSEL*) gene (r = 0.36, 95% confidence interval [CI]: 0.20–0.51, t[118] = 4.25, p_{FDR} < 0.05); cg23668631 located in the TSS1500 shore of the calcium/calmodulin dependent protein kinase kinase 1 (*CAMKK1*) gene (r = −0.35, 95% CI: −0.50 to −0.18, t[118] = −4.03, p_{FDR} < 0.05); cg18468844 located in the TSS1500 OpenSea of the platelet-activating factor receptor (*PTAFR*) gene (r = −0.35, 95% CI: −0.49 to −0.18, t[118] = −4.00, p_{FDR} < 0.05); and cg01211097 located in the TSS1500 shore of the ubiquitin-specific peptidase 10 (*USP10*) gene (r = −0.34, 95% CI: −0.49 to −0.17, t[118] = −3.87, p < 0.05). On the other hand, Pearson's product-moment correlations between predicted Levine's DNAm PhenoAge and liver function indices were nonsignificant (γ-GT: r = −0.01, t[114] = −0.06, p = 0.95; AST: r = 0.07, t[114] = 0.70, p = 0.49; and ALT: r = −0.04, t[114] = −0.41, p = 0.68).

The mixed linear model analysis of $DS_{Case@BaseVSLong}$ to predict the change in Levine's DNAm PhenoAge Δ over time showed a high total explanatory power (conditional R^2 = 0.86) with a significant interaction effect between time and group (β = 3.65, t[126] = 2.98, p < 0.01). While relapsers exhibited accelerated ageing by a mean (±SD) of 2.69 (±7.49) years during the 12-month period, abstainers were by a mean (±SD) of 4.79 (7.72) years below their expected chronological age (Figure 2 and Table 3). Pearson's product-moment correlation analysis of $DS_{Case@BaseVSLong}$ revealed only one CpG site—cg09809672 located in the TSS1500 shore of the ectodysplasin; a receptor associated death domain (*EDARADD*) gene—that was significantly associated with Δ after FDR correction (r = 0.35, 95% CI: 0.19 to 0.49, t[130] = 4.24, p_{FDR} < 0.05) (Data S5).

The calculated PRS did show a significant association with alcohol dependence at p-value threshold p = 1.0 for both AUD (NKr^2 = 0.0497, p = 0.0457) and PAU (NKr^2 = 0.1138, p = 0.0022). For p-value thresholds p = 0.05 and p = 0.001, see Data S7.

There was no significant association between PRS and Levine's DNAm PhenoAge Δ. There was also no significant association between the SNP rs916264 in APOL2 and Levine's DNAm PhenoAge Δ.

4 | DISCUSSION

Only two studies to date have investigated epigenetic age acceleration in patients with AUD.[10,13] Our results confirm the earlier finding that AUD is associated with accelerated ageing, albeit to an even greater extent than previously reported (4.7 vs. 2.2 years). However, we are the first to report the potentially recovering effects of long-term abstinence from alcohol and the potential influence of polygenic risk on epigenetic ageing.

In this study, we investigated epigenetic ageing in two patient cohorts. Results from $DS_{CaseVSCon}$ supported those of Luo et al.,[13] revealing a difference between AUD patients and CON subjects in terms of the discrepancy between epigenetic age and true

TABLE 2 $DS_{CaseVSCon}$—Levines DNAm PhenoAge Δ analysis of variance

Parameter	Sum of squares	df	Mean square	F	p	Partial η^2	VIF
AUD/CON	304.76	1	304.76	11.10	0.00	0.09	1.05
Sex	0.97	1	0.97	0.04	0.85	0.00	1.15
CD8T	49.28	1	49.28	1.79	0.18	0.02	1.09
CD4T	586.62	1	586.62	21.36	0.00	0.16	1.21
NK cells	20.55	1	20.55	0.75	0.39	0.01	1.13
B cells	35.18	1	35.18	1.28	0.26	0.01	1.19
Monocytes	55.78	1	55.78	2.03	0.16	0.02	1.27
Residuals	3075.67	112.00	27.46				

Note: Granulocytes were removed as predictor due to excessive collinearity with the other predictors. Variance inflation factors (VIFs) of the remaining predictors <1.5.

Abbreviations: AUD, alcohol use disorder patients; B cells, B lymphocytes; CD8T, CD8+ cytotoxic T lymphocytes; CD4T, CD4+ cytotoxic T lymphocytes; CON, control subjects; DNAm, DNA methylation; NK, natural killer.

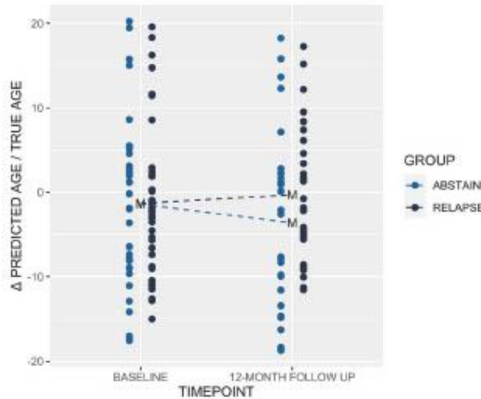

FIGURE 2 Interaction diagram for abstainers and relapsers in DSCase@BaseVSLong (M = mean). Differences in Levines DNAm PhenoAge Δ at baseline and at the 12-month follow-up were observed

chronological age. In line with the previous findings, differences in age acceleration were detected with Levine's DNAm PhenoAge only and not with chronological DNAm clocks (Horvath clock and Zhang's age predictor). This is expected given the variations in methodologic approaches for the two types of DNAm clocks. Chronological clocks are designed to predict true chronological age in very large heterogeneous samples[5]; possible biological markers are not included, as these large training samples include people with preexisting conditions, and therefore, only CpG sites that change uniformly over time independent of environmental influences are considered. Also, Zhang estimate is tailored to estimate chronological age as precisely as possible and explicitly not to be affected by mortality nor cell composition in ideal case. In contrast, biological DNAm clocks (including Levine's clock) favour the inclusion of CpGs that are altered in response to environmental factors. Thus, our results support the previous assertion[13] that significant results can only be expected for DNAm clocks measuring biological age. Contrary to the results of Luo et al., we found no association between liver transaminases and predicted Levine DNAm PhenoAge, which underscores the fact that the molecular mechanisms of epigenetic clocks are complex and poorly understood.

Our second cohort of relapsing and abstaining patients ($DS_{Case@BaseVSLong}$) yielded conflicting findings. The difference in predicted age Δ of 7.5 years between relapsers and abstainers from baseline to the 12-month follow-up was highly significant, indicating that abstinence from alcohol has beneficial effects on biological ageing in AUD patients. However, our results did not show an absolute positive age difference between predicted and actual age at baseline; an absolute age deviation only became apparent for relapsing patients at the 12-month follow-up. A possible reason for these unexpected results is the lower quality of measurements in the $DS_{Case@BaseVSLong}$ sample compared with the repeated measurements for $DS_{CaseVSCon}$, which allowed necessary corrections and is also reflected in the lower correlations between the Horvath clock or Zhang's age predictor and true chronological age (Table 1). Given this limitation of our study, replication of our results is necessary. Another limitation is that in $DS_{CaseVSCon}$ and at baseline in $DS_{Case@BaseVSLong}$, all AUD patients had been detoxified from alcohol for at least 3 but up to 21 days before measurements. Moreover, all patients had abstained from alcohol for at least 30 days until the first relapse and had had AUD (according to DSM-IV criteria) for a minimum of 3 years before enrollment. Given the clinical course of abstainers in $DS_{Case@BaseVSLong}$ over the 12-month period, we suspect that the actual epigenetic age difference between actively drinking AUD patients and CON subjects is even greater than what was shown by our analysis. It would therefore be highly valuable to repeat these experiments with active drinkers prior to their detoxification.

Previous studies have reported associations between epigenetic ageing and leading causes of death and disease burden.[31] The consumption of alcohol is linked to epigenetic changes that may contribute to these long-term consequences.[32,33] Several candidate genes associated with AUD were identified in a large-scale epigenome-wide

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TABLE 3 $DS_{Case@Base VSLong}$—Levines DNAm PhenoAge Δ mixed linear model

Parameter	Coefficient	95% CI		*t*	*df*	*p*	Std. coef.	Fit	VIF
(Intercept)	15.66	7.17	24.16	3.65	126	0.00	0.03		
Group	−0.53	−4.39	3.33	−0.27	126	0.79	−0.06		1.13
Time	−1.92	−3.69	−0.15	−2.15	126	0.03	−0.22		2.49
Sex	−6.59	−11.79	−1.40	−2.51	126	0.01	−0.27		1.02
CD8T	−12.42	−44.19	19.34	−0.77	126	0.44	−0.04		1.89
CD4T	−14.56	−43.54	14.42	−0.99	126	0.32	−0.07		3.71
NK cells	−21.02	−47.67	5.63	−1.56	126	0.12	−0.06		1.17
B cells	−138.09	−189.70	−86.48	−5.30	126	0.00	−0.28		2.26
Monocytes	−9.80	−43.01	23.41	−0.58	126	0.56	−0.03		1.62
Group × time	3.65	1.23	6.07	2.98	126	0.00	0.43		2.69
R^2 (conditional)								0.86	
R^2 (marginal)								0.21	

Note: Granulocytes were removed as predictor due to excessive collinearity with the other predictors.
Abbreviations: B cells, B lymphocytes; CD8T, CD8+ cytotoxic T lymphocytes; CD4T, CD4+ cytotoxic T lymphocytes; CI, confidence interval; DNAm, DNA methylation; NK, natural killer; Std. coef., standardized coefficient; VIF, variance inflation factor of the remaining predictors <1.5.

study using a cross-tissue/cross-phenotype approach.[34] Our exploratory investigation of the (epi)genetic background of age deviations in AUD with a series of correlations for every individual CpG in the Levine DNAm PhenoAge model identified significant and large effects for CpG sites associated with four genes—namely, *DSEL*, *CAMKK1*, *PTAFR* and *USP10*.

Polygenic risk is a potential indicator of genetic liability in AUD; thus, we intended to explore its effect on the epigenetic ageing process. Both PRS scores based on Zhou et al.[16] showed a significant association with alcohol dependence. However, we did not find an effect of the PRS on age acceleration, an exploratory single SNP analyses of ADH1B (rs1229984) and ALDH2 (rs671) on our epigenetic clocks failed due to the low number of risk alleles. We could also not replicate the previously reported association of epigenetic ageing with APOL2 (rs916264).[13] With respect to sample size and construction of the Levine DNAm PhenoAge,[12] which has been designed with a certain robustness against genetic influences, a zero-finding is comprehensible. However, with respect to the fact that the interplay between genetic variables and epigenetic ageing remains poorly understood,[35] we suggest to apply the same approach in a bigger sample.

In the $DS_{Case@Base VSLong}$, we identified one CpG associated with accelerated epigenetic ageing in relapsers compared with abstainers located in the shore of the *EDARADD* gene. As we limited the correlation analyses to differences in age acceleration detected using Levine clock CpG sites, it is not surprising that the genes with the highest correlations are related to metabolic functions and cell regeneration. While these analyses are limited by the number of CpGs included in the Levine DNAm PhenoAge model, the significant CpG sites or their associated genes are of interest for subsequent investigations on the (epi)genetic background of accelerated ageing in AUD. Given the limited size of our two cohorts, our results should be considered as preliminary. In the genome-wide association study carried out by Luo et al., a single nucleotide polymorphism in the apolipoprotein L2 (*APOL2*) gene was implicated in accelerated epigenetic ageing.[13] However, the study had a cross-sectional design and did not consider the effects of long-term abstinence and relapse. On the other hand, tissue-specific associations between alcohol dependence and epigenetic age were observed with the Horvath and Hannum clocks,[10] although these are considered less effective in reflecting environmental influences on the ageing process.

In summary, DNAm clocks serve as valuable biomarkers of biological ageing. It will be interesting to define subgroups of AUD patients that are more prone to epigenetic age acceleration or more responsive to the positive effects of abstinence. Genetic determinants are one of many possible confounders for associations with changes in transaminases and other tissue-specific effects. Subsequently, the complex interplay between genetic and environmental factors poses an additional challenge to interpret the data and determine causal relationships.

Epigenetic measures of ageing have potential utility in clinical settings as a complement to gold-standard methods for disease assessment and management.[31] For example, our results may be used in clinical practice to motivate patients with AUD to take the difficult path of long-term sobriety and associated alcohol withdrawal, as it can lead to measurable biological recovery. Finally, DNAm clocks may provide novel targets for pharmaceutical interventions, as demonstrated in other disease such as bipolar disorder[36] and schizophrenia.[37]

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICS APPROVAL

Ethics approval for the study was obtained from the ethics committee of Charité-Universitätsmedizin Berlin (EA1/157/11) and Universitätsklinikum Dresden (EK228072012). Participants received a monetary compensation of 10 €/hour for study participation.

AUTHOR CONTRIBUTIONS

TZ and EF conceived the study; TZ, EF, HF, LF, IV, AN and HW drafted the manuscript; TZ, EF, HF, LF, IV, AN, SA, SR and HW analysed and interpreted the data; SA und SR calculated the PRS, and TZ created the figures; HF supervised the study; and TZ, EF, HF, LF, IV, AN, SA, SR and HW revised the manuscript for important intellectual content. All authors contributed to manuscript revision, and read and approved the submitted version.

DATA AVAILABILITY STATEMENT

This manuscript contains previously unpublished data. The name of the repository and accession number(s) are not available due to patient confidentiality and participant privacy. Methylation array data can simultaneously identify individuals and convey protected health information 43434343434343. Patients did not provide written, informed consent for the publication of individual methylation profiles. Requests to access the datasets should be directed to Eva Friedel (eva.friedel@charite.de).

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2.5 Der Einfluss von Batch-Korrekturen auf falsch positive Befunde bei epigenetischen Untersuchungen

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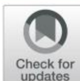
Simulating ComBat: how batch correction can lead to the systematic introduction of false positive results in DNA methylation microarray studies



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Abstract

Background: Systematic technical effects—also called batch effects—are a considerable challenge when analyzing DNA methylation (DNAm) microarray data, because they can lead to false results when confounded with the variable of interest. Methods to correct these batch effects are error-prone, as previous findings have shown.

Results: Here, we demonstrate how using the R function ComBat to correct simulated Infinium HumanMethylation450 BeadChip (450 K) and Infinium MethylationEPIC BeadChip Kit (EPIC) DNAm data can lead to a large number of false positive results under certain conditions. We further provide a detailed assessment of the consequences for the highly relevant problem of *p*-value inflation with subsequent false positive findings after application of the frequently used ComBat method. Using ComBat to correct for batch effects in randomly generated samples produced alarming numbers of false discovery rate (FDR) and Bonferroni-corrected (BF) false positive results in unbalanced as well as in balanced sample distributions in terms of the relation between the outcome of interest variable and the technical position of the sample during the probe measurement. Both sample size and number of batch factors (e.g. number of *chips*) were systematically simulated to assess the probability of false positive findings. The effect of sample size was simulated using $n = 48$ up to $n = 768$ randomly generated samples. Increasing the number of corrected factors led to an exponential increase in the number of false positive signals. Increasing the number of samples reduced, but did not completely prevent, this effect.

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Conclusions: Using the approach described, we demonstrate, that using ComBat for batch correction in DNAm data can lead to false positive results under certain conditions and sample distributions. Our results are thus contrary to previous publications, considering a balanced sample distribution as unproblematic when using ComBat. We do not claim completeness in terms of reporting all technical conditions and possible solutions of the occurring problems as we approach the problem from a clinician's perspective and not from that of a computer scientist. With our approach of simulating data, we provide readers with a simple method to assess the probability of false positive findings in DNAm microarray data analysis pipelines.

Keywords: DNA methylation, Simulation, EPIC array, 450 K array, Illumina, Batch effects, ComBat

Background

In the last two decades, the field of epigenetics has opened up new perspectives on complex medical questions [1–3]. DNA methylation (DNAm) is assumed to be modulated both by heritable factors [4] and by environmental conditions [5, 6]. DNAm has received considerable attention in the field of epigenetics research as a source of potential disease-related biomarkers and as one of the missing translational links between nature and nurture [7, 8]. Despite numerous successful findings of disease related biomarkers [2, 8, 9] and the enthusiasm within this novel and promising field of study, it is important to obtain a more nuanced picture of the biological processes related to the biomarkers being assessed, and to develop a more comprehensive and commonly accepted framework for their analysis. At present, researchers in this field have to cope with new technological possibilities and an insufficient understanding of the methylome at the same time [4, 10, 11].

Microarray platforms such as the Infinium BeadChips have played a big role in making cost-effective genome-wide measurements of DNAm possible. However, while these platforms can be used to investigate methylation sites across the genome, they assay less than 4% of the CpG methylation sites in the human genome [12] and subsequently confront researchers with numerous study design pitfalls: While the large number of measurement points (up to 850,000 on the Infinium MethylationEPIC BeadChip Kit) results in "big data", the number of measured samples often remains relatively small. This disproportionate number of samples relative to the number of measurement points represents a massive challenge for subsequent statistical analyses [13]. Another challenge is caused by so-called "batch effects", which include a series of effects caused by technical variability due to the time, place, and materials used (*batches*). Batch effects are almost impossible to avoid: This is because the individual samples are measured on *chips* - in case of an Infinium MethylationEPIC BeadChip Kit array - with 8 individual samples on each *chip*. These samples are arranged in *rows* at distinct positions (*sample wells*) on the *chips*. The *chips* in turn are mounted on a plate (*samples plate*) that has space for 12 chips (96 samples). The term "batch effect" is referring to the systematic measurement errors between the *sample wells* in *rows*, the *chips* and the *samples plates*. Additional batch effects can be caused for instance

by different times of measurement, different sample handling, different sites in multicenter studies and possible undiscovered effects due to the novelty of the technologies [14]. Because they are linked to the materials used to measure the samples, the batch factor number increases linearly with each additional sample (e.g. for each *chip*). Batch effects can dramatically reduce the accuracy of measurements [14] and can produce false positive effects if the sample distribution during the measurements is not uniformly distributed with regards to the *outcome of interest* (*unbalanced sample* – Fig. 1a). This can lead to significant group differences caused by measurement errors being wrongly attributed to the outcome of interest [15, 16].

To make matters even more complicated, there is no validated and commonly accepted framework for the analysis of genome-wide epigenetic data. Based on this lack of consistency in data analyses, there is little empirical knowledge about what outcomes to expect, which makes it extremely difficult for researchers to assess and replicate new findings [11].

With the R package *ChAMP* [17], an important attempt has been made to integrate the different steps necessary for the analyses of Infinium HumanMethylation450 BeadChip (450 K) and Infinium MethylationEPIC BeadChip Kit (EPIC) data into a comprehensive analysis pipeline. *ChAMP* addresses the problem of batch effect correction with the ComBat method [18], which uses an empirical Bayesian approach to avoid overcorrection—a critical feature to use with small sample sizes. ComBat has been heavily praised as being the most effective method for counteracting batch effects [19] when they are known [20]. It is implemented in the *sva* package [21], which itself is integrated into the *ChAMP* pipeline.

While ChAMP offers only a few settings for ComBat, the direct sva function call offers more options for using ComBat. Among other options there is the possibility to

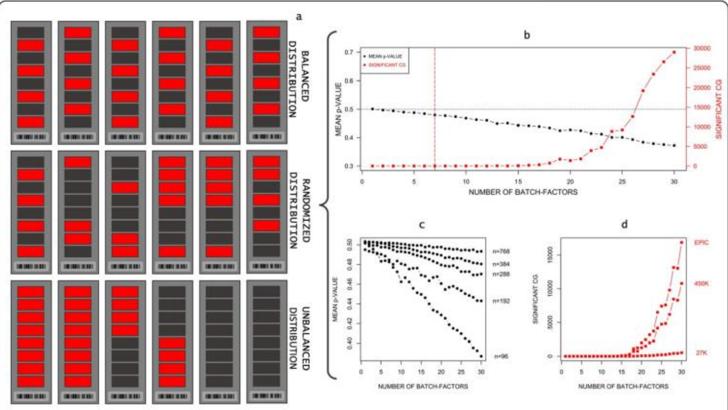

Fig. 1 a Different case and control sample distributions of $n = 48$ on six chips (red, case; dark grey, control). **b** Systematic increase of the number of factor levels for one corrected factor. The vertical red line shows the factor level with the first false FDR-significant result. **c** Mean p-value decrease for different sample sizes in combination with a systematic increase of the number of factor levels. **d** Number of FDR-significant CpG sites with a systematic increase of factor levels for the different Illumina arrays

specify a model matrix being used *("mod")* with covariates or a *outcome of interest* variable besides the batch factors. Champ automatically passes the *outcome of interest variable* to ComBat.

In an earlier study with 69 subjects, we found a high number of CpG-sites with significantly differing methylation levels between two groups using the *ChAMP* analysis pipeline. When we used the ComBat method for batch correction provided in *ChAMP* to correct for *row* and *chip batches*, we became aware of previously reported problems with this approach: Two cautionary case reports were published in 2018 [22] and in 2014 [23] reporting problems experienced when ComBat was used to correct for batch effects in *450 K* data. After applying ComBat to adjust for a non-biological signal, Prince and Robinson reported that roughly 10 k to 20 k significant CpG sites (false discovery rate (FDR) < 0.05) emerge. These sites had not been present before ComBat correction and were not replicable under a revised analysis design and use of ComBat. In an analogous way in an earlier report, Buhule found 25 k differentially methylated CpG-sites (FDR < 0.05) before batch correction, but around 100 k significant CpG-sites after correction. Both studies analyzed data from pilot studies with limited sample sizes (n = 30 in [22]; and n = 92 in [23]) and used the Illumina *450 K* array. While these case reports should warn researchers from blindly applying ComBat or similar methods to remove batch effects from an unbalanced sample, it remains unclear to what extent this effect occurs and how this effect relates to varying sample sizes and to the different Illumina arrays. Furthermore, as Price and Robinson aptly stated: "[…] it is alarming that thousands of false discoveries might have been claimed if the analysis had been limited to standard processing pipelines".

In this report, we aim to provide a detailed assessment of consequences of applying the frequently used ComBat method for the highly relevant problem of p-value inflation, which results in subsequent false positive findings [18]. We use this approach as a hands-on example of how to systematically investigate the methods used in multi-step microarray platform analysis pipelines.

We further provide researchers with a simulation-based quantification of ComBat-introduced false signal induction under various configurations, and a simple tool to assess the probability of false positive findings in DNAm microarray data analysis pipelines.

Methods

As clinicians, we have become increasingly aware of the problems associated with using multi-step analysis pipelines without being able to realistically verify the source code of all the methods used. This has led us to the conclusion that a simulation should be performed to investigate these problems, without the interference of possible real biological signals. Our simulation was created using the packages discussed, namely *sva* [14] and *ChAMP* [17], which are implemented in the R (3.6.1) and Bioconductor (3.9) environments on Windows 10.0.18362.

The basis for our simulation were 758,289 mean and standard deviation values from probes on an *EPIC* array, based on data from 69 patients collected in our laboratory.[1]

Based on this data, random numbers were generated from a normal distribution using the "rnorm" function with the "Mersenne-Twister" algorithm [24] to generate

[1]More information about laboratory data DNA methylation quantification and quality control is available in Supplementary Document - Section A

simulated methylation beta values for every CpG site with a mean and standard deviation corresponding to the natural CpG sites.[2] Data from our laboratory were not used in any further analyses.

The data generated in this way cannot contain any signal, and therefore cannot contain any batch effect either. In our opinion, this is the best way to investigate the effect of ComBat on the data. The alternative approach of investigating the effects of ComBat on real data would always carry the risk of producing true positive results, which would therefore make an accurate analysis of false positive findings much more difficult.

The resulting data from our simulation share many properties with their biological CpG counterparts, such as the distribution of differences in type-I and type-II CpG-sites which occur in Illumina arrays for technical reasons [25].[3] This enabled us to follow the *ChAMP* pipeline as planned and in the next step to normalize the data for type-I and type-II differences, as required by the manual for the *sva* package [21] using the beta-mixture quantile normalization (BMIQ) method [26]. BMIQ serves as an intra-sample normalization procedure, correcting the deviation of type-II probe values, and is implemented within the "champ.norm" function.

Because it can be argued that a test without batch effects is not very naturalistic and offers the risk of being a special case, we added in a second step artificial batch effects to our data. For this purpose, we based the batch effect simulation on the preliminary work of Wen Bin Goh and Wong [15] by adding randomly generated effects between 1% and – 1% to the previously generated data. This intended to simulate a simple technical brightness offset during measurement. Each sample was added with the respective systematic errors for *row* and each *chip*.

Following this, the basic effects of *balanced*, *unbalanced*, and *random sample distributions* were tested on 48 randomly generated samples. Testing was applied with and without batch effects and with 100 simulation repetitions each. This part of our analysis set the starting point for our simulations. It was performed using the "champ.runCombat" function, as well as with a direct call to the function provided within in the *sva* package, while the subsequent analyses used the "champ.runCombat" function. The function provided by the *sva* package was additionally executed with and without the use of a "model matrix for *outcome of interest* and other covariates besides batch" (*mod*) option [14].

After this basic analysis, effects of ComBat were systematically tested for *randomly* distributed samples under varying conditions. We consider the *balanced* and *unbalanced sample distributions* to be the exceptions, with the random sample distribution the normal case for most studies. Accordingly, the functions provided by the *ChAMP* pipeline were encapsulated in simulation loops.

At first, $n = 96$ simulated samples—corresponding to size of one *samples plate*—were used to gradually increase the number of ComBat-processed factor levels. In a second simulation, the number of batch factors was increased with two equal-sized randomly distributed factor levels each. In a second simulation, we investigated the effect of increasing the sample size in five steps from $n = 96$ (one *samples plate* / 8 *chips*) up to

[2]Data and the R code for the simulation are available as a Supplementary Material (MethylationSimulationScript.ZIP)

[3]A more thorough description of the simulation and the resulting simulated value distributions can be found in Supplementary Document – Section B

n = 768 samples. After these simulations with respect to the Illumina *EPIC* array containing 850,000 CpG-sites, the effects of ComBat on older and smaller arrays (Illumina Infinium HumanMethylation27 BeadChip (*27 K*) and *450 K*) with n = 96 samples were examined. *27 K* and *450 K* data were created by sampling from the *EPIC* data accordingly.

As a final analysis a simulated sample of n = 48 normal distributed probes were enriched with 2000 CpG sites with significant/trend wise (uncorrected) group differences with respective p-values from 10^{-1} to 10^{-20}. Following this step, the Dataset again was added with systematic batch effects for *row* and *chip*.

In all simulations, the average p-value and the number of significant CpG-sites after FDR correction were analyzed with the "champ. DMP" function. This function uses the *limma* package to calculate the p-value for differential methylation by applying a linear model. If ComBat batch correction works as expected, neither an increase of significant CpG-sites nor a deviation from a mean p-value of 0.5 would be expected for the normally distributed data without simulated batch effects. In the first simulation with and without simulated batch effects, the p-value distribution was additionally analyzed using Q-Q plots and using the genomic inflation factor λ for all corresponding sample distributions and ComBat variants. The genomic inflation factor λ is defined as the ratio between the medians of the ComBat-corrected distribution of the test statistic and the expected statistic without ComBat correction. This therefore quantifies the magnitude of the bulk inflation and of the excess false positive rate [27].

In order to validate the results of our systematic simulations relative to real research results, we simulated the preliminary results of two studies [22, 23] according to their factor structure using our *ChAMP*-based simulation.

Results

While researchers have previously suspected that unwanted effects would only occur in unbalanced study designs [22, 23, 28], our simulation of n = 48 samples over 100 simulation runs showed[4] a considerable undesired effect of ComBat on all sample distributions (Fig. 1a). The test statistics were examined using the mean p-value and λ, which both showed considerable deviations from their expected values (expected: mean p = 0.5; $\lambda = 1$) for all variants and distributions.[5] The smallest deviation from the expected values was obtained by simulating the random sample distribution in conjunction with ComBat, which was implemented by the *sva* package without the *"mod"* option enabled (mean $p = 0.49$; $\lambda = 1.11$). In accordance with the previously published reports, the *unbalanced* sample distribution (Fig. 1a) showed the highest simulated deviation using the ComBat implementation of *ChAMP* (mean $p = 0.31$; $\lambda = 3.26$), resulting in $M_{\mathrm{FDR}} = 109,097$ FDR–significant and $M_{\mathrm{BF}} = 1293.38$ Bonferroni-corrected (BF) CpG sites. In comparison to these results, the sampled random distribution (Fig. 1a) showed much smaller distortion of the test statistics (mean $p = 0.41$; $\lambda = 1.81$), resulting in $M_{\mathrm{FDR}} = 5597.48$ FDR-significant and $M_{\mathrm{BF}} = 23.81$ BF false significant sites. As expected, the balanced sample design showed the smallest p-value reduction (mean $p = 0.41$; $\lambda = 1.72$). However, contrary to previous reports, the observed impact of ComBat on the sample was enough to yield $M_{\mathrm{FDR}} = 3159.64$ FDR ($M_{\mathrm{BF}} = 16.06$) false significant CpG sites.

[4]Supplementary Table 1 provides a full report of the simulation results
[5]Supplementary Figure 1 provides Q-Q plots for test statistics

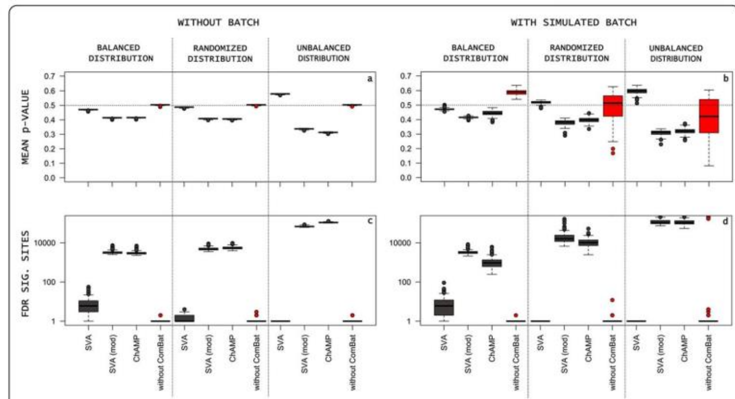

Fig. 2 "mod" refers to a model matrix for the outcome of interest. **a** Boxplots of *p*-value distributions without added batch effects and under various conditions. Dotted line indicates expected mean *p*-value of 0.5. **b** Boxplots of *p*-value distributions with simulated batch effects and under various conditions. **c** Boxplots of FDR false significant sites without batch. **d** Boxplots of FDR false significant sites without batch. Boxplots for "*without ComBat*" are indicating the false positive sites due to uncorrected batch effects for the respective sample distributions

These effects could be replicated in comparable sizes for the *sva* package implementation of the ComBat function with the *"mod"* option enabled[4]. While all the simulated distortions observed were in the direction of an enhanced significance, the opposite effect was observed in the *sva* package implementation using the unbalanced sample distribution without the target variable option (mean $p = 0.58$; $\lambda = 0.41$). While this substantial deviation did not generate any false significant CpG sites, it could in theory result in a substantially elevated number of false negative sites. While all the simulated test statistics showed undesirable deviations, the resulting number of false significant sites was negligible for the random and unbalanced sample distributions when the *sva* package implementation without the *"mod"* option was used. Any other combination resulted in a substantially elevated number of false significant CpG sites. It is important to note that for this variant of ComBat, the balanced sample distribution generated a considerable number of false significant sites, with up to 55 FDR-significant sites.

The corresponding simulation with added simulated batch effects[6] showed the same trends concerning the relative distortion of the mean *p*-values (Fig. 2c). In addition to investigating the effect of ComBat, Fig. 2d (red) shows the potential for false positive results due to uncorrected batch effects: The uncorrected, unbalanced simulation with batch effects showed a very high potential of false FDR significant CpG ($M = 41{,}912.5$, $SD = 111{,}629.7$) due to batch effects.

A subsequent systematic investigation of ComBat-introduced effects (based on the ComBat implementation in *ChAMP*) showed a considerable effect of ComBat on the results—the mean *p*-value decreased as the number of batch factor levels increased

[6]Supplementary Table 2 provides a full report of results from the simulation with added batch effects

(Fig. 1b) and as the number of batch factors increased. The first FDR- and BF-significant CpG sites appear at a batch factor number of 7 and these increase exponentially in number with increasing factor levels from this point on. Using this approach, we are able to demonstrate that even a small number of factor levels or factors can lead to a considerable number of false positive FDR- or even BF-corrected results. The number of such FDR-corrected significant sites increases exponentially with the number of factor levels.

A systematic simulation of the influence of sample size (Table 1) on the reported effects showed that an increase in sample size reduced the decrease in mean *p*-value, but that this effect was nevertheless detectable in every sample size (Fig. 1c). Using correction for the row and chip with a random sample distribution resulted in 118 FDR-significant sites at a sample size of $n = 768$. For the balanced sample, this resulted in a consistent reduction in the number of false positive sites with increasing sample size, whereby false positives were not observed until the sample size reached $n = 786$. For the unbalanced sample distribution, however, the exact opposite process was observed, with a steady worsening of the deviation of the test statistic, resulting in an extreme inflation of the number of significant sites, with up to 500 K FDR-significant sites.

Comparison between *27 K*, *450 K*, and *EPIC* results showed a similar picture for the *EPIC* and *450 K* array sizes, but a considerably smaller effect on *27 K* array size (Fig. 1d).

A close examination of the false significant CpG sites introduced showed that neither extreme values (close to 0 or 1) nor outliers played a role in the creation of false significant CpG sites. It was primarily the CpG sites showing randomly assigned high group differences before ComBat correction that were affected. A closer look at the individual values showed that all values were slightly changed and that this change was not evenly distributed across both groups.[7]

The analysis of simulated significant/trend wise (uncorrected) group differences showed that ComBat in all his variants has a positive effect on the detection of significant differences (Fig. 3). An exception is the application of Combat (SVA without *mod*) to an *unbalanced* sample. Here the detection of significant sites is strongly deteriorated, which is in strong correspondence with the results of the previous simulations (Fig. 2a), where an increased mean *p*-value has been shown for this use case.

Next, we validated our findings with a simulation based on data from real studies: Price and Robinson [22] found 9612 differentially methylated CpG sites in their first comparison, and 19,214 sites in their second comparison. A simulated result with 50 runs yielded $M = 11{,}270.74$ ($SD = 221.70$) differentially methylated CpG sites for the first comparison, and $M = 23{,}477.06$ ($SD = 382.67$) for the second comparison.

For the factor structure of Buhule et al. [23], our simulation with 50 runs predicted $M = 103{,}188.70$ ($SD = 481.84$) differentially methylated CpG sites, while 94,191 false significant CpG sites were reported in the study.

[7]Supplementary Figure 2 provides a scatterplot of a false significant CpG site before and after ComBat correction.

Table 1 ComBat (ChAMP) applied to increasing sample sizes

sample size	batch factors	balanced				random				unbalanced			
		mean-*p*	λ^+	FDR*	BF**	mean-*p*	λ^+	FDR*	BF**	mean-*p*	λ^+	FDR*	BF**
$n = 48$	8 rows + 6 chips	0.4131827	1.720126	3540	15	0.3735538	1.990719	27,057	132	0.2872	3.880235	156,084	2803
$n = 96$	8 rows + 12 chips	0.4395739	1.465350	265	4	0.4254060	1.538365	1412	19	0.2609	4.999723	214,009	11,048
$n = 144$	8 rows + 18 chips	0.4487983	1.391675	84	2	0.4308005	1.453891	1218	8	0.2335	6.362690	273,773	25,522
$n = 192$	8 rows + 24 chips	0.4533012	1.355385	47	6	0.4438228	1.428494	218	2	0.2133315	7.746447	318,618	42,427
$n = 240$	8 rows + 30 chips	0.4565824	1.334509	27	1	0.4424769	1.419485	226	4	0.1978271	9.738140	352,805	59,949
$n = 288$	8 rows + 36 chips	0.4588096	1.320805	9	2	0.4443718	1.406706	181	4	0.1854536	11.319961	379,740	77,903
$n = 336$	8 rows + 42 chips	0.4580821	1.310937	11	2	0.4453380	1.388399	144	4	0.1733198	13.128076	406,856	97,847
$n = 384$	8 rows + 48 chips	0.4589804	1.303460	28	1	0.4512571	1.370874	41	3	0.1574975	15.871418	440,828	127,703
$n = 432$	8 rows + 54 chips	0.4609161	1.296584	2	0	0.4532645	1.360342	84	4	0.1565864	16.385741	442,872	130,896
$n = 480$	8 rows + 60 chips	0.4629813	1.290615	0	0	0.4545829	1.352150	77	3	0.1495949	18.068155	457,389	146,684
$n = 528$	8 rows + 66 chips	0.4640470	1.286172	4	1	0.4541513	1.347141	89	4	0.1444482	19.435062	469,246	159,929
$n = 576$	8 rows + 72 chips	0.4643993	1.282560	5	0	0.4562415	1.346690	67	3	0.1392911	21.116253	480,228	174,133
$n = 624$	8 rows + 78 chips	0.4647209	1.279449	1	1	0.4546367	1.351416	97	3	0.1319790	22.583813	495,269	193,145
$n = 672$	8 rows + 84 chips	0.4646304	1.277031	10	0	0.4550306	1.344902	99	1	0.1285275	24.769395	501,777	201,233
$n = 720$	8 rows + 90 chips	0.4629008	1.275070	5	1	0.4552286	1.342806	94	3	0.1250251	26.836670	509,759	213,140
$n = 768$	8 rows + 96 chips	0.4631314	1.273107	0	0	0.4545927	1.341437	118	3	0.1209156	28.445529	517,941	224,289

[+] Genomic Inflation Factor λ

[*] Significant CpG sites with False Discovery Rate 5%

[**] Significant CpG sites with Bonferroni correction 5%

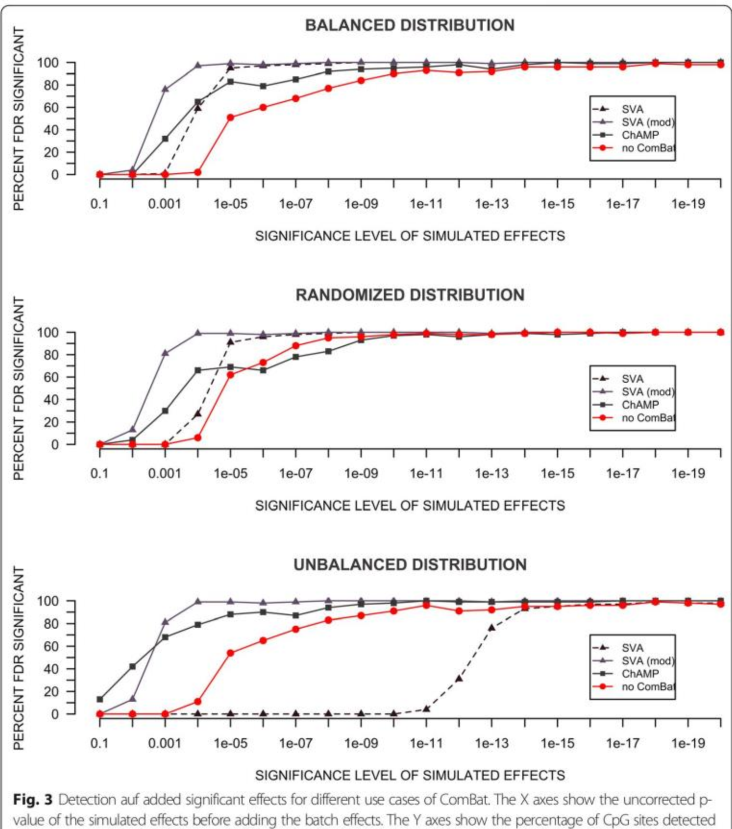

Fig. 3 Detection auf added significant effects for different use cases of ComBat. The X axes show the uncorrected p-value of the simulated effects before adding the batch effects. The Y axes show the percentage of CpG sites detected as FDR $p < 0.05$ after adding the batch effects for the different forms of batch effect correction

Assessment of ComBat in previously published empirical studies

Because of the potential implications of our results, we conducted a systematic search for previously published empirical studies. We used the full text search provided in Google Scholar to successfully identify studies which employed ComBat in their statistical analysis. Using this approach, we were able to access 54 papers published since 2018 that used *Illumina 450 K* and *EPIC* arrays in combination with ComBat for their analyses. Unfortunately, none of the identified authors provided sufficient detail regarding their batch factor structure, sample distribution, and the subsequent application of ComBat to enable us to recreate their complete analyses sufficiently. 72.73% of authors merely indicated that ComBat was used, but not to what extent, while the remaining studies specified the corrected factors, but were missing the exact number of factors or did not specify the sample distribution. Some authors tried to mitigate the inflation in the occurrence of significant sites by reporting only those CpG sites which had a minimum methylation rate difference between groups.

Discussion

When technical factors related to DNAm analyses, such as the *chips*, the position of the samples on the *chips*, or even the date of processing, are confounded with the dependent variable of interest, this can very easily lead to disastrously false results (Fig. 2d) [29, 30]. However, as indicated above, the use of batch effect reduction methods can potentially lead to equally large problems. The results of our simulation study reveal a fundamental problem: in addition to the impossibility of identifying significant effects after correction for multiple testing with small sample sizes, it is just as impossible with increasing sample sizes to avoid severe batch effects due to technical (e.g. *samples pl*ate and *chip* size) or practical (multicentric studies) reasons.

Moreover, our results show that the use of ComBat for batch effect mitigation can lead to any number and magnitude of false significant results, which occur in a variety of different use cases. We were able to successfully replicate the results of the earlier studies by Buhule et al. and by Price and Robinson [22]. The results reported here strongly support the important warnings made by other authors to double-check every step of a DNAm analysis and to be skeptical about the results obtained using ComBat for batch effect mitigation on unbalanced samples [22, 23, 28, 31]. Furthermore, our results imply that these warnings should be extended to balanced samples too. At this point it is important to note that in our results the combination of a *randomized* sample distribution and ComBat usage without the use of covariates or a *outcome of interest* variable was least prone to error. This is also the usage variant shown in the official tutorial of sva, even if the manual of sva suggests other possible use cases. However, in reality the use of ComBat often seems to be a different one due to seemingly better results as the integration in ChAMP suggests.

Furthermore, the positive effect on the actual removal of batch effects with ComBat and the subsequent better detection of actual real effects can be replicated (Fig. 3) in accordance with previous studies [22, 23].

Unfortunately, the precise extent of the consequences of our simulation results remains unclear, since most authors do not report their correction methods in detail. Because of this, we were unable to confirm the results of other previously published studies which used ComBat. Therefore, we created Fig. 4, which is intended to provide a rough guideline for evaluating studies. It is provided without any guarantee of correctness, because a precise evaluation would depend on the exact factor structure and ComBat configuration used by the study. We therefore appeal to authors to describe their use of analysis pipelines in greater detail and to provide all of the information necessary to replicate a finding. In this case, the R and Jupyter Notebooks might be a good starting point, because they are capable of combining theoretical considerations with the practical implementation.

The limitations of this study were that the effects presented were restricted to the use of ComBat, which is only one (however frequently used) possible method out of many for correcting for batch effects in whole methylome data. The simulation of effects applied here needs to be further validated using real data and can only be interpreted as an approximation of the real effects. Additionally, the results presented here are limited to probe-wise (DMP) analyses, because the naturally

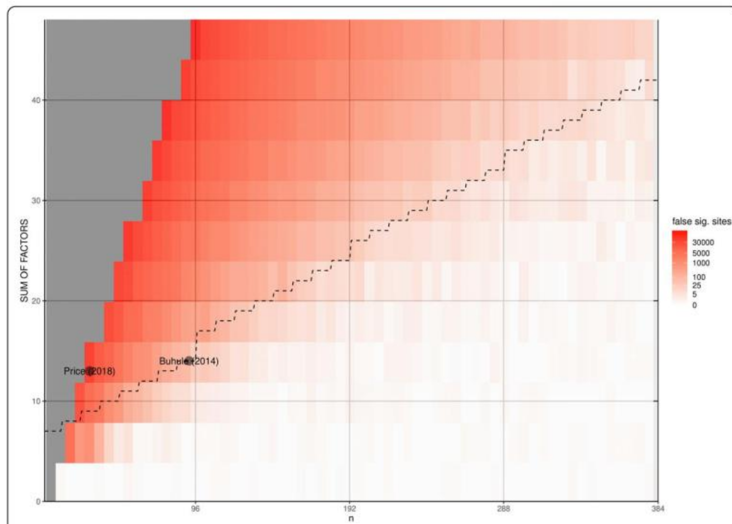

Fig. 4 Heatmap of FDR-significant CpG sites after ComBat application. The dotted line indicates the number of corrected factors when "correcting" for a technical batch. Grey tiles were not assessed due to the sample–factor ratio. The redder the cells are, the more false significant CpG sites were found after applying ComBat

occurring intercorrelation of CpG sites is not accounted for in our simulation approach.

Furthermore, it is important to note that our batch effect simulation was very basic. It is plausible that many different and more complex batch effects exist, which are not sufficiently covered by this study and could potentially produce different results. Therefore, it is important that the structure of batch effects itself is analyzed as accurately as possible in further research.

Conclusions

Even if our work suggests otherwise at first glance, ComBat can be a very good method for batch effect removal. But it is crucial not to use this method blindly and to be very skeptical of positive findings that cannot be found without correction. In this context we want to emphasize the importance of the sample distribution. The distribution of the samples during the measurement is fundamental for the creation of batch effects and, as we have shown, their removal. This is why the distribution of samples should be presented in full detail in future studies. We want to recommend visual illustrations like Fig. 1a following the example of Buhule et.al [23]. for this purpose. Furthermore, contrary to previous research results, we can only advise against using any kind of non-random sample distribution. Our simulation suggests that balanced samples with respect to the *outcome of interest* pose new problems that have been underestimated in the literature so far.

While in our view, efforts to integrate and standardize DNAm analysis in packages such as *ChAMP* are an essential step toward extending the replicability and comparability of results, it must be noted that this integration means that *ChAMP* alone

depends on 233 separate packages. Therefore, it is impossible in practice for researchers to check all of these packages with their respective source codes for correctness and correct usage. This is particularly problematic because statistical hypothesis testing ("*p*-value statistics") only works correctly if all requirements for these methods are met in the pre-processing and the structure of the data. While the results of our simulations do not allow conclusions to be made about other correction methods, similar problems are possible with other methods. This means that a simple method of verifying results is required. Therefore, we would encourage researchers to apply our deliberately simple yet effective method for checking for false positive results to their own analysis pipelines using the R code provided in the Supplementary Material.

Summarizing the lessons learned from our results, we strongly recommend not blindly trusting the analysis pipelines discussed above. Testing them with random data without the possibility of real significant results is a simple way to test the pipelines and sample distributions in advance. In addition, the scientific community urgently needs to develop a standardized way to adequately present complex statistical analysis methods.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12859-020-03559-6.

Additional file 1. Supplementary Document with two sections: A. DNA methylation quantification and quality control. B. Assessment of the simulated data. Methylation Simulation Script. Contains all descriptive data necessary for the simulation as well as simple R code to create any number of simulated *EPIC* methylomes.

Additional file 2: Supplementary Figure S1. Q-Q plots of simulation run 1 with 48 samples.

Additional file 3: Supplementary Figure S2. Scatterplot of one false significant CpG site from simulation run 1 with and without application of ComBat.

Additional file 4: Supplementary Table S1. Full report of the simulation results with $n = 48$ without simulated batch effects

Additional file 5: Supplementary Table 2. Full report of the simulation results with $n = 48$ with simulated batch effects.

Abbreviations

DNAm: DNA methylation; 450 K: Infinium HumanMethylation450 BeadChip; 27 K: Illumina Infinium HumanMethylation27 BeadChip; BF: Bonferroni-corrected; BMIQ: Beta-mixture quantile normalization; EPIC: Infinium MethylationEPIC BeadChip Kit; FDR: False discovery rate; mod: Possibility to specify a model matrix being used with covariates or a outcome of interest variable besides the batch factors in the ComBat implementation of the SVA package

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Authors' contributions

TZ and EF conceived the study; TZ and EF drafted the manuscript; TZ, EF, and HF analyzed and interpreted the data; TZ created the figures; HF was the mentor for the study; HF, AN, and SB revised important intellectual content in the manuscript. All authors contributed to manuscript revision, and have read and approved the submitted version.

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The founding sources had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials

All datasets for this study are included in the manuscript and in the supplementary files.

Ethics approval and consent to participate
Ethical approval for the study was obtained from the ethics committee of Charité-Universitätsmedizin Berlin (EA1/157/11) and Universitätsklinikum Dresden (EK228072012). Participants received a monetary compensation of 10 €/hour for study participation. All participants signed a written informed consent before enrolment.

Consent for publication
Not applicable; manuscript does not contain any individual person's data in any form.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2.6 Wie Stresserfahrung und kognitive Geschwindigkeit bei Entscheidungsprozessen interagieren

ORIGINAL RESEARCH
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How Accumulated Real Life Stress Experience and Cognitive Speed Interact on Decision-Making Processes

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Rationale: Advances in neurocomputational modeling suggest that valuation systems for goal-directed (deliberative) on one side, and habitual (automatic) decision-making on the other side may rely on distinct computational strategies for reinforcement learning, namely model-free vs. model-based learning. As a key theoretical difference, the model-based system strongly demands cognitive functions to plan actions prospectively based on an internal cognitive model of the environment, whereas valuation in the model-free system relies on rather simple learning rules from operant conditioning to retrospectively associate actions with their outcomes and is thus cognitively less demanding. Acute stress reactivity is known to impair model-based but not model-free choice behavior, with higher working memory capacity protecting the model-based system from acute stress. However, it is not clear which impact accumulated real life stress has on model-free and model-based decision systems and how this influence interacts with cognitive abilities.

Methods: We used a sequential decision-making task distinguishing relative contributions of both learning strategies to choice behavior, the Social Readjustment Rating Scale questionnaire to assess accumulated real life stress, and the Digit Symbol Substitution Test to test cognitive speed in 95 healthy subjects.

Results: Individuals reporting high stress exposure who had low cognitive speed showed reduced model-based but increased model-free behavioral control. In contrast, subjects exposed to accumulated real life stress with high cognitive speed displayed increased model-based performance but reduced model-free control.

Conclusion: These findings suggest that accumulated real life stress exposure can enhance reliance on cognitive speed for model-based computations, which may ultimately protect the model-based system from the detrimental influences of accumulated real life stress. The combination of accumulated real life stress exposure and slower information processing capacities, however, might favor model-free

strategies. Thus, the valence and preference of either system strongly depends on stressful experiences and individual cognitive capacities.

Keywords: chronic stress, model-based learning, model-free learning, decision making, cognitive speed, real-life events

INTRODUCTION

Habitual responding to rewards and the pursuit of goals are key to human decision-making. Such habitual (automatic) vs. goal-directed (planned) control of behavior is associated with distinct neural systems for valuation and decision-making (Dolan and Dayan, 2013). Computational attempts to understand these behavioral systems assume that both reflect different computational strategies during reinforcement learning, namely model-based (goal-directed) vs. model-free (habitual) behavior (Daw et al., 2005, 2011; Dolan and Dayan, 2013). Therefore, there is a close association between the theoretical concepts of goal-directed and model-based behavior (Friedel et al., 2014; Gillan et al., 2015; Sjoerds et al., 2016). A key difference between the two behavioral strategies is that model-based behavior strongly demands higher cognitive functions to plan actions prospectively based on an internal model of the environment. Model-free behavior on the other hand relies on simple retrospective evaluation of cached reward values and is cognitively less demanding (Daw et al., 2005; Otto et al., 2013a; Schad et al., 2014). The conditions under which each of these systems controls behavior have been of particular interest in neuroscience and psychiatry, in part because the (im)balance between model-free and model-based behavior is believed to be a key factor in a number of psychiatric disorders. Psychiatric conditions characterized by increased model-free behavior at the cost of model-based performance are e.g., addiction, binge eating disorder and obsessive compulsive disorder (Sebold et al., 2014; Voon et al., 2015; Gillan et al., 2016; Heinz et al., 2017). Among situational factors that influence reward-based decision making, stress is a key candidate for biasing the balance of the two systems toward more habitual decision making (Schwabe and Wolf, 2009, 2012) and might thus be of relevance for the development and maintenance of these disorders.

One key factor in the arbitration between model-based and model-free behavior may be the interaction between stress and cognitive functioning, as stress is known to exert strong influences on cognition and learning (Baumeister et al., 2002; Garrett et al., 2010; Otto et al., 2013b). The magnitude and valence of this influence crucially differs depending on the operationalization (e.g., pain, social stress), the timing and duration of acute vs. chronic stress exposure (Lupien et al., 2009) and the specific cognitive function. Acute stress is known to impair goal-directed choices (Schwabe and Wolf, 2009) and executive functions underlying model-based behavior (Otto et al., 2013a). Executive cognitive functions that have been strongly associated with model-based behavior are processing speed (Schad et al., 2014) and working memory capacity (Otto et al., 2013b; Smittenaar et al., 2013; Schad et al., 2014).

Recent reports of decision-making under stress (for review see Starcke and Brand, 2012) primarily focused on effects of acute stress (Schwabe and Wolf, 2009; Otto et al., 2013b; Buckert et al., 2014). For example, it has been shown that acute stress, as indicated by a transient cortisol response of the neuroendocrine system to a laboratory stressor disrupts context-dependent memory (Schwabe et al., 2009), induces a shift from more goal-directed towards habitual strategies (Schwabe and Wolf, 2009) and impairs model-based behavior (Otto et al., 2013b). Crucially, high working memory capacity protects individuals from this disruption (Otto et al., 2013b), suggesting that stress interacts with executive functions underlying model-based control.

On a neurobiological level the mesostriatal dopamine system is prominently implicated in reinforcement learning in humans with neural signals in the ventral striatum covarying with prediction error signaling during reinforcement learning (Kurniawan et al., 2013). We have previously shown that the neural correlate of reinforcement learning in the ventral striatum is moderated by cognitive functioning and chronic (accumulated real life) stress experience (Friedel et al., 2015). This signal in the ventral striatum has also been shown to be influenced by acute stress (Robinson et al., 2013) and changes in cortisol levels during an acute stressor were correlated with increases in striatal responses during a decision-making task (Dedovic et al., 2009). On the behavioral level, stress facilitates a shift from flexible cognitive to more rigid habit memory systems (Schwabe and Wolf, 2009). On the neural level this is in line with the idea of reduced prefrontal cortex functions such as working memory and attention, promoting a switch from "thoughtful "top-down" control by the prefrontal cortex to "bottom-up" control by the amygdala and related subcortical structures" (Arnsten, 2009; Yu, 2016).

However, while negative influences from acute stress on decision-systems are well documented, little is known about how accumulated real life stress exposure affects cognitive functions underlying model-based choice. Evidence from animal studies indicates that chronically stressed rats turn towards habitual behavior (Dias-Ferreira et al., 2009) and one study suggests that this finding might be translated to human decision-making (Soares et al., 2012). A recent study reports that acute and chronic (accumulated real life) stress may interact: acute stress exposure reduce model-based behavior, but only in subjects earlier exposed to high levels of chronic (accumulated real life) stress (Radenbach et al., 2015). These findings underline the importance of chronic stress in behavioral control. However, the cognitive and computational mechanisms underlying such influences are still insufficiently understood.

Here, we used the Social Readjustment Rating Scale (Holmes and Rahe, 1967) to study how accumulated real life stress interacts with cognitive speed on model-based vs. model-free decision-making in a sample of healthy subjects. First, we explored contributions of model-based vs. model-free decision-making via statistical analysis of choices in a sequential decision-making task. Following up on statistical findings, we tested our primary hypothesis that stress and cognitive speed interact with model-based and model-free decision-making as indicated by the interaction between transition frequency (common vs. rare) and reward.

MATERIALS AND METHODS

Subjects and Screening Instruments

A group of $N = 95$ right handed healthy adults ($N = 16$ females) with a mean age of 43.62 years ($SD = 11.0$; range: 21.4–66) and an average of 11.3 years of school education ($SD = 1.5$ years) was recruited in a longitudinal German two-center study on learning and alcohol dependence (LeAD, see www.lead-studie.de; Clinical Trials identifier NCT01744834). The reported sample of healthy subjects had been matched to a sample of alcohol dependent subjects according to their age, smoking status and gender. We excluded one subject based on implausible high stress values (SRRS = 635 > 3 SD of the group's mean of 122). The study consisted of 2 days of testing, including 1 day of psychopathological assessment, neuropsychological tests, and questionnaires, and a second day involving fMRI scanning during two experimental learning tasks. Neuropsychological testing included the digit symbol substitution test (DSST, Wechsler, 1997) as a measure of cognitive speed and working memory capacity as assessed with the digit symbol backwards test (Wechsler, 1997; Aster et al., 2006) which had previously been associated with model-free and model-based control (Otto et al., 2013b). Moreover, we have assessed the German version of the verbal knowledge test (MWTB, Lehrl, 2005), which relates to crystallized IQ (Schad et al., 2014) and the trial-making-test (TMT) -A and B, which assesses executive functioning (Corrigan and Hinkeldey, 1987; Sánchez-Cubillo et al., 2009).

On the first day, written informed consent was obtained from all participants before they underwent the neuropsychological testing. On the second day, participants completed the below-described sequential decision making task (Daw et al., 2011). After completing the task, participants received monetary compensation for their participation. Ethical approval for the study was obtained in accordance with the Declaration of Helsinki from the Medical Ethics Committees of Charité–Universitätsmedizin Berlin (EA/1/157/11) and Technische Universität Dresden (EK 228072012). Not included were subjects with Axis I psychiatric disorders except nicotine dependence, alcohol abuse and specific phobia according to DSM-IV as measured with the Composite International Diagnostic Interview (CIDI, Wittchen and Pfister, 1997; Jacobi et al., 2013), subjects with DSM-IV personality disorders (SAPAS screening; Moran et al., 2003), and subjects with MRI contraindications (for further details see Sebold et al., 2016).

Measures of Accumulated Real Life Stress and Cognitive Speed

Accumulated Real Life Stress

In the Social Readjustment Rating Scale (SRRS, Holmes and Rahe, 1967) participants indicated whether any of 43 potentially stressful life events occurred to them within the last 12 months. Each life event is associated with a specific amount of life change units (LCUs) based on ratings by a large sample of participants, ranging from 100 LCUs for "death of a spouse" to 11 LCUs for "minor violation of the law". The LCUs for each of these life events were added up, providing a measure of past-year stress load for each participant, which has been proven to be a reliable indicator of overall (and lifetime) accumulated real life stress (Holmes and Rahe, 1967; Scully et al., 2000).

Cognitive Speed

Cognitive speed was assessed with the DSST (Wechsler, 1997), see **Figure 1**. The DSST is a neuropsychological test measuring general processing speed (Salthouse, 1992), writing speed, and short-term-memory (Laux and Lane, 1985). Subjects are provided with a code table assigning nine different abstract symbols to the digits 1–9 and are then given a table presenting a list of digits in each top row and empty boxes in each bottom row. They are then instructed to sequentially draw as many of the 133 (maximum score) corresponding symbols underneath the digits as possible in 120 s. Standardized values corrected for age according to the manual (DSST, Wechsler, 1997) resulted in scores from 2 to 19 which were used for subsequent analyses.

Two-Step Task

We adapted the Two-Step decision task (Daw et al., 2011, **Figure 2**) for MATLAB with the Psychophysics Toolbox Version 3 extension (Brainard, 1997; Pelli, 1997) in order to assess model-based vs. model-free decision making. We used a new set of colored stimuli, but the same transition structure and outcome probabilities as in the original Two-Step study (Daw et al., 2011). Participants had to choose one out of a pair of abstract grayscale stimuli leading to another colored stimulus pair for choice at stage 2 (stage 1 and 2 at **Figure 2A**). Instructions emphasized reward maximization. Importantly, the win probability for each of the four stage 2 stimuli varied over time according to a slow and independent random walk (chances of winning money at **Figure 2A**). The probability to be presented with a specific stimulus pair at stage 2 depended on the choice at stage 1 and was constant over time; there was a common (70%) and a rare (30%) transition for each stage 1 stimulus. After the experiment, one third of all rewards (with a fixed minimum of 3 EURO and maximum of 10 EURO) was additionally paid out to increase the motivation of the participants. The instructions provided detailed information about the structure of the task; specifically concerning the varying outcome probabilities at stage 2 and about the constant transition probabilities between stage 1 and 2. In addition, there were 50 practice trials prior to the main experiment. The distinction between model-based and model-free performance primarily depends on the use of the transition probability: a

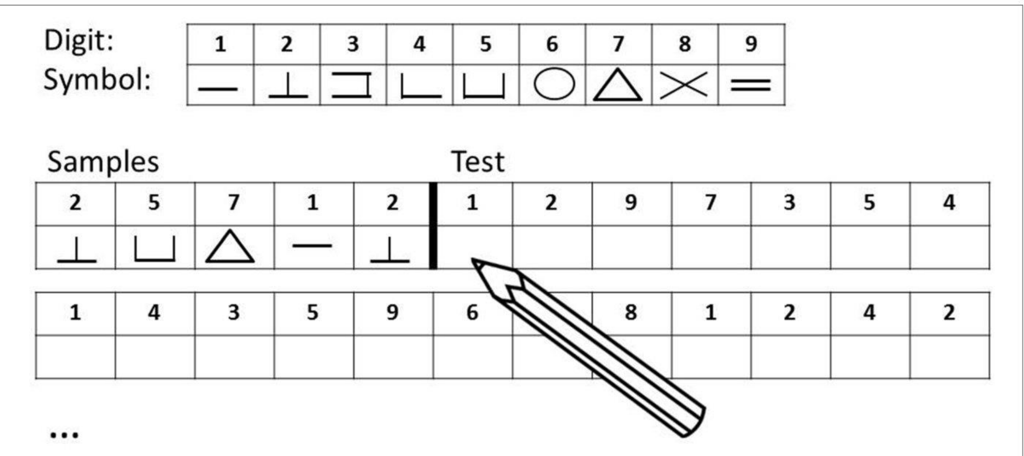

FIGURE 1 | Digit Symbol Substitution Test (DSST, Wechsler, 1997): the DSST consists of a code table displaying pairs of digits and symbols, and rows of double boxes with a digit in the top box and an empty space in the bottom box. The task for the subject is to use the code table to determine the symbol associated with each digit, and to write as many symbols as possible in the empty boxes below each digit within 120 s.

purely model-free learner would repeat a decision that led to a rewarded choice ignoring the transition frequencies, resulting in a main effect of reward on selection of the first stage; whereas a purely model-based learner would repeat a decision that led to a rewarded common choice but most unlikely repeat a decision that led to a rewarded rare choice, resulting in an interaction between transition frequency (common vs. rare) and reward (see **Figure 2B**).

Data Analysis

The final sample consisted of 95 subjects with a mean DSST score of 10.8 (*SD* = 3.1) and a mean SRRS score of 115.7 (*SD* = 94.9). All regression analyses were conducted using linear models implemented in the stats package of the R programming language, version 3.1.2 (cran.us.r-project.org). For orthogonal contrasts (rewarded vs. unrewarded/common vs. rare), we used effect coding [−0.5 0.5]. The level of statistical significance was set to $p < 0.05$.

We were specifically interested in how model-free and model-based control were related to stress and cognitive speed. For this purpose, we calculated two individual scores, one for model-free (% rewarded common + % rewarded rare − % unrewarded common − % unrewarded rare, see **Figure 2B**, left plot) and one for model-based behavior (% rewarded common + % unrewarded rare − % rewarded rare − % unrewarded common, see **Figure 2B**, middle plot), as previously described (Sebold et al., 2014). Individual model-free and model-based scores were extracted from the raw data of the Two-Step task, where the percentage of individual first stage repetitions was calculated based on the previous trial's outcome (rewarded vs. unrewarded) and transition frequency (common vs. rare, see **Figure 2B**). Model-free effects describe the individual main effect of reward, whereas individual scores for model-based control reflect the interaction between transition frequency and reward. In line with our previous research (Friedel et al., 2014; Sebold et al., 2014), we chose this analysis strategy because we aimed to extract individual model-free and model-based scores in order to subsequently predict differences in both scores from stress and cognitive speed.

The scores (both approaching normal distribution) then served as criterion variable in two subsequent linear regressions, in which the interaction between stress and cognitive speed was tested on each of these scores. We computed median splits of DSST score (which were normally distributed) and subjects were assigned to a high or low cognitive speed group (low ≤ 11, high > 11), before entered into the regression model. SRRS scores were z-transformed before they were entered into the regression models as a continuous variable. For further *post hoc* tests and illustrative purposes (see **Figure 3B**), we additionally assigned subjects to a low (≤ 101) and a moderate to high (>101) stress group based on the group's median split.

As previous research has indicated that differences in age could impact on the balance between model-free and model-based control (Eppinger et al., 2013; Sebold et al., 2016), our results could potentially have been confounded by age effects. In order to test this, we performed additional analyses, where we put age (z-scaled) as an additional nuisance regressor in the two previously described linear models.

In order to replicate previous studies, which demonstrated that subjects showed a mixture between model-free and model-based decision-making strategies in the Two-Step task, we

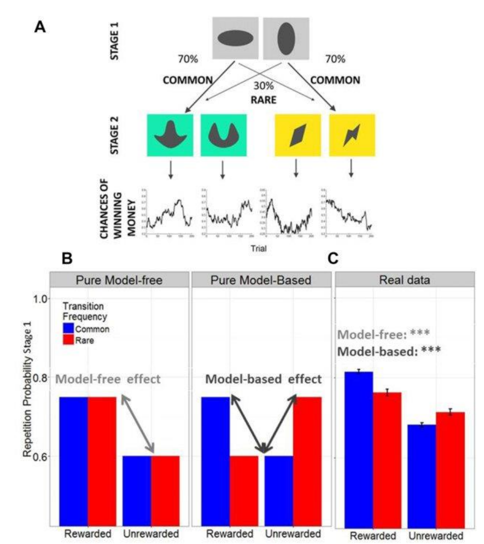

FIGURE 2 | (A) Structure of the Two-Step Task. **(B)** Simulated data of a pure model-free vs. a pure model-based decision-maker. Model-free and model-based strategies predict distinct response patterns on the first stage. In model-free decisions, first stages should be repeated whenever the outcome of the previous trial was rewarded, whereas they should not be repeated whenever choices were unrewarded. Therefore model-free decisions predict a main effect of reward on first stage repetition of the subsequent trial. In model-based decisions, the individual takes transition frequencies into account. Thus, for instance, when a trial from the rare transition frequency ended up in reward, the individual knows that in order to obtain reward in the next trial he/she should actually switch to the opposing first stage stimuli, because this one has a higher probability of ending up at the specific second stage stimulus pair that is now associated with high probability of reward. **(C)** Across all subjects ($N = 95$), model-free and model-based scores were significantly positive (as indicated with the *** both $p < 0.0001$), suggesting that subjects showed a mixture of model-free and model-based choice strategies.

performed one-sample t-tests, which tested whether each score was significantly larger than zero.

RESULTS

Across all subjects, model-free and model-based scores were significantly positive (both $p < 0.0001$), suggesting that subjects showed a mixture of model-free and model-based choice strategies (see **Figure 2C**).

In the linear model regressing cognitive speed, stress and their interaction on model-free control, no main effect was found for either stress ($p = 0.16$), nor cognitive speed ($p = 0.30$), while their interaction ($\beta = 0.5$, $F_{(91)} = 2.76$, $p = 0.022$) was significantly associated with model-free behavior. In fact, model-free control increased with increasing stress exposure when cognitive speed was low (see **Figure 3A**).

In the second linear model we again found no main effect of cognitive speed ($p = 0.1$) nor stress ($p = 0.86$) on model-based control, but an interaction between stress and cognitive speed which was negative, ($\beta = -0.07$, $F_{(91)} = 2.7$, $p = 0.04$), indicating that model-based behavior was reduced when stress exposure was high but cognitive speed was low (see **Figure 3A**).

Healthy controls reported rather low levels of accumulated real life stress, which led to a positive (left) skew of the data. When dichotomizing the SRRS score effects closely failed to reach significance (on a p-level of 0.05: interaction of stress and cognitive speed on model-free control, $p = 0.08$; interaction of stress and cognitive speed on model-based control, $p = 0.09$). This seems plausible, as the sample consisted of healthy control subjects, where most subjects reported a comparably little amount of accumulated real life stress. To maintain most of the variance of the SRRS predictor variables (which is reduced by performing median splits) and in line with previous research (Radenbach et al., 2015) we stuck with the above reported analyses. The assumptions for a multiple regression analysis were met.

The digit symbol backwards test (Wechsler, 1997; Aster et al., 2006), the German version of the verbal knowledge test (MWTB, Lehrl, 2005) and the TMT-A and -B (Reitan and Wolfson, 1985) did not interact with stress regarding either model-free or model-based control.

When age was added as additional covariate in our two linear models, we again found that the interaction between stress and cognitive speed was significant for model-free ($\beta = 0.05$, $F_{(90)} = 2.07$, $p = 0.022$) and model-based control ($\beta = -0.07$, $F_{(90)} = 2.05$, $p = 0.04$), suggesting that the interaction between stress and cognitive speed on model-free and model-based control was not caused by confounding age effects. Moreover, in these analyses, we found no main effect of age on model-free ($p = 0.78$) or model-based control ($p = 0.67$), suggesting that in our sample age did not impact these decision-making strategies.

Exploratory *post hoc* analyses, where we tested the influence of cognitive speed on model-free and model-based decision-making separately for low and high stress subjects revealed that cognitive speed increased model-based ($\beta = 0.06$, $p = 0.025$) but decreased model-free control ($\beta = -0.03$, $p = 0.04$) only in high stress subjects, whereas this effect was not significant in low stress subjects (model-based: $\beta = 0.02$, $p = 0.4$, model-free: $\beta = -0.13$, $p = 0.34$). Thus, differences between the influence of cognitive speed on model-free vs. model-based control were mainly driven by subjects who had experienced comparably high stress in the past year (see **Figure 3B**).

DISCUSSION

The main finding of our study is that self-reported life stress exposure during the past 12 months interacts with cognitive processing speed on human decision-making: in individuals with lower cognitive speed, accumulated real life stress was associated with reduced model-based performance and a shift towards model-free choice behavior. High levels of cognitive speed, on the

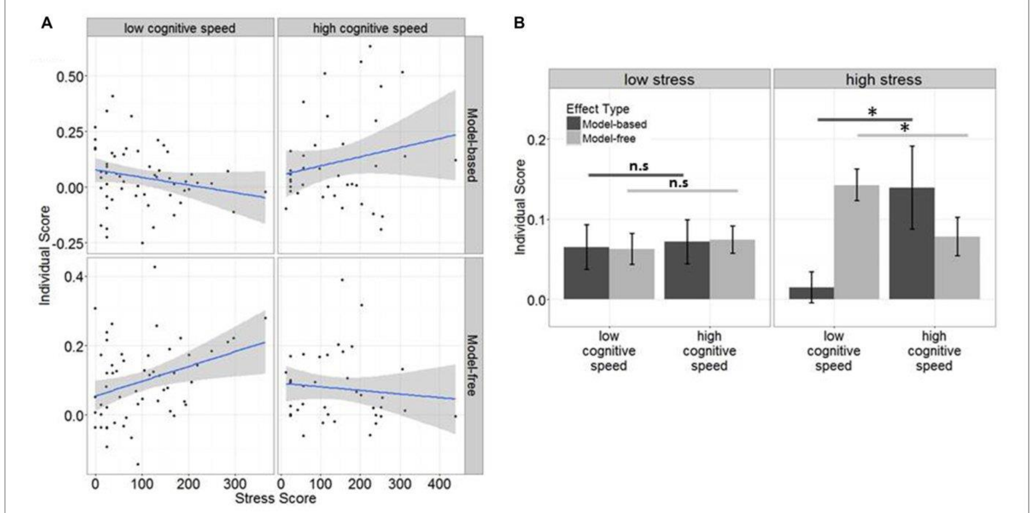

FIGURE 3 | (A) Effects of stress and cognitive speed on model-free vs. model-based control: subjects with low cognitive speed display an increase of model-free but a decrease of model-based behavior with increasing stress exposure, whereas subjects with high cognitive speed display an increase of model-based but a decrease of model-free behavior with increasing stress exposure. **(B)** The differential association of cognitive speed with model-free and model-based control was particularly evident in subjects who had experienced high stress (significant effects indicated with *), whereas there was no such effect in subjects who had experienced low stress.

other hand, seemed to protect individuals from such influences, as these were associated with an increased reliance on the model-based system, especially after high accumulated real life stress exposure.

Acute stress is known to induce a distinct decline of model-based choices, while leaving the model-free system unaffected (Otto et al., 2013b). High working memory capacity, a measure closely related to cognitive flexibility and processing speed, was recently shown to protect model-based choices from such deteriorating influences (Otto et al., 2013b). This suggests that the relative transition from model-based towards model-free decision-systems (Schwabe and Wolf, 2009; Otto et al., 2013b) observed after acute, laboratory-induced stress might be due to taxing of executive functions underlying model-based decision-making. The present findings replicate findings that high processing speed is related to model-based decision-making (Schad et al., 2014) and complement on previously reported interaction effects between acute stress reactivity and cognitive abilities (Otto et al., 2013b) by pointing to the influence of chronic, long-term real-life stress. With respect to the interaction of acute and chronic stress experience on model-based decision making, Radenbach et al. (2015) have shown that acute stress results in a decrease of model based performance only when chronic (accumulated real life) stress exposure was high. In addition to and complementing on this finding, we show that the association of real-life stress with model-based decision making is also modulated by cognitive speed.

An important question arising from our findings is which mechanisms underlie the association between long-term stress, cognitive speed and the balance between model-based vs. model-free control. Empirical evidence on the differential influence of acute vs. long term stress on cognitive functioning is in part controversial. Acute stress is known to exert a negative influence on memory. Schwabe and Wolf (2009) report that acutely stressed individuals become insensitive to the devaluation of a particular outcome accompanied by a significant decrease in explicit knowledge of action-outcome contingencies. For enduring past stress experience time-dependent effects on working memory processing, emotional memory and brain function in general have been reported (for a recent overview see Yu, 2016). Chronic enduring stress exposure in humans led to a compensatory upregulation of prefrontal functioning whereas acute stress and the quick influence of cortisol in combination with noradrenaline led to an increase of subcortical and a decrease of prefrontal functioning (Hermans et al., 2011, 2014). Findings from animal research demonstrate that chronically stressed rats become insensitive to devaluation of outcomes (Graham et al., 2010), together with atrophy of the medial prefrontal cortex and hypertrophy of the putamen (Dias-Ferreira et al., 2009) indicating a negative influence of long term stress exposure on cognitive processing.

Looking from an evolutionary perspective, Decker et al. (2016) suggest that the recruitment of model-based valuation systems relies on a critical cognitive component, which is

associated with the gradual maturation of goal-directed behavior. Whereas a model-free strategy was apparent in choice behavior across all age groups, a model-based strategy was absent in children, emerged in adolescents and matured in adults. This observation suggests that cognitive resources like processing speed may be decisive in coping with the influences of long-term real-life stress experience depending on brain maturation and age. In this context, the complexity and uncertainty associated with accumulated real life stress may pose difficulties and expose limits for the development of fully rational decision strategies and favor computationally less demanding model-free decision-making strategies (Daw et al., 2005; Otto et al., 2013b).

One important aspect of the mechanisms associated with an overreliance on model-free strategies is their association with the development and maintenance of various psychiatric disorders, such as addiction, obsessive compulsive disorder and binge eating disorder (Everitt and Robbins, 2005; Sebold et al., 2014; Voon et al., 2015). Gillan et al. (2016) used a transdiagnostic approach and applied the Two-Step task to about 2000 healthy individuals assessing a broad variety of symptoms (assessed via a self-report questionnaire containing 209 items), which have been associated with different psychiatric diagnostic categories (e.g., alcohol addiction, schizotypy, depression and social anxiety). Independent of the diagnostic category, the authors report a strong association of compulsive spectrum behavior with a decrease in model-based performance in the Two-Step task. Decision-making tendencies might thus be linked to certain trait markers that interact with the vulnerability for the development of compulsive spectrum disorders. Also, past stress exposure increases the risk for the development of psychiatric disorders, such as major depressive disorder and schizophrenia (Murgatroyd and Spengler, 2011a,b, 2012) and might further add up to an increased vulnerability with respect to its interaction with individual cognitive capacities and decision making strategies.

An important limitation to our findings is that they are correlational and from a cross-sectional design, hence no causal conclusions should be drawn and assumptions on the employment of cognitive abilities according to environmental circumstances are theoretical. However, the different directional associations are interesting to disentangle. The importance of our findings derives from the fact that we complement previous findings on acute stress (Otto et al., 2013b) with chronic (accumulated real life stress) exposure, strengthening the importance of both (past) environmental as well as cognitive variables in understanding human decision making. Coping abilities associated with high processing speed might be able to enhance model-based decision-making in response to stressful experiences. Further, especially longitudinal research is needed to disentangle effects of acute vs. chronic (accumulated real life) stress exposure and cognitive abilities on choice systems in different mental disorders, and to closely parse the cognitive and computational processes underlying the interaction of processing performance speed and past stress experiences in model-based decision-making.

To account for accumulated real life stress effects, we used the weighted sum of (positive and negative) life events reported by the subjects in the past 12 months with the SRRS. The generalizability of our results is thus limited, as we could not account for the interindividual differences in the experience of accumulated real life stress, which can potentially be influenced by an (im)balance of personal traits, resources and the demands placed upon an individual by social and occupational situations. However, an interesting feature of the SRRS is that it spans a broad range of events and their estimated potential to elicit readjustment processes, including events that are usually related to positive affect (such as pregnancy, marriage and outstanding personal achievement). Crucially these events may be regarded positively by some and negatively by others, depending on the context of change (such as changes in residence, changing to a different line of work, major changes in responsibility at work). Moreover the SRRS includes negative events (such as death of spouse, death of a close family member). All of these events have been rated and evaluated according to their need for social readjustment on large independent samples (Holmes and Rahe, 1967; Scully et al., 2000).

Due to a lack of statistical power we could not assess the influence of sex on decision making and its interaction with accumulated real life stress. However, this aspect is worth mentioning, as there is recent evidence that sex differences are important modulators of stress-related reward sensitivity and decision making (for a recent overview see Yu, 2016). It was found that stress led to greater reward collection and faster decisions in males but less reward collection and slower decisions in females (Lighthall et al., 2012). One study showed that mild psychological stress resulted in a significant decrease in reward-related responses in the medial prefrontal cortex without affecting ventral striatal responses in women (Ossewaarde et al., 2011).

We did not assess physiological measures of stress. A biological correlate of chronic stress such as hair cortisol, which could give some important information on the hypothalamic–pituitary–adrenal (HPA) axis in the months before assessment, should be considered in future studies. Given the effects of cortisol on cognition, this could have an effect on model-based/model-free learning as well (Otto et al., 2013b; Radenbach et al., 2015).

Altogether, our findings suggest that the cognitive abilities and processes underlying model-based decision-making may not be fixed (Schad et al., 2014), but are rather flexibly employed according to environmental circumstances. While model-based computations build on executive resources and processing speed, especially when past experiences have already demanded flexible adaptation to ever changing environments (such as stress induced through a high need for social readjustment), other settings seem to foster model-free processes. A preference for model- free strategies might prevail after high stress exposure especially when experience has shown that the possibility of a fast flexible adaptation has been insufficient (e.g., due to low processing speed).

AUTHOR CONTRIBUTIONS

AH conceived the study. AH, HW, MR, MNS, USZ and FS designed the study protocol; EF, MS, SK-P, SN collected the data; EF, MS and IMV analyzed the data; EF, MS, SK-P, SN, IMV, FS, USZ, MNS, MR, HW and AH interpreted the data and wrote the manuscript. All authors viewed the final version of the manuscript prior to submission and are accountable for all aspects of the work. EF and MS contributed equally.

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3 Diskussion

Ziel der aufgeführten Studien soll ein besseres Verständnis für die Entstehung und Aufrechterhaltung von abhängigem Verhalten sein. Wie kommt es dazu, dass ein ursprünglich als angenehm erlebtes, sporadisch gezeigtes Verhalten, wie z. B. der Konsum von einem Glas Wein, in der Folge zu einem für den Einzelnen schädlichen bis zerstörerischen Verhalten wird, das oft nicht mehr dem erlebten und formulierten Willen (z. B. zur Abstinenz) entspricht? Obwohl damit zahlreiche negative Konsequenzen wie der Verlust von sozialen und beruflichen Beziehungen, körperliche Erkrankungen und eine reduzierte Lebensqualität verbunden sind, wird das schädigende Verhalten aufrechterhalten. Und obwohl zahlreiche Variablen mit süchtigem Verhalten in Verbindung gebracht werden konnten, sind die kausalen Mechanismen nur unzureichend verstanden. In den oben dargelegten Forschungsarbeiten wurden drei Aspekte von süchtigem Verhalten untersucht: 1) Veränderungen im PIT-Effekt und deren neuronale Korrelate, 2) (epi)genetische Korrelate eines anhaltenden Alkoholkonsums und deren methodische Besonderheiten und 3) die Rolle von chronischem Stresserleben auf belohnungsabhängiges Lernen in Abhängigkeit von kognitiven Funktionen. Die Bedeutung dieser Arbeiten im Kontext eines besseren Verständnisses für die Entstehung und Aufrechterhaltung der Alkoholabhängigkeit wird im Folgenden diskutiert.

3.1 Veränderungen im PIT und deren neuronale Korrelate

Eine grundlegende These der Suchtforschung besagt, dass süchtiges Verhalten initial einem zielgerichteten Verhalten folgt (80). Ziel sind positive, belohnungsassoziierte Aspekte des Konsums. Die Natur des Verhaltens verändert sich aber durch den anhaltenden Konsum und wird in der Folge habituell, mit einem zwanghaften, von den eigentlichen Bedürfnissen der Patient*innen losgelösten Charakter. Reize, die mit der Substanz assoziiert sind, können ein Verhalten evozieren, unabhängig davon, ob damit weiterhin positive Folgen gekoppelt sind. Zwanghaftes Verhalten wird in diesem Kontext definiert als die fehlerhafte Verhaltensinhibition auf einen Reiz trotz aversiver Konsequenzen. Ursachen für dieses maladaptive Verhalten wurden unter anderem in aversiven Motivationszuständen („*spiralling distress*“), z. B. bei Entzugserscheinungen (81), medikamenteninduzierter Sensibilisierung (82) und einem Verlust der Top-Down-Regulation auf neuronaler Ebene (83, 84) nachgewiesen. Le Moal und Koob (1997) sprechen von einer „hedonisch homöostatischen Dysregulation“ als kontinuierlichem

Prozess substanzinduzierter, biosozialer und genetischer Veränderungen, die das gesteigerte Verlangen nach der Substanz und die damit verbundenen Veränderungen im Verhalten und Erleben beeinflussen. Interessant sind die Parallelen zu einer stressinduzierten Negativspirale, die sich sozialpsychologisch im Verhalten und gleichzeitig über die Veränderung neuronaler Schaltkreise und Reaktionsmuster im Gehirn nachweisen lassen (81). Auf Ebene der Neurotransmitter ist die Besonderheit in der Wirksamkeit von Ethanol seine Interaktion mit mehreren Neurotransmitterrezeptorsystemen, die zu den positiv verstärkenden Wirkungen von Alkohol beitragen (81). Die beteiligten Neurotransmitter und Rezeptorsysteme umfassen Wirkungen auf die γ-Aminobuttersäure- (GABA), Glutamat-, Dopamin-, Serotonin- und Opioidpeptidsysteme, die sich alle im mesokortikolimbischen Dopaminsystem und seinen Verbindungen zum NAC und der Amygdala befinden.

Neu hinzugekommen sind Aspekte aus tierexperimentellen und Humanstudien, welche die Bedeutung Pawlowscher Konditionierungsprozesse nachweisen konnten (85). Während die veränderte Ansprechbarkeit auf alkoholassoziierte Hinweisreize bei AUD bereits gut untersucht ist , besteht eine aktuelle Herausforderung für das Feld der Suchtforschung darin zu verstehen, wie diese Pawlowschen Hinweisreize tatsächlich verhaltensrelevant werden und so zwanghaftes Verhalten im Verlauf des Substanzkonsums erklären könnten. Hier konnten tierexperimentelle Studien die Bedeutung des PIT bestätigen (50). Wir konnten erstmalig nachweisen, dass die neuronale Signatur des PIT-Effekts im NAC mit dem Rückfallgeschehen bei AUD-Patient*innen assoziiert ist (21). Auf der Verhaltensebene zeigten die Gruppe der Patient*innen mit AUD einen stärkeren PIT-Effekt im Vergleich zu der Kontrollgruppe. Auf neuronaler Ebene zeigte sich eine Zunahme der NAC-Aktivität ausschließlich bei AUD-Patient*innen, die innerhalb von drei Monaten einen Rückfall in den Alkoholkonsum erlitten. Diese erhöhte Aktivierung war zudem prädiktiv für die Schwere des Rückfalls. Die Ergebnisse belegen die Bedeutung und Validität tierexperimenteller Befunde (86–88) hinsichtlich des PIT-Effektes bei Patient*innen mit Alkoholabhängigkeit. Der NAC als vordefinierte Zielregion unseres Effektes leitet sich aus zahlreichen Vorbefunden ab (88–95). Der PIT-Effekt aktivierte zusätzlich verschiedene neuronale Substrate außerhalb des NAC, insbesondere in präfrontalen Bereichen (96), die a. e. zielgerichtete Steuerungssysteme im Rahmen des PIT-Effektes ansprechen, Annäherungsverhalten modulieren und so ggf. auch dem Rückfall in der Gruppe der abstinenten Patient*innen entgegenwirken konnten (93). Die Zunahme des neuronalen PIT-Effekts bei Patient*innen mit Rückfall kann auch mit der Tatsache assoziiert

sein, dass Geld einen hohen Wert (und damit eine hohe Salienz) für Rückfälle aufgrund wirtschaftlicher Schwierigkeiten in dieser Gruppe hat und auch eine generell erhöhte Sensitivität auf Belohnungen und Bestrafungen in dieser Gruppe besteht (97).

Wendet man im PIT alkoholassoziierte Hintergrundstimuli an (Probanden wählen vorab aus drei Alternativen ihr Lieblingsgetränk Wein, Bier oder Schnaps und sehen ein Bild dieses Getränks im Hintergrund während des Transfer-Teils), zeigen die Ergebnisse eine unterdrückte Annährungsantwort (also einen negativen PIT-Effekt, d. h. weniger Tastendrücke beim Einsammeln einer Muschel mit Alkohol im Hintergrund) ausschließlich bei in der Folge abstinenten Patient*innen (98). Das neuronale Korrelat dieser Verhaltensinhibition lag erneut in der Aktivierung des NAC. Die Tasache, dass vor allem abstinente und weniger schwer erkrankte Patient*innen eine Veränderungen im PIT auf alkoholassoziierte Stimuli zeigten, weist neben der mechanistischen Bedeutung von Annärungsverhalten auch auf eine therapeutische Implikation hin. Die Entgiftung von Alkohol ist mit ausgeprägten aversiven emotionalen (Angst, Depression, innere Unruhe) und körperlichen (Zittern, Schwitzen, Übelkeit bis zu Entzugskrampfanfällen) Zuständen gekoppelt. Diese aversiven Zustände können zu einer aversiven Konditonierung der Substanz beitragen und so die Inhibition des aufsuchenden Verhaltens fördern, eine Art „Alkoholvermeidungstraining“, das auf neuronaler Ebene mit einer zunehmenden Aktivierung der Amygdala gekoppelt ist (99). Die Amygdala ist ein wesentliches neuronales Korrelat aversiver Emotionsverarbeitung (100). Eine Zunahme der Aktivierung kann auf neuronaler Ebene einer zunehmenden negativen emotionalen Salienzzuschreibung bedeuten. Dies ist ein Mechanismus, der bei reduzierter Ausprägung zu einem erhöhten Rückfallrisiko führen kann, indem alkoholassoziierte Stimuli unzureichend gemieden, also die Annährungsantwort nicht unterdrückt wird. Gleichzeitig kann dieser Mechanismus genutzt werden, um über eine Stärkung der Verhalteninhibition Abstinenz zu unterstützen, mit widersprüchlichen Ergebnissen in der Rückfallprävention bei Patient*innen mit AUD (99, 101–104). Während die im Jahr 2016 in einer Metaanalyse zusammengefasste Wirksamkeit von insgesamt 16 Interventionen bei AUD keinen direkten Einfluss auf süchtiges Verhalen zeigte (104), berichten einzelne Studien Effekte auf die Abstinenzerhaltung: Über ein Annährungs-Vermeidungstraining („Approach-Avoidance-Task“) wurden z. B. stationäre Patient*innen einer Alkoholentzugsklinik während des siebentägigen Entgiftungsprogramms implizit geschult, Vermeidungsbewegungen als Reaktion auf Bilder von alkoholischen Getränken und Annäherungsbewegungen als Reaktion auf Bilder von alkoholfreien Getränken

zu machen. Die Ergebnisse zeigen eine Reduktion der Rückfallraten und des Verlangens nach Alkohol 14 Tage nach Entlassung in der Gruppe mit den meisten Trainingseinheiten im Vergleich zur Kontrollgruppe, einschränkend muss aber erwähnt werden, dass die empirische Untersuchung in eine rehabilitative Maßnahme eingebettet war und daher nicht in allen Aspekten mit weiteren Studien vergleichbar ist (102). Mit einer ähnlich aufgebauten sogenannten *„Stop Signal Task“* zeigten Sjoerds und Kollegen (101) bei Patient*innen mit AUD auf der Verhaltensebene eine positive Korrelation zwischen Schwere der Erkrankung und reduzierter Fähigkeit zur Verhaltensinhibition. Auf neuronaler Ebene war die reduzierte Fähigkeit zur Verhaltensinhibition mit einem *shift* von der Aktivierung kortikaler (primärer motorischer Kortex) hin zur Aktivierung subkortikaler Regionen (u. a. Striatum und Thalamus) verbunden. Die Fähigkeit einer kortikalen Kontrolle scheint mit der Dauer und der Schwere der Erkrankung abzunehmen. Das entspricht Ergebnissen von Magrabi et al. (105), die eine negative Korrelation zwischen modellbasiertem Verhalten und striataler Aktivierung bei Patient*innen mit Alkoholabhängigkeit nachweisen konnten.

Dass subkortikale Regionen bereits bei schädlichem Alkoholkonsum relevante Biomarker sind, konnten wir auf behavioraler und neuronaler Ebene mit PIT-Untersuchungen beobachten (20, 96). Bei jungen 18-jährigen sozialen Trinkern zeigte sich bei Hochrisikotrinkern im Vergleich zu Niedrigrisikotrinkern auf der Verhaltensebene ein erhöhter PIT-Effekt, der auf neuronaler Ebene mit einer Zunahme subkortikaler Aktivierungsmuster (Amygdala) assoziert war. Die Stärke des PIT-Effektes war außerdem positiv mit dem polygenetischen Risikoscore für Alkoholabhängigkeit korreliert (20). Hochrisikotrinker zeigten eine höhere Anfälligkeit für Pawlowsche Hinweise, insbesondere wenn sie mit instrumentellem Verhalten in Konflikt standen. Eine erhöhte Aktivität im ventralen Striatum (bottom-up) und eine verminderte präfrontalte Reaktion (top-down) und ihr verändertes Zusammenspiel können so zu dysfunktionalem Verhalten bei den Hochrisikotrinkern beitragen (96).

In der Zusammenschau der dargestellten Befunde konnte wir die empirischen Hinweise auf einen spezifischen Endophenotypen (106, 107) für die Entstehung und Aufrechterhaltung der Alkoholabhängigkeit mit wichtigen Ergebnissen zum PIT-Effekt ergänzen. Dieser Endophenotyp besteht auf neuronaler Ebene aus einer Abnahme kortikaler und Zunahme subkortikaler Aktivierungsmuster, auf Verhaltensebene zeigt sich die Zunahme des Einflusses Pawlowscher Konditionierungsreize auf das Verhalten. Die zugrunde liegenden Konditionierungsprozesse haben wiederum ihre genetische Basis in verschiedenen Bereichen,

die im folgenden Abschnitt diskutiert werden. Die dargestellten Befunde liefern wichtige Implikationen für Interventionsstudien, die zur Prävention und Therapie der Alkoholabhängigkeit genutzt werden können.

3.2 (Epi-)Genetische Korrelate eines anhaltenden Alkoholkonsums und deren methodische Besonderheiten

Der oben beschriebene Endophänotyp für die Entstehung und Aufrechterhaltung der Alkoholabhängigkeit basiert auf genetischen und epigenetischen Grundlagen, die das Verständnis über die jeweils zugrunde liegenden Mechanismen erweitern. Im Folgenden soll die genetische Grundlage der beschriebenen Lernmechanismen 1) über den PRS, 2) über den single SNP OPRM1, 3) über epigenetische Veränderungen im gesamten Genom diskutiert werden und 4) auf alkoholassoziierte Alterungsprozesse als Korrelat der Erkrankung und deren potenzielle therapeutische Implikationen eingegangen werden. Ein kritischer Fokus liegt auf der fehlenden Replikation epigenetischer Befunde und dem Risiko falsch positiver Befunde durch sog. *Batch*-Korrekturen.

1) Garbusow et al. (20) berichten, dass die Zunahme des PIT-Effektes bei Hochrisikotrinkern mit einem PRS für die Alkoholabhängigkeit assoziiert ist. Der Vorteil des PRS liegt darin, dass alle relevanten SNPs gewichtet anhand der Effektstärke für die Assoziation mit dem relevanten Merkmal (hier Menge des Alkoholkonsums) in die Berechnung miteinbezogen werden (108). Die Ergebnisse genomweiter Assoziationsstudien (GWAS) bestätigen die Polygenität von AUD und problematischem Alkoholkonsum (54, 109–111), um beispielsweise Personen mit erhöhtem Risiko für die Entwicklung einer AUD zu identifizieren (112). Eine kürzlich durchgeführte Längsschnittstudie zeigte eine signifikante Vorhersagefähigkeit von PRS für Trinkmenge, Einnahmehäufigkeit und gefährliches Trinken, aber diese Ergebnisse sind durch geringe Mengen an erklärter Varianz gekennzeichnet, die von ~0,5 bis ~1,7 reichen (113). Ältere Studien berichten ähnliche Ergebnisse (59, 112). Jeder der genannten Autoren kommt zu dem Schluss, dass die prädiktive Validität von PRS für Aspekte der Alkoholabhängigkeit von der Größe der zugrunde liegenden Entdeckungsstichprobe aus der entsprechenden GWAS abhängt. Der von Garbusow et al. (2019) gezeigte wesentliche Befund eines erhöhten PIT-Effekts bei Hochrisiko-Trinkern legt nahe, dass starke Auswirkungen von Pawlowschen Hinweisreizen auf das instrumentelle Verhalten einen zentraler Mechanismus des riskanten Alkoholkonsums

darstellen, dessen genetische Basis sich zumindest in Ansätzen im PRS wiederfindet. Über den beschriebenen Endophänotyp aus neuronalen Aktivierungsmustern und Veränderungen im PIT bei AUD ist es gelungen, eine Assoziation zwischen einem zentralen Mechanismus auf Verhaltens- und neuronaler Ebene und dem polygenetischen Fundament der Erkrankung herzustellen. Wie Fullerton & Nurnberger (114) in ihrem Review aufzeigen, kann der PRS klinische Fälle von Kontrollen auf Bevölkerungsebene unterscheiden (108), aber auf individueller Ebene oft nur unzureichend den Erkrankungsstatus identifizieren. Der PRS hat daher (bislang) nicht das Potenzial eines therapierelevanten Biomarkers, sondern soll dazu dienen, die genetisch determinierten behavioralen und neuronalen Mechanismen besser zu verstehen.

2) Des Weiteren zeigen die oben dargestellten Ergebnisse (75) auf der SNP-Ebene einen Zusammenhang zwischen dem OPRM1-Genotyp, der die Verfügbarkeit und Funktion der Opioidrezeptoren reguliert, und der Stärke des PIT-Effekts. Träger des OPRM1-Risikoallels (G+) zeigten den größten PIT-Effekt mit den höchsten Rückfallraten unter der Gruppe der alkoholabhängigen Patient*innen. Der OPRM1-Polymorphismus zeigte in Vorstudien neben einer Assoziation mit der Alkoholerkrankung insbesondere auch Zusammenhänge mit dem Annäherungsverhalten auf konditionierte Reize (115–117), sodass die Assoziation mit PIT plausibel erschien. Tatsächlich war der OPRM1-Genotyp in unserer Studie selbst nicht direkt mit der Rückfallrate assoziiert, erst die Kombination mit der Größe des PIT-Effektes machte eine Vorhersage des Rückfalls möglich. Die Moderatorfunktion auf Verhaltensebene macht deutlich, wie wichtig die Identifikation zentraler behavioraler Mechanismen ist, um differenziertere Aussagen zu genetischen Determinanten treffen zu können.

Der OPRM1-Polymorphismus wurde zudem als potenzieller Biomarker für die Wirksamkeit der Naltrexon-Behandlung indentifiziert (115, 118). Zu den wenigen pharmakologischen Möglichkeiten der Abstinenzerhaltung bei AUD zählen mit Nalmefen und Naltrexon zwei Modulatoren am Opioidrezeptor. Weber und Kollegen (119) konnten eine Reduktion der reizinduzierten Antwort als Folge einer Blockade von sowohl Dopamin als auch der Mu-Opioid-Rezeptoren bei Menschen nachweisen: In einem randomisierten, doppelblinden Design wurde gesunden Probanden entweder der Dopamin-D2/D3-Rezeptorantagonist Amisulprid, der Opioidrezeptorantagonist Naltrexon oder Placebo verabreicht und das Reiz-Reaktions-Verhalten u. a. über ein PIT-Paradigma erfasst. Im Vergleich zu Placebo unterdrückten sowohl Amisulprid als auch Naltrexon signifikant die reizinduzierte Reaktion. Diese Ergebnisse unterstreichen die Bedeutung des PIT-Effektes als zentralen Mechanismus der AUD, der sowohl auf pharmakologische

(119) als auch verhaltensbasierte Interventionen (101-103) anspricht. In Ergänzung zu Weber et al. (119) konnten Garbusow et al. (2016) die Bedeutung des PIT-Effektes auch für sekundäre Verstärker (z. B. Geld im Gegensatz zum primären Verstärker Essen) nachweisen. Ebenfalls interessant ist der Zusammenhang zwischen der Wirksamkeit von Naltrexon und der Belohungserwartung: Berichtet wird eine erhöhte Wirksamkeit von Naltrexon bei einer Untergruppe von Patient*innen, die den Konsum von Alkohol vor allem mit Belohnung assoziierten (120, 121) und solchen, die sehr hohe positive Erwartungen an den Konsum koppelten (122).

Die in Sebold et al. (2021) dargestellten Daten bestätigen die Bedeutung des OPRM1-Polymorphismus für den PIT-Effekt und dessen potentielle Relevanz für die Entstehung und Behandlung einer Alkoholabhängigkeit.

3) Neben Single-SNP-Analysen und GWAS rücken auch immer mehr empirische Untersuchungen zum Einfluss epigenetischer Veränderungen für die Entstehung und Aufrechterhaltung der Alkoholabhängigkeit in den Fokus (123, 124). In zahlreichen Studien konnten über Querschnittserhebungen Unterschiede im Epigenom zwischen Proband*innen, die Alkohol konsumieren (starke Trinker und Patient*innen mit Alkoholabhängigkeit) und gesunden Kontrollen nachgewiesen werden (124–130). Die verwendeten Stichprobengrößen schwanken allerdings stark in ihrer Größe zwischen n = 20 (130) bis zu n = 13.317 (127). Problematisch ist die häufig fehlende Replikation einzelner CpG-Stellen, in denen bereits alkoholassoziierte Veränderungen nachgewiesen wurden. In der erst kürzlich von Lohoff und Kollegen (131) durchgeführten bis dato größten Einzelkohorten-Epigenomweiten-Assoziationsstudie (EWAS) zum Alkoholkonsum (n = 8161) wurde neben 2504 signifikanten mit Alkoholkonsum assoziierten CpG-Stellen zudem in Analogie zu dem von Garbusow et al. (20) verwendeten PRS ein *DNA Methylation Risc Score* (MRS) berechnet, der einen prädiktiven Wert für den Krankheitsstatus hatte. Diese Ergebnisse unterstreichen die Bedeutung epigenetischer Veränderung für die Alkoholabhängigkeit und legen eine kombinierte Auswertung genetischer und epigenetischer Befunde zur Vorhersage komplexer Krankheitsbilder wie der Alkoholabhängigkeit nahe.

Ein häufiges Problem bei der Publikation epigenetischer Befunde ist, dass die Kolleg*innen zwar die neuen Befunde und ggf. auch eine Liste der besten 10–20 CPG-Stellen (im Sinne eines hohen p-Wertes) berichten, oft aber keine Auskunft über die Replikation früherer Methylierungsbefunde (auch z. B. aus der eigenen Arbeitsgruppe) und insbesondere ausgebliebener

Replikationen geben. Der Fokus auf der Identifikation neuer Befunde ist zwar innovativ und erleichtert die Publikation von Ergebnissen, ist aber wenig hilfreich bei der Identifikation falsch positiver Befunde, wie sie u. a. durch die Anwendung von *Batch*-Korrekturen entstehen können. Dies ist nur eines von vielen Beispielen, welche die Problematik der Replizierbarkeit von (epi)genetischen Studienergebnissen aufzeigen. In der von uns durchgeführten Studie (23) konnten wir keine der zuvor als signifikant berichteten CpG-Stellen replizieren. Dies lag sicher auch an der vergleichsweise kleinen Stichprobengröße von n = 69 und einer fehlenden gesunden Kontrollgruppe, die durch unser vor allem auf den Längsschnitt ausgerichtetes Design zustande kam. Neben den fehlenden Replikationen fanden wir auch keine signifikante Veränderung an einzelnen CpG-Stellen im Genom zwischen Patient*innen, die rückfällig wurden und solchen, die abstinent blieben. Was wir fanden, war eine negative Assoziation zwischen der mittleren Methylierung und dem Alkoholkonsum, ein Ergebnis, das sich nur zum Teil in vorherigen Publikationen wiederfindet (132–134). Ein Grund für die Heterogenität der Befunde liegt vermutlich in der Tatsache, dass sowohl die Stichprobenzusammensetzung (Patient*innen vs. starke Trinker und Kontrollen) als auch die Länge des Beobachtungszeitraums stark schwanken (21 Wochen bis 10 Jahre). Das heißt, kurzfristige Veränderungen, die während und kurz nach dem Entzug stattfinden, sind ggf. nicht mehr nachweisbar, während langfristige Veränderungen über einen so langen Zeitraum bislang noch nicht erhoben wurden oder durch eine zu kleine Stichprobengröße nicht identifiziert wurden. Eine weitere Schwierigkeit ergibt sich daraus, dass DNA-Methylierungsstudien an Patient*innen mit AUD, insbesondere Methylierungsmuster des gesamten Genoms, sich im Vergleich zu Single-SNP-Analysen und GWAS in einem relativ frühen methodischen Entwicklungsstadium befinden (135, 136). Wir konnten die Ergebnisse früherer Studien (137, 138) replizieren, die ihre Warnung bei der Anwendung von *Batch*-Korrekturen vor allem für unbalancierte Stichproben aussprechen (23). Unsere Daten zeigen zusätzlich, dass dieses Risiko auch bei balancierten Stichproben besteht. Obwohl es bei den aufgezeigten Analysen um ein sehr umschriebenes und technisches Problem geht, zeigt sich daran pathognomonisch die Vulnerabilität in der Reliabilität epigenetischer Befunde.

4) Eine weitere Methode, um sich epigenetischen Veränderungen bei Patient*innen mit Alkoholabhängigkeit zu nähern, sind Alterungsprozesse, die durch den Alkohol an der Zelle auch im peripheren Blut nachweisbar sind (73, 74). Indem nur ausgewählte CpG-Stellen in die Analyse einbezogen werden, die bereits einen Zusammenhang mit Alterungsprozessen und AUD

gezeigt haben, ist diese Methode sehr viel spezifischer und erheblich weniger anfällig für falsch positive Befunde im Vergleich z. B. zum Whole-Epigenome-Ansatz. Wir haben die eingangs beschriebenen Daten aus (14, 23) erneut ausgewertet, diesmal ergänzt um eine Kontrollstichprobe und eine unabhängig erhobene zweite Kohorte von AUD Patient*innen (76). Die angewandte Analyse beschränkt sich auf das Modell der epigenetischen Uhr (70–72). In einem zweiten Schritt wurden die epigenetischen Befunde in Kombination mit einem PRS für Alkoholkonsum untersucht. Während wir vorangegangene Befunde zum epigenetischen Altern bei Patient*innen mit AUD replizieren konnten (73, 74), zeigen wir nun zusätzlich und erstmalig, dass dieser Prozess bei Patient*innen, die abstinent von Alkohol blieben, deutlich rückläufig war, also potenziell reversibel ist. Die Verwendung von epigenetischen Uhren könnte einen neuen Biomarker mit therapeutischen Implikationen sowohl auf der Verhaltens- als auch auf der molekularen Ebene für die Diagnostik und Therapie von AUD darstellen. Die Möglichkeit potenziell reversibler Alterungsprozesse am Methylom kann ggf. auch dazu genutzt werden, Patient*innen zur Abstinenz zu motivieren und den Erfolg der Abstinenz direkt an der Zelle zu monitoren. Es gilt zu klären, welche Subgruppen besonders sensitiv auf epigenetische Veränderungen sowohl bei Konsum als auch bei Abstinenz von Alkohol reagieren und inwiefern psychosoziale Faktoren und dysfunktionale Lernmechanismen sich in Veränderungen der epigenetischen Uhr abbilden lassen. Therapeutische Implikationen der dargestellten Befunde könnten sich zum einen aus der Möglichkeit ergeben, besonders vulnerable Gruppen zu identifizieren, z. B. Patient*innen, die mit starken Veränderungen im Methylom reagieren, oder auch pharmakogenetisch neue Ziele an den entsprechenden CpG-Stellen für medikamentöse Therapien zu etablieren.

3.3 Die Rolle von chronischem Stresserleben auf belohnungsabhängiges Lernen in Abhängigkeit von kognitiven Funktionen

Neben den oben dargestellten Einflussfaktoren aus dysfunktionalen Lernmechanismen, neuronalen, genetischen und epigenetischen Faktoren, soll im letzten Teil dieser Habilitationsschrift der Einfluss von chronischem Stresserleben auf die oben beschriebenen Lernprozesse und deren Implikation für die Entstehung und Aufrechterhaltung der AUD diskutiert werden. Akutes und chronisches Stresserleben kann Rückfälle begünstigen (139) und es ist unumstritten, dass die Mehrzahl von AUD Patient*innen in Ihrem Leben häufig anhaltenden Stresserfahrungen ausgesetzt war (140). Wir konnten nachweisen (13), dass gesunde Probanden in

Abhängigkeit von ihrer kognitiven Leistungsfähigkeit und dem Ausmaß vorangegangener chronischer Stresserfahrung unterschiedlich stark mit einem Abfall modellbasierten Lernmuster, erfasst über die sequenzielle Entscheidungsfindungsaufgabe *two step*, reagieren. Gillan und Kollegen (141) haben ebenfalls über die *two step*-Aufgabe den Anteil an modellfreien und modellbasierten Reaktionstendenzen an 2000 gesunden Proband*innen über zahlreiche psychiatrische Diagnosekategorien hinweg erfasst. Die Autoren berichten unabhängig von der diagnostischen Kategorie (z. B. Alkoholabhängigkeit, Schizotypie, Depressionen und soziale Angstzustände) eine starke Assoziation zwischen Verhalten auf dem kompulsiven Spektrum (d. h. Störungen, die mit der Wiederholung von Verhaltensweisen trotz negativer Konsequenzen assoziiert sind) und einer Abnahme im modellbasierten Lernen. Chronisches Stresserleben ist ein Faktor, der die Balance zwischen modellfreien und modellbasierten Reaktionstendenzen weiter in Richtung eines habituellen Verhaltens verschiebt (142) und so potenziell zur Entstehung und Aufrechterhaltung der AUD (43) und weiterer psychiatrischer Krankheitsbilder beitragen kann.

Chronische Stressbelastung in der Vergangenheit erhöht das Risiko für die Entwicklung psychiatrischer Störungen (143, 144) und könnte als moderierende Variable zum einen den erhöhten Einfluss individueller kognitiver Fähigkeiten auf Entscheidungsfindungsstrategien begünstigen und gleichzeitig modellfreie Lernprozesse verstärken. Das Training kognitiver Fähigkeiten stellt eine mögliche therapeutische Intervention dar, die vor dem Einfluss von Stresserfahrung auf Entscheidungsfindungsprozesse schützt. Otto und Kollegen (79) berichten einen deutlich reduzierten Abfall modellbasierter Entscheidungsfindungsprozesse unter akuter Stresserfahrung, wenn eine hohe Arbeitsgedächtniskapazität vorhanden war. Radenbach und Kollegen (145) berichten zudem, dass akuter Stress nur dann zu einem Abfall modellbasierter Strategien führt, wenn er in Kombination mit einer anhaltenden chronischen Stressbelastung auftritt. Ziel therapeutischer Interventionen sollte daher auch die Reduktion akuter Stressoren und das Einüben von Entspannungstechniken sein, um darüber die Balance der entgegengesetzten Entscheidungsprozesse zu verstärken.

Auf neurobiologischer Ebene kommt hier erneut dem mesostriatalen Belohnungssystem eine wesentliche Bedeutung zu. Die funktionelle Aktivierung im Striatum als neuronales Korrelat von belohnungsabhängigem Lernen wird moderiert durch kognitive Faktoren wie die fluide Intelligenz und chronisches Stresserleben (146) sowie durch akute Stressoren und damit in

Verbindung stehenden Veränderungen der Kortisolkonzentration während der Entscheidungsfindung (147). Auf Verhaltensebene findet eine Verschiebung weg von kognitiv aufwendigen Abwägungsprozessen hin zu automatisierten Reaktionstendenzen statt. Eine weitere wichtige Moderatorvariable in der Bedeutung dysfunktionaler Lernmechanismen für die AUD ist die Erwartungshaltung gegenüber der Wirkung von Alkohol. Sebold und Kollegen (148) berichten für eine Gruppe von AUD-Patient*innen und gesunde Kontrollen, dass vor allem die Interaktion von einer sehr hohen positiven Erwartung an die Wirkung von Alkohol und wenig modellbasierten Lernprozessen den Rückfall bei Patient*innen mit AUD prädizieren konnte. Diese Interaktion war gekoppelt an eine Abnahme der präfrontalen Aktivierung im Rahmen der Entscheidungsfindung. Dazu kommt, dass im Rahmen der Alkoholabhängigkeit die Toxizität des Alkohols selbst zu einer Abnahme der kognitiven Leistungsfähigkeit führt, wobei hier dosisabhängige Effekte diskutiert werden (für eine Übersichtsarbeit (149)).

Zusammenfassend scheint die Abnahme modellbasierter Strategien die Entstehung und Aufrechterhaltung der AUD zum einen über Umweltfaktoren wie akute und chronische Stresserfahrung und zum anderen über kognitive Variablen wie die Arbeitsgedächtniskapazität, die kognitive Geschwindigkeit und den fluiden IQ zu moderieren. Die Adressierung dieser Komponenten in der Behandlung der AUD bietet daher wichtige therapeutische Implikationen.

Ausblick und zukünftige Forschung

Die dargestellten Studien bestätigen die Komplexität der Mechanismen, die zur Entstehung und Aufrechterhaltung der AUD beitragen können. Neben der genetischen Vulnerabilität beeinflussen zahlreiche Faktoren das individuelle Risiko und den Verlauf der AUD. Zu den wesentlichen Faktoren zählen die Erfahrungen, die wir mit Alkohol gemacht haben, die Erwartung, die wir an seine Wirkung koppeln, die Bedingungen, unter denen wir konsumieren und nicht zuletzt mit welchen kognitiven Ressourcen wir neue Reize und Informationen verarbeiten können, um etablierte Verarbeitungsprozesse zu verändern. Die Wirksamkeit medikamentöser Therapien z. B. mit einem Opioid-Rezeptor-Antagonisten hängt womöglich von den genannten Faktoren ab. Der Vorteil ist ein dynamisches System, das durch Veränderung der einzelnen Faktoren verändert werden kann. Die optimale Behandlung eines/einer Patient*in mit AUD sollte daher im besten Fall die Gesamtheit aller genannten Einflussvariablen adressieren. Die Hinweise aus den dargestellten Forschungsarbeiten zu einer dynamischen Balance von

Lernmechanismen, die abhängig von externen und internen Faktoren in eine Dysbalance geraten und damit die Entstehung und Aufrechterhaltung von Abhängigkeitserkrankungen fördern können, liefern Argumente für die Verwendung eines eher dimensionalen Krankheitsbegriffes der AUD, wie er z. B. in der neusten Ausgabe des DSM verwendet wird (8).

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Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

April 2023

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