# Unravelling the relationship between cigarette smoking and language development

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## Papers associated with this thesis

**Peixinho, J**., Toseeb, U., Mountford, H. S., Bermudez, I., & Newbury, D. F. (2022). The effects of prenatal smoke exposure on language development-a systematic review. *Infant and Child Development*, e2331. (See appendix).

Pradhan, A., Mountford, H., **Peixinho, J**., Rea, E., Prekovic, S., Maxwell, S., Webster, R., Beeson, D., Unravelling the molecular interactions between α7 nicotinic receptor and a RIC3 variant associated with backwards speech. *Cellular and molecular life sciences.* Under review. (See appendix).

# Abbreviations

ACh- acetylcholine

apFRET- acceptor photobleaching fluorescence resonance energy transfer

ASD- autism spectrum disorder

BGT- bungarotoxin

BMI- body mass index

CC- coiled-coil

CS- cigarette smoking

eGFP-enhanced green fluorescent protein

ER: endoplasmic reticulum;

fMRI- functional magnetic resonance imaging

FRET: Försters resonance energy transfer

FSIQ- Full scale IQ

GABA-γ-aminobutyric acid

mCherry- monomeric cherry fluorescent protein

MCS-multiple cloning site

nAChRs- nicotinic acetylcholine receptors

RIC3- resistant to inhibitors of cholinesterase 3

SCZ-schizophrenia

SES- socioeconomic status

SERT- Serotonin transporter

SS- signal sequence

TM- Transmembrane

VIQ- verbal IQ

WM- working memory

WT- wild-type

# Abstract

Cigarette smoking (CS) is a leading cause of mortality and morbidity. Despite increasing knowledge regarding the health threats of CS, its global use remains a problem, even among pregnant women, with 8.1% of pregnant women smoking. In addition to maternal health, prenatal CS has been linked to neurodevelopmental disorders, such as ADHD and ASD, which include deficits in language skills. However, there is little research on CS specific effects on language skills. Nicotine, the addictive component of tobacco, exerts its cognitive effects by binding to the neuronal nicotinic acetylcholine choline receptors (nAChRs), among which the subtypes α7 and α4β2, have been linked to cognitive functions such as working memory (WM). Moreover, recent work linked a rare variant in Resistant to inhibitors of cholinesterase 3 (RIC3; NM_024557.4:c.262G>A, NP_078833.3:p.G88R) to a unique ability to speak backwards, a language skill with hypothesised association with exceptional WM capacity. Could RIC3 variants be a potential link between CS and offspring language outcomes via effects on nAChRs? First, using PubMed and Web Of Science, we systematically reviewed existing literature considering prenatal CS exposure and child language outcomes. Then, we compared the effects of RIC3A26S, RIC3V196FS, and RICT177S on the function of function of human α7 receptors using fluorescently tagged α7 nAChR and Forster's resonance energy transfer (FRET) microscopy imaging. Our systematic review reported negative effects of prenatal CS exposure on offspring language outcomes in 13 of 14 studies reviewed. Our apFRET experiments found the RIC3 variants studied introduced did not affect the interaction of RIC3 and α7 nAChRs in HEK cells. Conversely, in α4β2 experiments, introducing the V196F variant to RIC3 led to significantly increased α4 and β2 expression, as measured by fluorescence. The results of this study reinforce that prenatal CS exposure negatively impacts offspring language outcomes. None of the variants introduced to RIC3 increased interaction with α7, however, the significantly increased α4 and β2 expression in the presence of RICV196F, suggests that effects of RIC3 on language could lie in its effects on α4β2 expression.

# Chapter 1- Introduction

Language is a distinctively human trait, at the very heart of what it means to be human. There are various aspects of language, usually characterised into expression or the ability to communicate thoughts and ideas and, reception, the ability to understand what is communicated to you. These aspects can be realised through diverse modalities such as spoken language, signed language, written language, all of which require the careful recruitment and orchestration of different brain regions and neuron types. Thus, language is essentially a function of the brain that is executed using diverse regions and functions of the brain. However, language is not just an additive exercise of various brain functions but rather the result of the unique recruitment and coordination (temporal and spatial) of diverse higher brain functions (audition, motor control, vision, memory, learning), including their inter- and-intra connectivity pathways. Some of these pathways may be overlapping signalling pathways whilst other may be exclusively used by language. Deciphering the structural, cellular and molecular mechanisms underpinning and modulating this pivotal human trait is a key task of molecular neuroscience.

## 1.1 Models of language development

Overtime, numerous models have been proposed to help explain the process of language development. The traditional, or classical, neurological model of language development used studies of patients with brain injuries that impair language and identified two primary centres of language in the brain, both located on the left side of the brain. These are the Broca's area (located in the inferior frontal gyrus of the left hemisphere of the brain) and the Wernicke's area (located close to the lateral sulcus, near the junction between the parietal and temporal lobes) (Binder et al., 2009) (figure 1.1). The two regions are linked through subcortical white matter, particularly the arcuate fasciculus (Binder et al., 2009). Put simply, Broca's area is involved in planning and executing speech and writing movements, whilst Wernicke's area acts as a receptive area for mapping of auditory stimulus to meaning. Non-auditory stimuli

require additional regions such as the visual cortex for written language (Mayeux and Kandel, 1985). However, damage to the Broca's and/or Wernicke's regions does not account for all types of language disorders. Damage to Broca's area alone has not been found to cause Broca's aphasia or chronic aphasia (Mohr, 1976; Mohr et al., 1978; Alexander and Crutcher., 1990) but rather has been linked to damage at both Wernicke's and Broca's areas (Fridriksson et al., 2015). Similarly, chronic Wernicke's aphasia has not been associated with damage to Wernicke's area (Bogen and Bogen, 1976), but rather to the middle temporal gyrus and surrounds (Dronkers and Baldo, 2009). Inconsistencies remain regarding whether damage to the arcuate fasciculus is responsible for conduction aphasia (Bernal and Ardila, 2009, Anderson et al., 1999; Quigg and Fountain, 1999; Hickok and Poeppel., 2000; Quigg et al., 2006; Dronkers and Baldo, 2009; Buchsbaum et al., 2011). Moreover, research has shown that the language network stretches well beyond classical Broca's and Wernicke's area and involves multiple white matter tracts and subcortical circuits (Ojemann, 1983; Dronkers et al., 2004; Catani et al., 2005; Price, 2012; Fridriksson et al., 2018).

The Hickock-Poeppel dual-stream model aimed to address some limitations of the classical model above as well as other limitations. Namely, when speech perception was measured using syllable discrimination tasks instead of comprehension, severe deficits were found following unilateral left hemisphere lesions (Caplan et al., 1995). It was found that syllable discrimination and word comprehension double dissociate, even when both tasks required differentiating between the same phonemes (Miceli et al., 1980). Essentially, someone unable to reliably differentiate between similar syllables, for example /ba/ and /pa/ was able to hear the word bear and reliably point to a picture of a bear while avoiding a closely- matched phonemic distractor.

The new model hypothesised interconnections between cortical regions by two streams: a dorsal stream supporting auditory-motor integration for speech production and a ventral stream processing speech signals for conceptualization and understanding (Hickok & Poeppel, 2000; 2004; 2007; 2015). The dorsal stream maps sound to articulatory representations and is strongly left-lateralized, projecting from the posterior superior temporal to the inferior frontal cortices. The ventral stream is bilaterally organised and projects from the posterior

middle and inferior temporal gyrus to the anterior middle temporal gyrus (Hickok & Poeppel, 2000; 2004; 2007; 2015). Evidence for bilateral organisation comes from findings that left hemisphere disruption due to stroke (Baker, Blumsteim, & Goodglass, 1981; Rogalsky, et al., 2011; Rogalsky et al., 2008) or functional deactivation in Wada procedures (Hickok, et al., 2008) did not result in a notable decline in the ability of patients to process speech sound information during comprehension tasks (Hickok & Poeppel, 2000, 2004, 2007). Bilateral lesions involving the superior temporal lobe do, however, result in severe speech perception deficits (Buchman et al., 1986; Poeppel, 2001). In short, this multi-domain language architecture indicates that the development and production of language involves most of the higher centres of the brain and that their appropriate inter- and intra-connectivity must be essential for language.

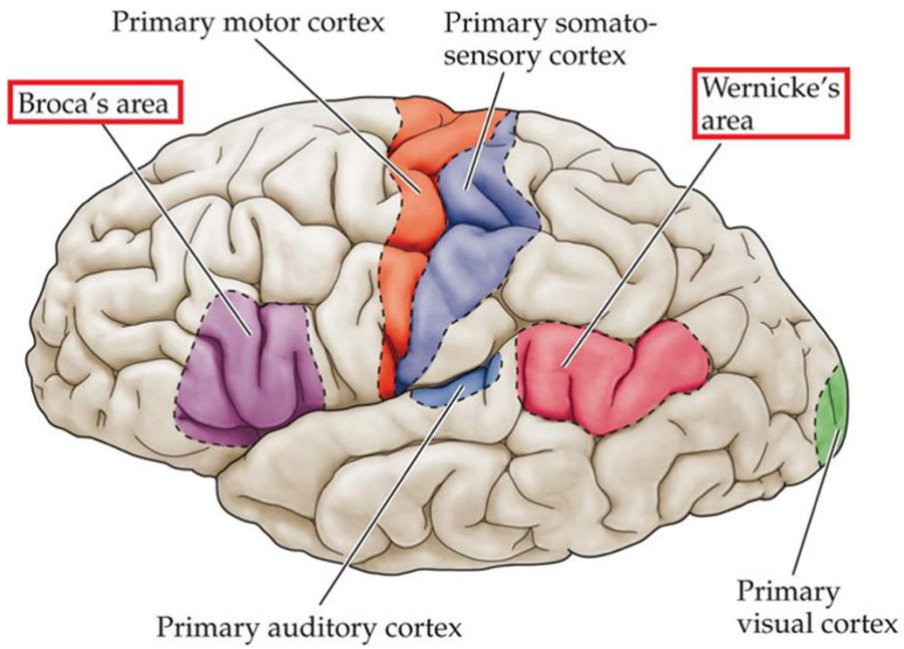


*Figure 1.1 Diagram of the main regions of the brain involved in language.*

So, considering the importance of neural networks on language development, are intact neural networks the most important factor? Recently, some models have shifted their focus to the malleability of language development and acknowledge that, whilst as humans we possess innate brain structures for language learning ability (Chomsky, 2005), the importance

the opportunities afforded by caregiver–child interactions cannot be underestimated (Ford et al 2020). This is to say that adequate language development is complex and not only requires the presence of adequate brain circuitry but also environmental stimulus for optimal development.

Given the complex brain architecture of language, it is likely that language uses multiple neurotransmitter systems. However, most neurotransmitters in the brain exert pleiotropic effects across several brain regions during development as well as in the mature brain, making it difficult to identify specific language neurotransmitters as well as deciphering the molecular mechanisms underlying their "language roles". This problem is illustrated with the neurotransmitter serotonin (5HT). Linkage studies have implicated *Slc6a,* which codes for SERT, in Autism Spectrum Disorder (ASD) (Weiss et al., 2006; Carneiro et al., 2008). Moreover, it has been reported that genetic variants of the promoter region of the serotonin transporter gene (*SERT*) are involved in delayed language onset and intellectual disability in individuals with ASD (Sutcliffe et al., 2005; Hervas et al., 2014; Beitchman et al., 2006). The serotonin transporter removes serotonin from the synaptic gap of serotonergic synapses, thereby determining the strength and duration of synaptic signalling at this type of synapse. The importance of the transporter in serotonergic synapses is demonstrated by the physiological and behavioural effects of social (amphetamines, cocaine) and therapeutic (e.g., Prozac) drugs that inhibit the transporter (Squire et al., 2008). It is not known how the brain serotonergic system relates to reduced verbal communication in individuals with ASD; however, studies that have used positron emission tomography (PET) with a serotonin transporter tracer have shown a reduction in levels of the transporter not only in the brain stem -where the nuclei of brain serotonergic neurones is located, but also in the neocortex, frontal cortex, parietal cortex, rostral middle frontal, insular cortex, anterior cingulate cortex, posterior cingulate cortex, nucleus accumbens, and putamen in individuals with autism (Andersson et al., 2021). Thus, despite that serotonergic presynaptic terminal from brain stem serotonergic release serotonin throughout the brain, only specific regions appear to be affected in those with ASD, including those contributing to language.

This specificity could stem from, for example, the receptor subtype(s) present on the serotonergic synapses. Most neurotransmitters, regardless of their nature (e.g., ligand-gated or G-protein coupled receptors), act on families of receptor types. For example, serotonin receptors comprise seven families of G-protein coupled receptors (5HT1 to 5HT7) and one ligand-gated ion channel receptor (5HT3 receptor). Furthermore, each of the G-protein coupled receptor types is subdivided into subtypes (i.e., 5HT1A to 5HT1) (https://www.guidetopharmacology.org/). The type of receptor present in postsynaptic neurones is defined during development through processes that imply not only cell migration but also target-recognition and dendritic pruning.

The glutamatergic system could also contribute to language due to its extensive involvement in higher cortical functions and its contribution to synaptic plasticity and memory formation (Li and Selkoe, 2020). However, although glutamate signalling is dysregulated in Alzheimer's disease, Fragile X associated Tremor/Ataxia Syndrome, and Aphasic Stroke Syndromes, three conditions in which language is severely impaired (Li and Selkoe., 2020), it is not known how glutamatergic signalling may relate to language dysfunction in these pathological states. Other neurotransmitters that *likely* contribute to language due to their presence in language-relevant regions of the brain (e.g., pre-frontal cortex, gyruses) and role in cognition are the catecholamines (eg. Dopamine, noradrenaline) and acetylcholine (https://www.guidetopharmacology.org/). Both catecholaminergic and cholinergic agents have been shown to influence executive cognitive functions as well as mnemonic encoding and retrieval processes (reviewed by McNamara and Albert, 2004), all of which are needed for language skills such as vocabulary and grammar.

### 1.2 Language is affected by genetic and environmental factors

Language development shows inter-individual variation suggesting that rare genetic variants play a role in language. For example, a twin study which considered the effects of genes on early reading skills, found that language development, as assessed by parameters such as words that are understood, varies in rate of word acquisition as well as in the types of words acquired (DeThorne et al., 2010). Furthermore, several gene variants, or chromosome regions have been implicated in dyslexia and/or speech-related disorders (Fisher et al., 1998; Alarcón

et al., 2008; Vernes et al., 2008; St Pourcain et al., 2014) Since genes as well as the processes in which they are involved are influenced by intrinsic (e.g., hormones) or extrinsic (e.g., environmental toxins, chemicals) factors, a long-standing question in language research is how these genetic and environmental factors affect language, particularly whether these factors operate throughout the development of language (in utero, early infancy, etc.) or at specific stages (e.g., only in utero or early infancy), their molecular targets and language domains (e.g., semantics, phonology, etc). Understanding these distinctions will help deciphering not only the brain domains involved in language but, critically, the molecular mechanisms underlying language and its development in utero and after birth. Therefore, this study focuses on the potential influence of maternal CS exposure and genetic variants that may contribute to language.

### 1.3 Environmental factors

Environmental factors that have been suggested to affect language development include parental input (e.g., quantity and quality of maternal speech) (Hart, 2004), long-exposure times to television (Tomopoulos et al., 2010) and, in utero exposure to alcohol (McGee et al., 2009) and/or cigarette smoking (reviewed by Peixinho et al., 2022). It is not known how these factors, particularly those related to family environment, relate to language development: are the effects language-specific or secondary to other effects such as the all-important brain development? Environmental factors could affect the intra- or inter-connectivity of language-relevant brain regions by acting during brain development in utero or after birth, and these processes could be modified directly by environmental factors by acting on neurones (e.g. activating or inhibiting neurones) or by affecting pivotal brain development events such as neuronal migration or dendritic tree pruning. An example often mentioned of how gene-environment interactions may affect biological functions is a repeat length polymorphism (5-HTTLPR) in the promoter region of the *SLC6A4* mentioned above in relation to autism. The *SLC6A4* has been shown to affect the rate of serotonin uptake (Murphy and Moya, 2011) and clinical studies have suggested that the those carrying 2 copies of the S allele of 5-HTTLPR may be more likely to be affected by life stress contributing to depressive responses to adversity (Caspi et al., 2003). However, other studies have not found strong interactions

between stress and the variant contributing to depression (Culverhouse et al., 2018). The authors hypothesised that if the S allele of 5-HTTLPR increases risk of depression only in stressed individuals, as found by Caspi et al., 2003, then it must not be broadly generalizable effect, but must instead be of modest effect size and only observable in limited situations (Culverhouse et al., 2018). Moreover, a recently published systematic review concluded that there is no consistent evidence of an association between low serotonin levels and depression (Moncrieff et al., 2023). These discrepancies are not easy to resolve but highlight the difficulties in establishing gene-environment relationships, particularly in clinical studies, which often cannot be replicated due to differences in the experimental design (e.g., prospective versus retrospective studies).

Environment-gene interactions are particularly difficult to identify in language, mostly due to the interconnectedness of this function with diverse higher functions of the brain, such as learning and memory. A well-known example of external environmental factors that affect language development is exposure to alcohol in utero. The most distinguishing characteristic of individuals that have been exposed to alcohol in utero is impaired cognitive and behavioural function, which is directly associated with damage to the central nervous system (CNS). In turn, damage to the CNS results in impairment of various aspects of cognition, including overall intellectual performance, executive function, learning and memory, visual-spatial ability, motor function, attention, and activity levels (typically, hyperactivity). Affected individuals also display behavioural problems, including adaptive dysfunction, academic difficulties, and increased rates of psychiatric disorders. In terms of language, some studies have suggested that prenatal alcohol exposure is associated with speech and language disturbances, such as reduced word comprehension (Mattson et al., 1998) and grammatical and semantic abilities (Becker et al., 1990). However, other studies have found no impairment in language skills in infants (Greene et al 1990). These discrepancies may stem from differences in levels of alcohol consumption or the timing of the exposure (eg., beginning of pregnancy *versus* throughout pregnancy), which would result in different levels of CNS damage and hence language impairment (McGee et al., 2009). Thus, the question that arises from these studies is whether the language impairment seen in children exposed to alcohol *in utero* is secondary to injuries to the CNS.

How could alcohol produce damage to the CNS? Firstly, alcohol is a well-known teratogen which has been shown by both clinical and animal studies to be able to cross the placenta, accumulating in the amniotic fluid (Brien et al, 1983). Alcohol affects every organ system of the developing foetus (Popova et al., 2016), especially the CNS. Studies of children with foetal alcohol syndrome using magnetic resonance imaging (MRI) or functional (fMRI) have shown that exposure to alcohol reduces the volume of the parietal cortex (Archibald et al., 2001), and this region is linked to the cognitive processing of language and in the sensorimotor control of writing (Brownsett and Wise, 2010), among other higher functions. Other brain regions, such as the temporal and occipital lobes are not affected, suggesting a region-specific effect of alcohol (Archibald et al., 2001). Autopsies of infants born to mothers that have consumed alcohol during pregnancy have shown devastating effects on the development of the brain, including incorrectly located neurones, reduced dendritic trees, poorly developed olfactory lobes and optic tracts, fewer cells in the dentate gyrus (which is part of the hippocampus, which is essential for memory), and fewer cells in the cerebellum (reviewed by Chen et al., 2003). It may be that these effects are the result of interactions between alcohol and its main brain targets, nicotinic acetylcholine receptors (nAChR) and GABA-A receptors, both of which contribute to neuronal migration and neuronal recognition during development (Liu et al., 2007). These data suggest that nAChRs may be an important link between environmental insults and cognition.

Nicotine, is an agonist of nAChR and is the major addictive component of tobacco, suggesting nicotine as a major contributor to the health burden associated to CS, including deficits in neuronal and language development. The content of nicotine in cigarettes varies depending on the brand, type of tobacco and cigarette production. However, on average most cigarettes will contain 12-15 (around 0.1-0.2% of the total weight of the cigarette) but on average, a person absorbs only 1-2 mg (around 6 -12 µg per cigarette (https://www.medicalnewstoday.com/articles/how-much-nicotine-is-in-one-cigarette#nicotine-amount). Effects on nAChR function occur as 20-100 nG of nicotine reaches the brain (Brody et al., 2006)

CS is another major environmental insult that not only occurs at similar prevalence to that of alcohol consumption during pregnancy but also often co-occurs with alcohol consumption (Cannon et al., 2012, Kvigne et al., 2003, May et al., 2000, Meschke et al., 2013, Viljoen et al., 2002). MRI Studies on 6-9 year old children exposed to prenatal CS have shown an association between CS and the thinning of cognitive control regions such as the superior frontal, parietal, lateral occipital, and precentral cortices, as well as smaller total brain, gray, and white matter, as well as thalamic volumes (El Marroun et al., 2014; Margolis et al., 2021). These data recapitulate animal study findings which have reported negative effects of prenatal exposure to nicotine on attention and inhibitory control (Bryden et al., 2016), suggesting that prenatal ETS exposure may adversely impact human brain development, and specifically cognitive control circuits. Together, these data suggest a potential link between CS and cognitive outcomes in general.

The picture that emerges from the studies mentioned above is that certain environmental insults, namely alcohol and CS exposure, affect primarily the structure of the brain, which likely underlies the cognitive deficit observed in children exposed to alcohol and/or CS in utero. What remains unresolved is if the observed language impairments are secondary to the effects on the cognition apparatus of the brain. Although language is not fully developed in-utero, there is evidence that some elements of this function are present prenatally such as the ability to recognise maternal sound and speech patterns and to differentiate these from other humans (Graven and Browne, 2008). Changes in areas relevant to language may produce injury that is not surmountable by subsequent developmental events.

### 1.4 nAChR chaperones and language

Evidence that genes influence language comes from twin studies (Dale et al., 2000; Eley et al., 2001) as well as from studies that have identified mutations in genes causing familial language disorders such as childhood apraxia of speech (Vernes et al., 2008), other disorders that present some language difficulties as one of many phenotypic features present (e.g., polymicrogyria (Smith et al., 2018). More recently, genetic studies of language traits such as backward speech have also shed light on the variety of different genes that may affect language, further highlighting the complexity of this CNS function (Prekovic et al.,2016).

Specifically, one such study involving a Serbian family with members reporting the ability to speak backwards, identified three novel coding variants in the *RIC3*, *RIPK1* and *ZBED5* genes that co-segregated with the trait in the family and were suggested to be functional (Prekovic et al., 2016). EEG and fMRI data from the proband showed that working memory (WM) areas of the brain (prefrontal cortex, fusiform gyrus) activate during backward-speech, suggesting that backward speech relies upon WM, a cognitive element with a limited capacity that can store information but transiently (Prekovic et al., 2016).

Of the three variants identified by Prekovic et al. (2016), the *RIC3* variant may be highly significant regarding cognition, and by extension WM. First, it has been suggested that variants in RIC3 are associated with cognitive dysfunction (Severance and Yolken., 2007). RIC3 was reported to be elevated in post-mortem brains of those with bipolar disorder (BD) as well as schizophrenia (SCZ), with RIC3 expression levels correlating strongly with α7 levels as well as α4 and β2 levels in unaffected controls and those with SCZ and with α7 in those with BD (Severance and Yolken, 2007). Strikingly, an early report has also hypothesised a link between SCZ and backwards speech (Critchley et al., 1928). As such, this highlights a potential link between α7 nAChRs and backwards speech.

Secondly, RIC3 facilitates nAChR assembly within the ER (Lansdell et al., 2005). Brain nAChRs, particularly the homomeric α7 and the heteromeric α4β2 nAChR are highly expressed in the hippocampus, thalamus, and cortex and contribute to cognition, attention, and WM (Thomsen et al., 2010; Wallace and Porter, 2011). Moreover, the α7 and α4β2 nAChRs affect neuronal migration and cell targeting during neuronal development (Role and Berg, 1996), when the foundations for building language are established. Thus, RIC3 may affect language by influencing the assembly and maturation of nAChRs, which may in turn influence language through their effects on cognition.

### 1.5 Aims of the study

As highlighted throughout this introduction, there is a lack of research considering verbal language outcomes in the context of CS. Firstly, we aim to address this gap in the literature by systematically reviewing published studies on prenatal CS and language development. This

review highlighted that there is a link between prenatal exposure to CS and reduced language skills, albeit it is not clear whether those effects are directly on language or indirect through reduced cognitive abilities. As such, in light of recent findings (Prekovic et al., 2016), this thesis also examined the impact of the nAChR chaperone, *RIC3* variants linked to cognitive performance in language by examining the effects of variants associated with cognitive traits on the expression of α7 and α4β2 nAChR expressed in *in vitro* cell systems using confocal microscopy FRET approaches. Individuals with potentially functional RIC3 variants were identified from genomic data from the UK10K and ALSPAC datasets.

# Chapter 2

## The effects of maternal smoking or exposure during pregnancy on language

## 2.1 Introduction

### 2.1.1 Prenatal environmental exposures can affect brain development

As discussed in the introduction, exposure to toxic chemicals before birth can affect brain development. An example of this is exposure to nicotine through maternal CS. Regardless of worldwide education and sanctions related to CS, it is estimated that 1.7% of pregnant women worldwide and 8.1% of pregnant women in Europe smoke (Lange et al., 2018) highlighting that maternal CS remains a prominent problem in today's society.

Despite there being over 3000 different chemicals in tobacco smoke, nicotine is the main psychoactive component of CS. Nicotine is an alkaloid naturally found in the nightshade family of plants, including the tobacco plant, and primary exposure to this chemical occurs through active and passive smoking (Fagerström, 2014). In the brain, nicotine binds to nicotinic acetylcholine receptors (nAChR) activating the reward system, and exerts its action in the brain through $\alpha 4\beta 2^*$ nAChR (*denotes possible assembly with other nicotinic subunits) (Tapper et al., 2004).

### 2.1.2 Nicotinic receptors and memory

Various studies have implicated nAChRs in cognitive and cortical functions such as learning and memory and cortical neurophysiology. In animal models, systemic nicotine administration has been found to be associated with the improvement of various memory domains, including working memory (WM), which we have previously mentioned is relevant to language in the context of backwards speech (Levin et al., 2006; French et al., 2006; Arendash et al., 1995; Socci et al., 1995; Prekovic et al., 2016).

### 2.1.3 Smoking and links to language and cognition

Maternal CS during pregnancy is known to exert direct negative effects on birth outcomes including low birth weight and preterm birth (Salihu and Wilson, 2007), ear infections, asthma, reduced cognitive function and behavioural difficulties (DiFranza et al, 2004).

Regarding neurodevelopment, exposure to CS in-utero has been associated with an increased risk of Attention Deficit Hyperactivity Disorder (ADHD) (Langley et al., 2005; He et al., 2020), Conduct Disorder (Ruisch et al., 2018), and with a 29% increased risk of Schizophrenia, (Hunter et al., 2020). In addition, prenatal smoking is associated with subtypes of ASD such as Pervasive Developmental Disorder (PDD) (Tran et al., 2013).

### 2.1.4 Language specific timescales

There are well defined timescales for language development and, indeed, for when parents expect their child to learn to speak. Word comprehension develops rapidly in the first year of life with most children developing active vocabulary by the age of 2 (Fenson et al., 2000). The initiation of school and introduction to teaching alters the linguistic input to which the child is exposed and by the age of 6 most children have a well-developed vocabulary and have complete phonological production ability (Hoff, 2009). From the age of 6 to the onset of puberty, at around 12 years, strategies for generating and integrating information emerge, including more sophisticated use of language through use of more complex sentences and grammar (Rosselli et al., 2014).

Despite the above, there is evidence of emergence of language related mechanisms during foetal development. During this time, the brain undergoes significant developmental changes mostly in the form of synaptogenesis (Kostović et al., 2010). Additionally, animal models have shown that rapid reorganization of the auditory cortex occurs soon after the onset of hearing in rats (Chang et al., 2003). In humans, this is thought to occur by 27 weeks of gestation (Hepper et al., 1994). These data are indicative of the presence of learning ability in humans even prior to birth (Vouloumanos et al., 2007; Moon et al., 2000). Consistent with this, studies have shown that foetuses become attuned to various features of the surrounding auditory environment including the native language of the mother or her environment (DeCasper and Fifer., 1980; Moon et al., 1993), familiar melodies (Hepper et al., 1993) or fragments of stories heard during pregnancy (DeCasper and Spence 1986), and even the mother's voice (Kisilevsky et al., 2003). The above data highlight foetal development as a particularly fragile and important time for language development. It is possible that environmental insults, such as CS, could be particularly damaging to neuronal functions, including language, with numerous

early studies highlighting a specific effect of CS on verbal memory and processing speed (Richards et al.,2003; Ott et al., 2004; Buschke and Fuld 1974). If this is the case, it is thus likely that such effects would be seen from the time where overt language development is expected, at the age of 2.

### 2.1.5 Confounders

Despite ever increasing research on the effects of smoking, potential confounders continue to be difficult to delineate and account for. Maternal IQ and socioeconomic status (SES) appear to be particularly relevant to this field of study. This is due to the fact that these factors have all been shown to increase the likelihood of smoking. Studies have found that smoking is more prevalent in those of lower SES (Hiscock et al., 2012). Additionally, over the last few decades, the difference in the prevalence of people smoking across different educational levels has become more marked with more of those of SES smoking (Drope et al., 2018). The above data suggest that IQ as well as SES influence CS uptake, making these factors particularly relevant to consider as confounders when relating smoking to language outcomes.

Overall, there is evidence that prenatal environmental factors have various negative health effects on the foetus. These vary from physical to cognitive and indeed there is indication that language may be one of the latter characteristics affected. To this end, we aimed to systematically review existing literature considering maternal SDP or exposure and language outcomes.

## 2.2 Methods

In order to retrieve existing literature, a systematic review of journal articles published between the years 2000 and 2020 was conducted. Web Of Science (https://clarivate.com/webofsciencegroup/solutions/web-of-science/) and Pubmed (https://pubmed.ncbi.nlm.nih.gov/) were searched using comprehensive search strategies and the search terms below:

(((((((Child*)) AND ((((((((((((((((Develop*)) OR ((Language))) OR ((languages*))) OR ((Language*))) OR ((Language[MeSH Terms]))) OR ((Neuro*))) OR ((Vocab*))) OR ((Grammar))) OR ((Attention deficit hyperactivity disorder))) OR ((Attention deficit hyperactivity disorder[MeSH Terms]))) OR ((Autis*))) OR ((Dyslex*))) OR ((Dysprax*))) OR ((Speech))) OR ((Speech[MeSH Terms])))))) AND (((((((((Nicotin*)) OR ((Cigar*))) OR ((tobacco products))) OR ((Tobacco products[MeSH Terms]))) OR ((tobacco))) OR ((tobacco[MeSH Terms])))))) AND ((((((((((Parent*)) AND ((Smok*)))) OR ((((Matern*)) AND ((Smok*))))) OR ((((Passive)) AND ((Smok*))))) OR ((((secondhand)) AND ((Smok*))))) OR (((secondhand) AND (smok*)))) OR ((((household)) AND ((Smok*))))) OR ((((household*)) AND ((Smok*))))))) AND ((((((((((((prenatal*)) OR ((prenatal))) OR ((pregnan*))) OR ((uterus))) OR ((utero))) OR ((uterus[MeSH Terms]))) OR ((Mother))) OR ((mothers))) OR ((mum))) OR ((Mothers[MeSH Terms])))))) AND ((Expos*)).

The same search terms were used in the Web Of Science, with the exception of the inclusion of MeSH terms as this is not available on this platform. Filters applied to both were that these studies must have been conducted in the last 20 years, and the study must be in English, and outcomes should be articles or letters. In Web Of Science, no measures were included at the search stage to exclude animal studies as there was no clear option in its search engine but any animal studies were excluded at further stages.

When examining the search results, firstly, myself and my supervisor, Dianne Newbury, considered only information in the title and abstract to exclude non-relevant results. A second stage considered more detailed information from the full text and screened for in-depth details of the study design. The same inclusion and exclusion criteria were employed across both stages (for full list of inclusion and exclusion criteria, see appendix table S1). At the end, quality assessment of the remaining studies was conducted according to the Kuyper, 1991)checklist. At this stage, studies were excluded if the language outcome was not verbal, if the age of child language assessment did not fall into the age range of interest, if the exposure did not specifically consider maternal SDP or exposure or if they did not meet the quality criteria. Where multiple studies in the final list used the same cohort, one study was selected on the basis of the relevance of outcomes studied and/or sample size (see figure 2.1 for a flow chart of the study process).

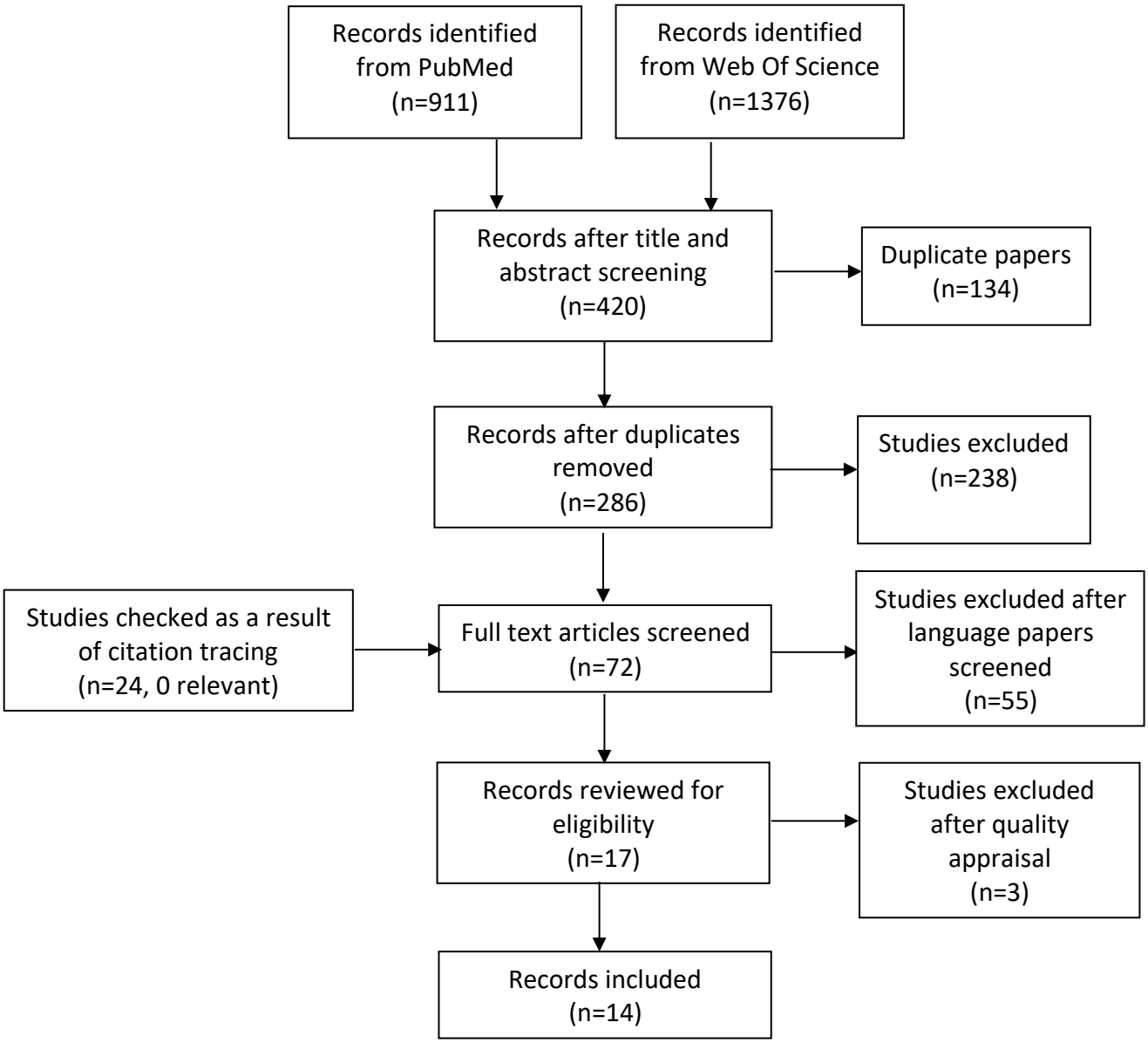


*Figure 2.1. Flow chart of study screening process.*

## 2.3 Results

### 2.3.1 Overall, CS exposure negatively affected language outcomes

Overall, our review found that CS exposure during pregnancy can negatively affect language outcomes. Of the 14 studies found after searching WOS and PubMed which met relevance and quality criteria (see Peixinho et al., 2022) which were included in the review, 13 (93%) reported negative associations between maternal pre-pregnancy CS, CS during pregnancy or exposure to smoke and childhood language outcomes (table 1; Alati et al., 2008; Eriksen et al., 2012; Gilman, Gardener, & Buka, 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur, Knox, & Lancashire, 2001; Mohamed, Loy, Lim, Al Mamun, & Jan Mohamed, 2018; Neumann et al., 2019; Polanska et al., 2017). Six of the 14 studies reported associations $p \leq 0.01$ (Alati et al., 2008; Gilman et al., 2008; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; MacArthur et al., 2001). and seven reported associations of $0.001<P<0.05$ (Eriksen et al., 2012; Heinonen et al., 2011; Julvez et al., 2007; Lee et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017) (figure 2.1, Table 1).

### 2.3.2 Language assessments differed across studies

Although all the studies in this review were screened and selected to consider child language development, the methods of ascertaining language ability varied between studies, as did the age of child assessment (Table 1). Five of the fourteen studies (36%) included in considered verbal IQ (VIQ) as a measure of language ability (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Julvez et al., 2007; MacArthur et al., 2001). Despite an overall negative effect of CS on language, different language components, did differ across the tests used. Measures of VIQ were more likely to report smaller P values ($p<0.001$) (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Julvez et al., 2007; MacArthur et al., 2001)

### 2.3.3 Assessment of CS exposure

Maternal CS exposure was investigated at various times during pregnancy, across studies, and using diverse methods. In our review, we considered mothers who had reported smoking data by six months post-partum, however, different studies gathered this data at different times and with varying degrees of frequency. Five studies (36%) only collected exposure data once (Alati et al., 2008; Eriksen et al., 2012; Lee et al., 2019; Moore et al., 2020; Neumann et al., 2019)) at various points in the pregnancy. Five further studies (36%) collected exposure data either within a week of delivery (Heinonen et al., 2011; Hsieh et al., 2008; MacArthur et al., 2001; Mohamed et al., 2018) or five months after birth (Huijbregts et al., 2006). The remaining four studies (29%) took repeated measures during the first, second, and third trimesters (Gilman et al., 2008; Hernandez-Martinez et al., 2017; Polanska et al., 2017) and every year up to 4 years postnatally (Julvez et al., 2007).

### 2.3.4 Nicotine dosage was associated with severity of offspring language outcomes

Seven (50%) studies (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017) explored the relationship between nicotine dosage and language and six of these reported stronger effects in the offspring of those who were exposed to CS more heavily during pregnancy (Alati et al., 2008; Eriksen et al., 2012, Heinonen et al., 2011; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017). This suggests that now only is exposure important in the link between, but also implicated the amount of CS exposure in the severity of these effects on language outcomes.

Categorisation of mothers according to CS also differed across studies. Some studies sub-categorised mothers by the number of cigarettes smoked daily (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011) whereas others used quantitative grouping according to levels cotinine found (Lee et al., 2019; Polanska et al., 2017). Other studies simply grouped the mothers into smokers or non-smokers (Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Moore et al., 2020; Neumann et al., 2019).

Different categorisation did not result in differences in the findings of these studies regarding significance or direction of effects.

Various methods were used to gather CS exposure data (figure 2.2). These methods included questionnaires (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Julvez et al., 2007; MacArthur et al., 2001; Neumann et al., 2019), as well as more direct measures via cotinine, which were measured using; urine (Lee et al., 2019; Moore et al., 2020), saliva samples (Polanska et al., 2017), cord blood (Hsieh et al., 2008), or hair samples (Mohamed et al., 2018). Nearly all studies using cotinine highlighted a direct ($P>0.001$), effect between nicotine exposure and language outcomes (Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017). Conversely, questionnaire-based studies found that their results were not altered by the time of data collection.

One difficulty found while considering nicotine exposure was whether this was active or passive. Maternal questionnaires only consider active exposure via self-reported CS whilst cotinine measurements consider both active and passive exposure. Out of the 10 studies which used questionnaires (figure 2.2), 6 attempted to address passive exposure through the use of paternal or home environment data (Alati et al., 2008; Eriksen et al., 2012; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Julvez et al., 2007; Polanska et al., 2017). One study (Hernandez-Martinez et al., 2017) reported non-significant effects of these environmental exposures while three studies reported significant effects (Alati et al., 2008; Eriksen et al., 2012; Huijbregts et al., 2006). The other two studies included these covariates in their models but did not report their significance.

| Author (Year) | PMID | Sample size<br>92-999<br>1000-9999<br>>10000 | Smoking measurement<br>questionnaire<br>direct measurement | Outcome measure | Significance<br>P<0.05<br>P<0.001<br>P<0.0001<br>NS | Effect<br>direct<br>indirect<br>no effect |
|---|---|---|---|---|---|---|
| MacArthur et al (2001) | 11213007 | | | VIQ 9-11yrs | | |
| Huijbregts et al (2006) | 28360824 | | | Vocab 3-5yrs | | |
| Julvez et al (2007) | 17550944 | | | VIQ 4yrs | | |
| Alati et al (2008) | 18670372 | | | VIQ 8yrs | | |
| Gilman et al (2008) | 18653646 | | | VIQ 7yrs | | |
| Hsieh et al (2008) | 18577398 | | | Language 2yrs | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Heinonen et al (2011) | 21397413 | | | Vocab & comprehension 4-5yrs | | |
| Eriksen et al (2012) | 23316364 | | | VIQ 5yrs | | |
| Hernandez-Martinez et al (2016) | 27465062 | | | Language & vocab 2-3yrs | | |
| Mohamed et al (2017) | 28803192 | | | Language 2 yrs | | |
| Polanska et al (2017) | 28714930 | | | Language 2 yrs | | |
| Lee et al (2019) | 30894196 | | | Language 2 yrs | | |
| Neumann et al (2019) | 30974313 | | | Vocab 4-5yrs | | |
| Moore et al (2020) | 31759580 | | | Language & vocab 4-5yrs | | |

*Figure 2.2 – Summary of broad study findings*

Table 1 – Fourteen studies included in systematic review (Results summary)

| Author (Year) | Title (PMID) | P-value (effect size, where given)[a] | Study conclusion | Confounders and effects |
|---|---|---|---|---|
| MacArthur et al (2001) | Effects at age 9 of maternal smoking in pregnancy: experimental & observational findings (11213007). | P<0.001 (Max VIQ change = -3.7) | Significant association between maternal smoking and VIQ (persistent vs stopped-smokers). Effect was indirect. | Parental factors (Mother's educational level and age), birth factors (birthweight, birth-length, head circumference, breastfeeding) and family/home factors (parity, home location, maternal employment) were independent predictors of VIQ. Association to smoking was accounted for by these variables. |
| Huijbregts et al (2006) | Interrelations between maternal smoking during pregnancy, birth weight & sociodemographic factors in the prediction of early cognitive abilities (28360824). | P<0.001 (B±SE= -0.17 ± 0.034) | Significant association between maternal smoking and vocabulary. Effect was indirect | Parental factors (maternal education), birth factors (birthweight, gestation, sex) and family/home factors (family income) were independent predictors of vocabulary. Association to smoking was accounted for by maternal education and birth weight. |
| Julvez et al (2007) | Maternal smoking habits & cognitive development of children at age 4 yrs in a population-based cohort (17550944). | P=0.03 (β=-0.59, 95%CI= -1.11 to -0.07) | Marginal association between maternal smoking and VIQ. Effect was direct. | Parental factors (maternal education) and family/home factors (social class) were independent predictors of VIQ. Association to smoking remained after adjusting for these effects. |
| Alati et al (2008) | Intrauterine exposure to alcohol & tobacco use and childhood IQ: Findings from a parental-offspring comparison within ALSPAC (18670372). | P<0.001 (Mean VIQ change = -2.63, 95%CI= -3.42 to -1.84) | Significant association between maternal and paternal smoking and VIQ Effect was indirect. | Parental factors (Parental education), child factors (sex) and family/home factors (social class, parity, home ownership and house crowding) were independent predictors of VIQ. Association to smoking was accounted for by parental education. |

| | | | | |
|---|---|---|---|---|
| Gilman et al (2008) | Maternal smoking during pregnancy & children's cognitive & physical development: a causal risk factor? (18653646). | P<0.001 (Max VIQ change = -0.77, 95%CI=-1.12 to -0.41, adjusted model) | Significant association between maternal smoking and VIQ Effect was indirect. | Parental factors (Mother's educational level, parental age, marital status, parental mental health) and family/home factors (social class, parity, maternal employment) were independent predictors of VIQ. Association to smoking remained after adjusting for these variables. |
| Hsieh et al (2008) | *CYP1A1* Ile462Val & *GSTT1* modify the effect of cord blood cotinine on neurodevelopment at 2 yrs of age (18577398). | P<0.0001 (β±SE= -10.15±2.24) | Significant association between maternal cotinine levels and language. Effect was direct | Parental factors (maternal education and ethnicity) and family/home factors (income) were independent predictors of language. Association to smoking remained after adjusting for these effects. |

Table 1 (continued) – Fourteen studies included in systematic review (Results summary)

| Author (Year) | Title (PMID) | P-value (effect size, where given)[a] | Study conclusion | Confounders and effects |
|---|---|---|---|---|
| Heinonen et al (2011) | Longitudinal study of smoking cessation before pregnancy & children's cognitive abilities at 56 months of age (21397413). | P<0.05 (β= −12.83, 95%CI=-21.30 to -4.35, pre-pregnancy smoking) | Marginal association between smoking >10 cigarettes/day before pregnancy and language comprehension. Effect was direct. | Parental factors (Parental education), birth factors (sex) and family/home factors (social class, parity, home ownership and house crowding) were independent predictors of comprehension. Association to smoking remained after accounting for these variables. |
| Eriksen et al (2012) | Effects of Tobacco Smoking in Pregnancy on Offspring Intelligence at the Age of 5 (23316364) | P<0.05 (max VIQ change = -2.5, 95%CI=-4.7 to -0.4) | Significant association between smoking >10 cigarettes/day and VIQ Effect was indirect. | Parental factors (parental education, maternal IQ, maternal age, maternal BMI), family factors (parity, smoke in house, parental marital status, home environment) were associated with child outcomes. Association to smoking was accounted for by these variables. |
| Hernandez-Martinez et al (2016) | Effects of prenatal nicotine exposure on infant language development: A cohort follow up study (27465062). | P=0.001 (mean Language Development Age change = -1.24) | Significant association between smoking and language development. Effect was direct | Parental factors (maternal age) and family/home factors (social class) were independent predictors of language. Association to smoking remained after accounting for these variables. |

| | | | | |
|---|---|---|---|---|
| Mohamed et al (2017) | Early life second-hand smoke exposure assessed by hair nicotine biomarker may reduce children's neurodevelopment at 2 yrs of age (28803192). | P=0.025 (β=-1.920) | Marginal association between hair cotinine level and communication. Effect was direct. | Parental factors (parental education), child factors (sex) and family factors (household income) were independently associated with communication. Association to smoking remained after adjusting for these variables. |
| Polanska et al (2017) | Environmental tobacco smoke exposure during pregnancy & child neurodevelopment (28714930). | P=0.009 (β=-5.19, adjusted model) | Marginal association between maternal cotinine levels in 1$^{st}$ and 2$^{nd}$ trimester and language development. Effect was direct. | Models were adjusted for parental factors (maternal IQ, maternal age, alcohol consumption), family factors (SES, parental marital status and parity) and birth factors (gestation, pregnancy complications, breastfeeding). Association to smoking remained after accounting for these variables. |

Table 1 (continued) – Fourteen studies included in systematic review (Results summary)

| Author (Year) | Title (PMID) | P-value (effect size, where given)[a] | Study conclusion | Confounders and effects |
|---|---|---|---|---|
| Lee et al (2019) | Exposure to prenatal second-hand smoke and early neurodevelopment: MOCEH study (30894196) | P=0.04 (β = − 2.73, 95% CI= -5.32 to -0.15, adjusted model) | Association between urinary cotinine and language development. Effect was direct. | Parental factors (maternal education, maternal age), birth factors (birthweight, breastfeeding), family factors (home location) and genetic factors (polymorphisms in *GSTM1/GSTT1* genes) were associated with development. Association to smoking remained even after accounting for these variables. |
| Neumann et al (2019) | A longitudinal study of antenatal & perinatal risk factors in early childhood cognition: Evidence from Growing Up in New Zealand (30974313). | P<0.05 (OR language below expected =1.28 (95% CI=1.04-1.57, adjusted model, pre-pregnancy smoking). | Marginal association between smoking pre-pregnancy and receptive language. Effect was direct. | Parental factors (maternal anxiety/depression and maternal diet) were independently associated with vocabulary outcomes. Association to smoking remained even after accounting for these variables. |
| Moore et al (2020) | Prenatal exposure to tobacco & offspring neurocognitive development in the healthy start study (31759580) | P=0.83, OR=1.8 (95%CI=-3.0 to 6.6, adjusted model) | No association between smoking and receptive vocabulary or communication difficulties. | Parental factors (maternal education, maternal age, maternal ethnicity), birth factors (birthweight and breastfeeding) and family factors (family income) were associated with language outcomes. |

[a] Effect sizes are reported with non-smokers as the baseline. In many papers, multiple comparison groups (e.g., different smoking levels) and different outcomes were considered. In these studies, the maximum effect is reported. Effect sizes will not be comparable

across studies. All effects reported are for unadjusted baseline models unless stated. See each individual paper for details of measures, models and effects

## 2.4 Discussion

Despite the vast literature regarding the effects of nicotine exposure on foetal health and child cognition, there is little research regarding its specific effects on language development. In this systematic review, we screened over 1000 papers focussed upon 14 papers that specifically considered language outcomes in relation to *in-utero* CS exposure. Thirteen of the 14 papers examined (93%) reported a negative association between CS or exposure and language outcomes (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017).

While there is limited research considering CS in regards to language, the negative overall trend between CS and language outcomes found in our review is somewhat expected, particularly when considering effects of other environmental exposures, namely alcohol consumption. In the introduction to this thesis, we mentioned that ethanol acts on nAChRs leading to alterations in brain areas associated with memory as well brain morphology and neuronal migration alterations (Chen et al., 2003). Similarly to alcohol, in regards to CS, it may be that these effects are the result of interactions between the nicotine in CS and its main brain targets, nAChRs which may consequently contribute to neuronal migration and neuronal recognition during development (Liu et al., 2007). Studies have shown that maternal smoking deprives the foetus of oxygen, causing hypoxia (Socol et al., 1982; Hutter and Jaegg., 2010). This has in turn been shown to disrupt neuronal migration leading to thinner cortical plates in affected mice (Zechel et al., 2005; Vasilev et al 2016). As mentioned previously, in humans, the cortex is highly important to language and as such, it Is possible that morphological alterations resulting from CS could impact language ability.

In line with previous reports, there was some inconsistency regarding the nature of the relationship between maternal CS or exposure and language development. Similarly to early reports which highlighted direct effects of CS exposure on overall cognition and verbal outcomes specifically (Fried et al. 1998), eight studies in this review also concluded that cs directly impaired early language (Heinonen et al., 2011; Hernandez-Martinez et al., 2017;

Hsieh et al., 2008; Julvez et al., 2007; Lee et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017). On the other hand, five concluded that the observed effects could be explained by confounding factors (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Huijbregts et al., 2006; MacArthur et al., 2001). These confounding factors varied between study designs making it hard to make a conclusion about the direction of effects. However, overall, more studies reported a direct effect thus suggesting that smoking, regardless of confounders likely has an effect on language outcomes.

Nevertheless, all of the studies in our review considered either the number of cigarettes the mother smoked, which is likely directly proportional to the amount of nicotine consumed by the mother, or considered the mother's cotinine levels. In light of this this and the consistent negative effect of CS or exposure to language outcomes seen in our review alongside the links between the sites of nicotine activation in the brain, nAChRs, and cognition in humans as well as animals (Newhouse et al., 2012; Levin et al., 1998) research on nAChR function poses an interesting mechanism to attempt to link CS and language outcomes.

Only five of the 14 studies in this review considered direct measures of nicotine, such as cotinine (Hsieh et al., 2008; Mohamed et al., 2017; Polanska et al., 2017; Lee et al., 2019; Moore et al., 2020). The other 9 studies used questionnaires to assess CS exposure levels. The latter poses a significant challenge in delineating what these results mean as that tobacco smoke contains over 3000 chemicals (American lung association., 2020). The fact that the majority of studies in this review used questionnaires means that associations found in these studies are not just directly considering the effect of nicotine on language but rather also all the other components of CS. Nevertheless, as nicotine is the main component of CS, it is possible to surmise that the exposure reported in questionnaires correlates to directly measured nicotine and thus is a valid first point of exploration of links between CS and later language outcomes

An important consideration is that the mother's nicotine levels do not always equate to those in the foetus. In humans, circulating foetal nicotine has been found to be 15% higher than in the mothers (Luck et al., 1985; Pastrakuljic et al., 1998). The latter suggests that correlating maternal CS or the foetal nicotine level is not always accurate adding another layer of complexity in discerning links between CS and later language outcomes. However,

considering that 1.7% of pregnant women worldwide and 8.1% of pregnant women in Europe smoke (Lange et al., 2018), and that 9.1% of women who smoke continue to smoke to the time of delivery (Nuffield Trust., 2019) focusing specifically on the mother's CS or nicotine levels is particularly relevant for consideration of the links between CS and language outcomes.

To conclude, our review found that exposure to cigarette smoking has a negative effect on the child's language outcomes. Whilst nicotine is well established as the main psychoactive component of tobacco smoke, there are various other chemicals present in tobacco smoke, many of which are teratogenic. Due to the complex nature of tobacco smoke and also due to the complex nature of language, delineating these results is exceptionally complex. However, what the results of this review do suggest is that, considering the fact that nicotine acts on nAChRs and nAChRs have been consistently linked to cognition, and that they play a role in neuronal migration during foetal development that factors affecting nAChR functions pose an interesting first step possible that at least some of the effects of CS on language are mediated through interactions between nicotine and nAChRs.

# Chapter 3

## Unravelling the effects of RIC3 variants associated with better language outcomes, on nAChRs

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## 3.1 Introduction

Overall, our review found that children exposed to CS prenatally were more likely to have poor language outcomes. Although nicotine comprises 0.1-0.2% of the total weight of a cigarette, the role of nAChR in cognition and neurodevelopment suggest that nicotine may contribute to the effects of CS on language development. To explore this possibility, the effect of RIC3 variants linked to cognitive performance on nAChR expression in clonal cell lines was investigated.

Nicotine has long been associated with the improvements in attention and WM. In particular, nicotine appears to lead to an 'inverted J response' with lower doses appearing to improve cognitive function while higher doses or persistent exposure lead to either no improvement or an impairment of cognitive function (Levin et al., 2013). Indeed, a meta-analysis which aimed to shed light on which aspects of human performance were more affected by nicotine and smoking found one of these to be specifically WM (Heishman et al., 2010). The latter highlights a specific effect of nicotine exposure via CS as particularly important regarding WM. Considering that the main component of tobacco smoke is nicotine and that that nicotine acts on nAChRs, it is possible to surmise that nAChR related mechanisms may be relevant to language.

### 3.1.1 nAChRs

nAChRs belong to the Cys-loop superfamily of ligand-gated ion channels. To date, 17 genes coding for nAChR subunits have been identified in vertebrates (Gotti et al, 2005). These genes encode for; alpha subunits, α2-α10, beta subunits (β2-β4), as well as β, δ, γ and ε subunits. All alpha subunits, apart from α5, contain a flexible extracellular domain loop, flanked by conserved aromatic residues and containing a cysteine-bridge on its tip. This is termed the c-loop and is key for agonist-binding, making the presence of α subunits a requirement for functionality (Albuquerque et al., 2009).

Considering the numerous subunits highlighted above, nAChRs may combine to form diverse, pentameric assemblies. They may assemble as homomers (such as α7* nAChRs) or heteromers (such as α4β2* nAChRs, where * corresponds to the possibility of different

subunits; figure 3.1; Gotti et al., 2005). The subunit composition of the pentamers define diverse properties of the receptors, including their ion permeability, assembly, chaperone interactions, trafficking, and cellular localisation (Corringer et al., 2000; Alburquerque et al., 2009; Wonnacott, 1997). and exhibit low affinity for acetylcholine (ACh) and nicotine, rapidly desensitize, and are involved in phasic synaptic responses, whereas α4β2 exhibit a high affinity for ACh and nicotine, desensitise slowly and are involved in tonic synaptic responses (Alkondon et al., 1993).

nAChR structure

nAChR subunits have a common ancestor and have been highly conserved throughout evolution, with the same subunit having 80% amino acid conservation across vertebrate species (Le Novere and Changeux., 1995). Hence, nAChR subunits have a well-established and similar structure, comprising a long extracellular N-terminus, three hydrophobic transmembrane domains (TM1-TM3), a cytoplasmic loop between TM3-TM4, another transmembrane region (M4), and a short extracellular C-terminus (figure 3.2).

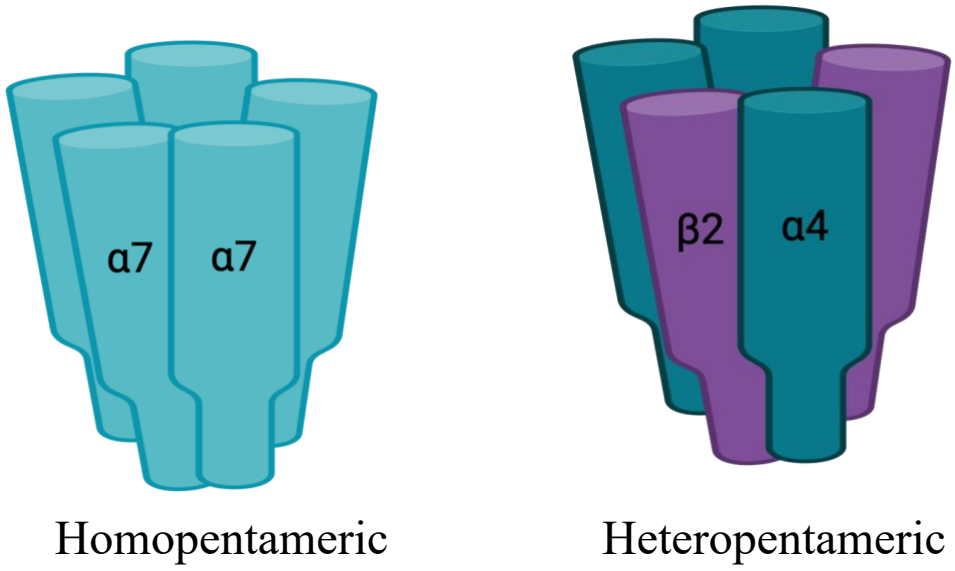



*Figure 3.1. Diagram showing the subunit composition of α7 and α4β2 nAChRs. Made using BioRender.* α7 assembles as a homopentamer, with 5 identical subunits, wheres as α4β2 consists of a combination of α4 and β2 subunits.

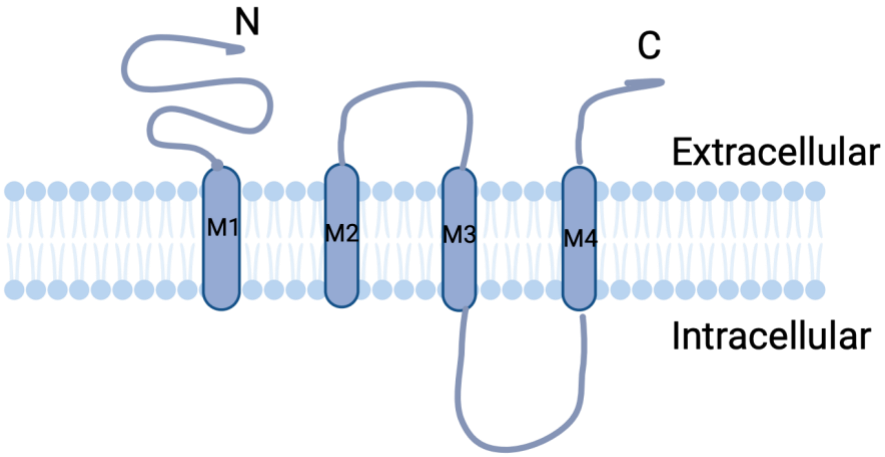



*Figure 3.2. Diagram showing the structure and membrane topology of a nAChR subunit. Made using BioRender.* Subunits each contain four transmembrane domains (M1-M4), an extracellular amino- and carboxy-terminus, and a M3-M4 intracellular loop of variable length.

### 3.1.2 NAChRs and cognition

In the mammalian brain, the most abundant nAChRs are α7 and α4β2. These receptors are expressed throughout the brain, but their expression is higher in cortical areas, the ventral tegmental area, the basal ganglia, thalamus and hippocampus (Millar and Gotti, 2009). Considering α7 nAChRs specifically, these can be postsynaptic, presynaptic (with a role in regulation of neurotransmitter release), or perisynaptic when they are involved in volume transmission (as seen in figure 3.3, left to right). In neurons, α7 receptors localise mostly perisynaptically on GABAergic and glutamatergic regions in the hippocampus and other regions to facilitate release of release of neurotransmitters such as glutamate, dopamine, GABA and ACh (McGehee et al., 1995; Gray et al., 1996; Kramer et al., 2022; Liu et al., 2022). This occurs when a cholinergic agonist binds to the outside of the channel, the channel opens, allowing the entry of cations. These cations further activate voltage-dependent calcium channels, allowing calcium entry (Benowitz 2009).

This ability of nicotine to activate a particular nAChR depends on the subunits which it consists of (see section 3.1.1; Dani and Bertrand 2007). This allows nAChRs to contribute to diverse brain functions such as cognition, memory, mood, reward, and motor functions (Millar and

Gotti , 2009). nAChR activity has also been linked to synapse development, maturation and neuronal migration (Berg and Conroy, 2002; Ballesteros-Yáñez et al., 2010; Lozada et al., 2012).

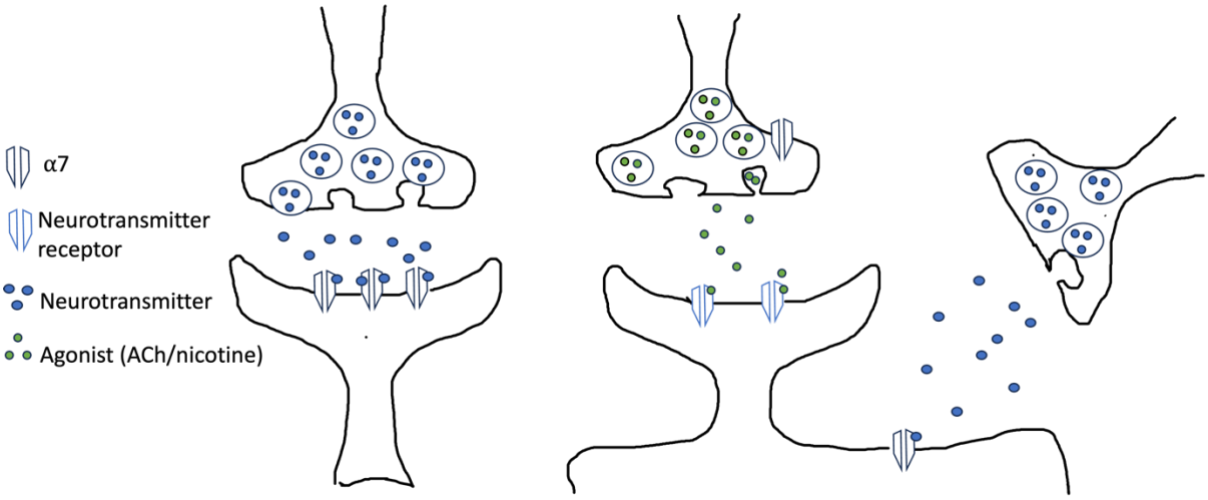



*Figure 3.3 α7 mediated neurotransmitter release in the mammalian brain*. α7 receptors can be postsynaptic, presynaptic (with a role in regulation of neurotransmitter release), or perisynaptic when they are involved in volume transmission. Adapted from Corradi and Bouzat, 2016.

How does nAChR signalling actually occur? For α7 signalling to occur, α7 must be present at the cell surface. The functional receptor's cell surface expression is largely dependent on its correct folding and oligomerization in the ER, where functional nAChRs are assembled and subsequently trafficked to the cell surface (Lansdell et al., 2005; Dau et al., 2013). Over the past two decades, research has highlighted that an ER resident chaperone, Resistant to Inhibitors of Cholinesterase 3 (RIC3), is involved in enhancing the folding and oligomerisation of α7 nAChR subunits prior to their trafficking to the cell surface (Halevi et al., 2002; Castelán et al., 2008; Lansdell et al., 2008; Lansdell et al., 2005; Wang et al., 2009). Indeed, the specific importance of RIC3 was highlighted in in vitro experiments in host cells not endogenously expressing RIC3, where exogenous RIC3 expression led to increased assembly and cell surface trafficking of α7 nAChRs (Dau et al., 2013), as measured by Forster's Resonance Energy Transfer (FRET), and increased functional α7 nAChR expression, as measured by, α-bungarotoxin (α-BGTX) binding, a nAChR antagonist (Halevi et al., 2002; Halevi et al., 2003;

Williams et al., 2005; Castelán et al., 2008 ). RIC3 also interacts with other nAChRs and the closely related 5-HT3 serotonin receptor but its effects, which can be positive or negative, depend on the identity of the receptor subunits, the host cell (Lansdell 2005; Haveli et al., 2003) and the ratio of receptor to RIC3 (Dau et al., 2013; Ben-David et al., 2016]. Although the role of RIC3 on the expression of $\alpha$7 nAChR *in vivo* is not fully understood (Deshpande et al., 2020), RIC3 expression has been linked to cognitive maintenance (Yokoyama et al., 2014) and, crucially, there is a good correspondence between RIC3 and $\alpha$7 nAChR expression in the rat hippocampus (Castelán et al., 2008). Furthermore, recent autoradiographic analysis of the brain of a RIC3 knock out mouse show a decrease in α-BGTX binding in the hippocampus and the cortex (Deshpande et al., 2020), brain regions that contribute to WM and language. Thus, it may be that RIC3 influences language skills through its effects on α7 nAChR expression (Lansdell et al., 2005), with FRET being the most recent experimental method used to consider this.

What is Förster's resonance energy transfer?

The concept of FRET was first introduced in the 1920's, when Jean-Baptiste Perrin and his son Francis Perrin explained the energy transfer process between two molecules in solution involving dipole-dipole intermolecular interaction (Sun et al., 2011). Förster published his first paper in 1946 where he established the correct distance over which this interaction occurred, 1-10nm (Forster 1996). It was not used experimentally until the 1990s; research into the full extent of its experimental uses as well as optimisation of this method remains ongoing. FRET microscopy measures the effects of energy transfer on the donor fluorophore, excited at a lower wavelength and, acceptor fluorophores, excited at a higher wavelength and this exchange of energy can be used to determine their spatial relationship over distances of up to 10 nm (fig 3.4) and thus confirm interaction. Acceptor photobleaching (ap) FRET differs slightly from FRET and takes advantage of the fact that donor fluorescence is quenched during FRET because some of the donor fluorescence energy is channelled to the acceptor. Photobleaching the acceptor fluorophore irreversibly eliminates the quenching effect and increases the level of donor fluorescence, if these fluorophores are <10 nm apart (Forster, 1948). This is hence a current and useful method for establishing how fluorescently tagged proteins in close proximity to each other, such as chaperones and nAChR subunits interact.

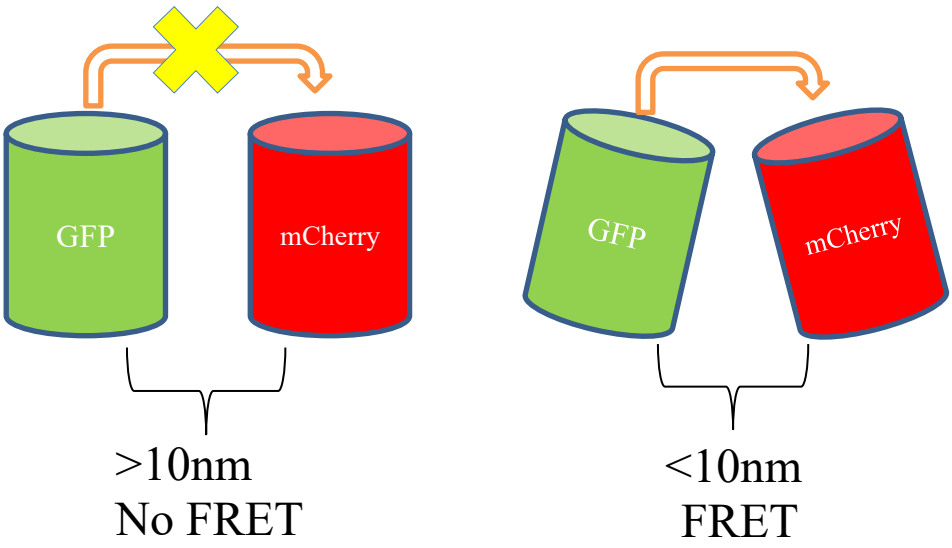



*Figure 3.4 Schematic of how FRET works. FRET occurs between complementary fluorophores are less than 10nm apart.*

### 3.1.3 What are the specific links between nAChRs and language?

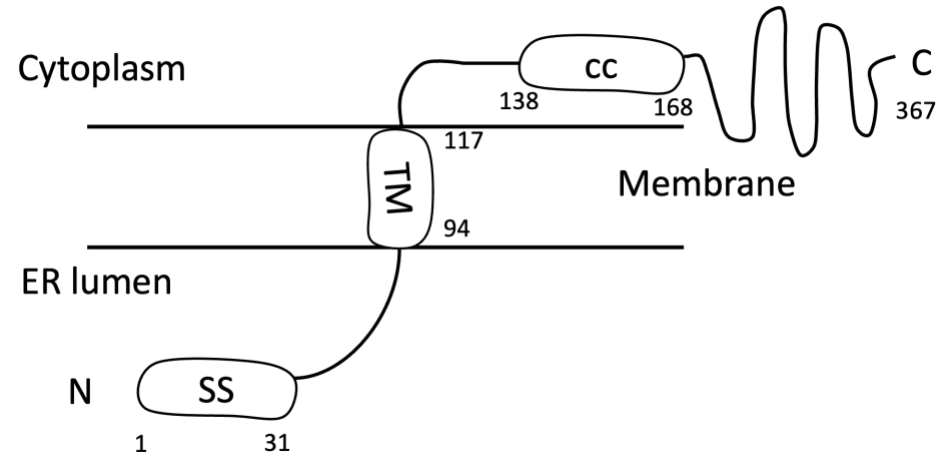
Although nAChRs contribute to neuronal development, there is limited direct evidence that this activity may impact language development, with the exception of the evidence that supports the view that in-utero CS exposure may impair the development of language skills. However, there is relatively recent evidence that a *RIC3* variant may affect the ability to speak backwards (Prekovic et al., 2016). A variant in the 88th residue of RIC3, leading to an amino acid change from Glycine to Arginine, G88R, was found in two family members with an innate ability to speak backwards (Prekovic et al., 2016). The authors hypothesised that this ability could be conferred by exceptional WM, served by cholinergic projections from the basal forebrain to the frontal cortex and that this, in turn, could be mediated by G88R (Prekovic et al., 2016). Support for this WM focused hypothesis comes from knock out mouse studies, which show a decrease in antagonist binding in brain regions that contribute to WM and language (Deshpande et al., 2020).

Focusing on the G88R variant identified by Prekovic et al, 2016, work in our lab considered whether the G88R variant conferred more efficient interaction between RIC3 and α7, thus contributing to better WM. Indeed, it was found that the variant form of RIC3, RIC3G88R, interacted significantly more efficiently with α7 compared to its WT form, as measured by apFRET (Pradhan et al., 2023, under review). Additionally, RIC3 expression has been shown to be highly correlated with α7 nAChR in post mortem brain tissues from diseased populations (Severance & Yolken, 2007) and RIC3 expression is upregulated in patients with schizophrenia and bipolar disorder (Severance & Yolken, 2007), both of which have language characteristics (de Boer et al., 2020; Dwyer et al., 2020; Rodriguez-Ferrera, McCarthy, & McKenna, 2001). Together, these data suggest that RIC3 may have some impact on WM and language development indirectly through its effects on α7 nAChR biogenesis and that specific variants in RIC3 may be a mechanism involved in this. Considering nicotine acts on nAChRs, this is a possible avenue by which CS can affect language. This also contributes to the rationale regarding why this study focuses specifically on RIC3 interactions with α7 nAChRs.

Given that α7 cell surface expression is a pre-requisite for its function, which in turn has been hypothesised to be affected by RIC3, over the last two decades, research has focused on identifying the structural domains of RIC3. So far, it has been found that RIC3 comprises of an N-terminal signal peptide, and a single transmembrane domain (TMD) and the N-terminal region containing two hydrophobic segments linked by a proline-rich linker, and a C-terminal region that contains either one (human, mouse, *Drosophila*) or two (*C. elegans*) coiled-coil motifs (Halevi et al., 2003). Moreover, in invertebrates, two TMD segments have been predicted whereas, in humans, the presence of these two TMDs is still debated with two models being proposed for the topology of RIC3; one where there is the presence of cleavable of a cleavable signal peptide in the N terminus, suggesting that RIC3 is a single-pass membrane with its N terminal located in the ER lumen (figure 3.4), and the C-terminal with its coiled-coil domain located in the cytoplasm (Wang et al., 2009). However, despite the proposed possibility of the presence of a signal sequence (SS) in the N-terminal, others found no evidence of a SS being cleaved during translation (Castillo et al., 2005; Castelán et al., 2008). The latter suggests that RIC3 is a type II transmembrane protein with the N- and C-

terminals in the cytoplasm (figure 3.4B). The latter topology is supported by the findings that the complete N-terminal is crucial for cell surface expression of α7 nAChRs in humans as well as invertebrates (Castillo et al., 2005; Lansdell et al., 2008; Castelán et al., 2008; Wang et al., 2009; Ben-David et al., 2016)

A




B

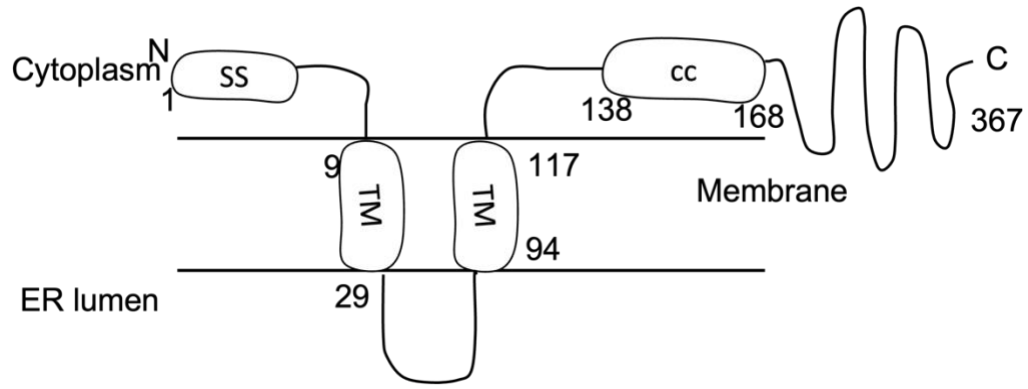



*Figure 3.4 Schematic of the proposed topology of RIC3.*
A)RIC3 protein that has a single transmembrane segment with the N terminus on the lumenal side and the C terminus on the cytoplasmic side of the ER. B) RIC3 protein that has 2 transmembrane domain segments with the N and C termini both on the cytoplasmic side of the membrane. SS, Signal sequence; TM, transmembrane domain; CC, coiled-coil domain.

The mechanism of nAChR assembly and maturation is a complex one which various studies have attempted to address. With regards to the specific functions of RIC3, an early model proposed that the coiled-coil domain is vital for the protein's role in α7 nAChR assembly and

maturation. The α7 MX helix has been proposed as the site of interaction between RIC3 and α7 (Jones, Buckingham, and Sattelle, 2010; Rudell et al., 2020.) Early studies hypothesised that each RIC3 protein associated with a single folded α7 subunit and that the pentamer would then be assembled through RIC3 dimerization at the C-terminal coiled-coil motif, pulling the subunits together (Wang et al., 2009). Conversely, other studies found that some isoforms of RIC3 which lack the coiled-coil domain but are still able to promote α7 assembly, highlighting that the coiled-coil domain was not required for RIC3 function and assembly.

One important aspect to note is that RIC3 is only one of multiple chaperones associated with the trafficking and assembly of nAChRs. Recently, Kweon et al., (2020) suggested a complex model involving a host of in which NACHO uses the rough ER and engages the α7 N-glycans and calnexin, and thereby indirectly mediates assembly of α7 TM domains with RIC3 engaging with the assembled α7 subunits (Kweon et al., 2020). How RIC3 engagement with α7 occurs at this later stage was not hypothesised by the authors, however, in light of previous findings, it's possible that this occurs via dimerization of the RIC3 coiled-coil domain at the later stages, after nascent α7 nAChR engagement with NACHO.

Summary and rationale

To summarise, the focus of this thesis on RIC3 stems from a recent, potential hypothesised role in language. Numerous studies have linked *CHRNA7*, which codes for α7, changes as a potential cause in those affected by speech and language difficulties. Furthermore, the G88R variant in *RIC3* has been linked to a unique ability to speak backwards, a language skill that is associated with exceptional WM capacity. In addition, increased *RIC3* expression has also been linked to schizophrenia and bipolar disorder, both of which include language related deficits as characteristics. Moreover, it has been found that one of the potential reasons why CS smoking incidence is more prevalent in those which SCZ, is because it is being used as a form of self-medication as those who smoke experience symptom alleviation (Leonard, Mexal and Freedman, 2007), likely due to its effects on nAChRs.

So far, there is little research specifically regarding the effect of RIC3 variants on the interaction of this chaperone with nAChRs. The above data highlight robust links between the

cholinergic system and that alterations in these could affect cognition. The systematic review in the previous chapter suggested that prenatal insults from cigarette smoking, potentially via nicotine, have negative effects on language. Nicotine's action on nAChRs, the presence of nAChRs in areas related to cognition and memory and the role of nAChRs in neurodevelopment strongly as well as the link of a RIC3 variant in the backward speech suggest a role for nAChR in language skills and thus a key role in the effects of CS in language development. Therefore, in the present study we examined the consequences of 2 variants in RIC3 associated with higher overall language scores, RICA26S and RICV196F, and RICT177S (control) on the interactions between RIC3 and $\alpha$7 nAChRs in HEK293 mammalian cells. We hypothesised that the variants of interest confer a more robust interaction between RIC3 and $\alpha$7 nAChRs compared to RICWT and the control variant. Using apFRET, however, we found that the above variants do not result in altered interactions between RIC3 and $\alpha$7 nAChRs.

## 3.2 Methods

### 3.2.1 Establishing RIC3 variants

In order to establish whether RIC3 variants conferred a more efficient interaction between RIC3 and α7 and thus establish if this was a mechanism affecting WM and hence cognition, the UK10K Cohorts project (http://www.uk10k.org/studies/cohorts.html) datasets were used. The UK10K catalogues the contribution of genome-wide genetic variation to a range of quantitative traits in 3,781 healthy individuals from 2 large UK population samples, ALSPAC and the TwinsUK registry thus allows the study of the contribution of low-frequency and rare variants on a various complex quantitative outcomes. Considering this and the fact that ALSPAC was also one of the studies included in the review in chapter 2, the UK10K dataset was searched for RIC3 variants.

To search the UK10K database, VCF tools (Danecek et al., 2011) were used to extract all variants in the RIC3 gene; all exonic or 5'UTR variants with a population (1000Genomes; 1000 Genome project consortium., 2012) frequency of <1% (N=14) were extracted. Variants seen in 3 or less individuals in the UK10K (N=9) were then selected. The main genetic (variant-

based) and individual (language-based) factors considered when choosing the variants of interest were the following; PolyPhen Score, Verbal IQ, vocabulary scores, and overall IQ, explanations for which are provided in table 3. The language variables were obtained from the ALSPAC dataset administering WISC tests, a standard IQ test to children which contains subsets designed to test various cognitive abilities (table 4). These are widely validated and used and thus make for easier comparisons and interpretation.

Table 3 - Variables from UK10K individuals with variants in RIC3

| **Protein based effects** | **Description** |
|---|---|
| Sorting Intolerant from Tolerant (SIFT) algorithm score | Predicts the impact of amino acid substitutions on protein function. Scores range from 0.0 (deleterious) to 1.0 (tolerated). |
| Polymorphism phenotyping v2 (Polyphen2) | Predicts the possible impact of an amino acid substitution on the structure and thus function of a protein. Scores range from 0.0 (tolerated) to 1.0 (deleterious). |
| Combined annotation dependent depletion (CADD) PHRED score | Scores the deleteriousness of single nucleotide substitution as well as insertion/deletions variants in the human genome. CADD predicts a continuous phred-like score that ranges from 1 to 99, with higher values indicating more deleterious cases. |
| phyloP100way Score | Measure evolution conservation at a single alignment site. Overall, this measure assumes neutral evolution. negative sign indicates faster-than expected evolution, while positive values imply conservation. |

Table 4- Key cognitive tests administered

| **Cognitive tests** | **Description** |
|---|---|
| WISC- verbal IQ | Derived from scores on six of the subtests: information, digit span, vocabulary, arithmetic, comprehension, and similarities. |
| WISC verbal comprehension index | Measures ability to access and apply acquired word knowledge |
| WISC- Freedom from distractability | A measure comprised of the sum of the scores on the Arithmetic and Digit Span subsets |
| WISC- Similarities scaled score | Measures verbal concept formation and reasoning. The child is asked to explain how two things are alike or similar. |

| | |
|---|---|
| WISC- Arithmetic scaled score | Measures numerical accuracy, reasoning and mental arithmetic ability. Mental arithmetic and story problems play an important part in the student's success. |
| WISC- Vocabulary scaled score | Measures verbal concept formation, knowledge, and expression. The child is asked to define a series of words. |
| WISC- Digit-Span scaled score | Measures working memory. The child is asked to repeat a series of numbers in the same order they were presented, or in reverse order. |
| WISC- Total IQ Score | Comprised of a score which considers all scores from all of the WISC subsets. A score of 100 is considered average. |

### 3.2.2 Cell culture

Human embryonic kidney 293 cells (HEK293T, supplied by ATTC UK) were maintained in Dubecco's Modified Eagle Medium (DMEM, 1X), supplemented with 10% foetal bovine serum (FBS). Cells were maintained in a T75 flask in an incubator at 37 degrees and 5% $CO_2$. Cells were grown to approximately 80% confluence before splitting in a 1:10 ratio. Before splitting or experimental work, adherent cells were treated with 500µl of trypsin for approximately 3 minutes at 37 °C.

### 3.2.3 Cloning RIC3 into pEGFP-c1

To express the variants in mammalian cells for confocal imaging and experiments, appropriate FRET fluorophore pairs were essential. The chosen pairs were GFP and mCherry. Standard PCR based cloning method was used for cloning RIC3 into the multiple cloning of a pEGFP-C1 expression vector. Wild type RIC3 (RICWT) cDNA (NM_024557) was amplified using primers containing EcoRI (5' TATTCGAATTCGCGTACTCCACAGTGCAGAGAGTCGCTCTGG 3') and KpnI (5' AATAAGGTACCTCACTCTAAACCCTGGGGGTTACGCTTCCTCAG 3') restriction sites. Annealing occurred at 55°C and the reaction took place for 31 cycles.

The fragment and the pEGFP-C1 (NovoPro Bioscience, Shanghai, China) were then digested with KpnI and EcoRI to clone RIC3 into the MCS (multiple cloning site) of the pEGFP-C1 plasmid to fuse the eGFP tag to the N-terminus of the RIC proteins. The digestion occurred at 37°C for 2 hours. The vector (pEGFP-C1) and insert (RICWT) were then ligated using standard T4 DNA ligase (Thermofisher UK) methods and placed on ice overnight.

Once the wild type fluorescent proteins had been assembled, we then introduced the variants of interest using site-directed mutagenesis with the primers on table 5. Primers were designed to contain the variant of interest approximately halfway through the primer (see table 5) and were used to introduce each mutation into RIC3.

Table 5 -Primers used for RIC3 mutagenesis

| | |
|---|---|
| RICA26S | Forward:GCTGCTGCCCAAG**T**CCTTCCTGTCCCG |
| | Reverse :CGGGACAGGAAGGACTTGGGCAGCAGC |
| RICT177S | Forward: TGGTGAGAGAGCACAGA**G**TGTGACTTCTGACCAAG |
| | Reverse: CTTGGTCAGAAGTCACACTCTGTGCTCTCTCACCA |
| RICV196F | Forward:CCGAGAAATCACCAGG**T**TCATGAAAGAAGGAAAAT |
| | Reverse: ATTTTCCTTCTTTCATGAACCTGGTGATTTCTCGG |

*Bold highlights the presence of the variants within the primers

### 3.2.4 Cloning mCherry into α7

Wild-type human α7 nAChR subunits were synthesised by GeneArt (Thermofisher UK) by inserting mCherry cDNA into the M3-M4 cytoplasmic loop of α7 at amino acid 391 (GeneArt, Thermofischer, UK). The positioning of the tag has previously been demonstrated to retain the functional properties of the receptor and the proteins reported to function normally (Nashmi et al., 2003; Drenan et al., 2008; Son et al., 2009; Murray et al., 2009). Subsequently, both the WT and fluorescent α7 nAChR subunit cDNA was then subcloned into the pCI expression vector (Promega, UK).

To obtain RIC3 and α7 DNA for experiments, DH5a competent cells were transformed with the ligated RIC3pEGFP-C1 and α7PSUnot-mCherry ligation products and spread onto agar plates containing either 50 μg $mL^{-1}$ kanamycin or 100 μg $mL^{-1}$ ampicillin respectively. The pEGFP-C1 vector contains a Kanamycin resistance gene whereas the α7-mCherry construct contains an ampicillin resistance gene. As such, successful ligation should lead to an intact resistance gene and thus colonies should only form if they contained the full, ligated construct containing the appropriate resistance gene. Colonies were then picked for DNA extraction.

### 3.2.5 DNA extraction

Minipreps and maxipreps were carried out to extract DNA according to manufacturer's (Qiagen) instructions.

### 3.2.6 FuGene Transfections

In order to consider the effect of the chosen variants on the interaction and thus the function of α7, HEK293 cells were transfected with the same amount of RIC3 and α7 as previous studies had highlighted an efficient interaction at this ratio (Dau et al., 2013). For transfections, HEK293 cells were grown to 80% confluency and seeded at a density of 160,000 -200,000 cells/ml into iBidiTreat dishes containing 2ml complete media at least 24 hrs before transfections. At least 24 hrs prior to imaging, cells were transiently transfected with FuGene, to allow protein expression to occur. The transfection reaction mix was prepared and contained; 95µl serum free media, 1µg DNA per reaction, (0.5µg RIC3 and 0.5µg α7 cDNA) 4.5µl FuGene reagent. After a maximum 15-minute wait, 100µl the DNA-complex mix was added to the dishes. Cells were kept in an incubator at and 5% $CO_2$.

### 3.2.7 FRET and apFRET

ApFret was used to detect the interactions between RIC3 variants and α7 nAChRs. Confocal microscopy was performed with a Zeiss LSM 880 confocal microscope, between 24 and 48 hours after transfection. Cell location and visualisation was done with Zeiss PlanApo 63x/1.4 NA oil immersion objective lense. In this study, eGFP was used as the FRET donor as this is excited at a lower wavelength, whereas, mCherry was the FRET acceptor. A previous paper had employed FRET with similar constructs as a means of considering the interaction between RIC3 and α7 (Dau et al., 2013). In terms of controls, pmCherry-eGFP (Addgene, plasmid#86639) was used as positive control, while mCherry-ER3 (Addgene, plasmid#55041) and LCK-GFP (Addgene, plasmid#61099) were used as negative controls for RIC3 and α7-mCherry, respectively. FRET between donor and acceptor was confirmed by bleaching of mCherry and monitoring the consequential increase in eGFP fluorescence. mCherry was excited with 561nm light and eGFP with 488 nm light. The capture wavelengths were 490nm-540nm for eGFP and 611-694nm for mCherry. The mCherry and eGFP laser transmissions

were kept at 1.5% and 0.8%, respectively, during scanning to avoid photobleaching; the mCherry (561 nm laser) was set at 100% during bleaching experiments. HEK293 cells expressing either just eGFP or mCherry were imaged with the ap-FRET settings to confirm minimal fluorophore crosstalk, and that the bleaching step did not reduce eGFP fluorescence. Five pre-bleach and five post-bleach scans of the eGFP and mCherry fluorescence were carried out in, mostly consistently sized ROI. Fluorescence intensity was monitored in the ROI and analysed using Microsoft Excel. For data analysis, the eGFP fluorescence intensity was normalized onto a percentage scale as described by (Graumann et al., 2007; Karpova et al., 2003). To calculate the percentage change in FRET efficiency ($E_F$), the following equation was used:

$$E_F = (\text{eGFPpost} - \text{eGFPpre})/\ \text{eGFPpre} \times 100$$

where eGFPpost is the average fluorescence intensity after the photobleaching and eGFPpre is the average fluorescence intensity before the photobleaching. Experiments were repeated five times.

### 3.2.8 tical analysis

Prior to statistical analysis, cells were double checked using the Range Indicator Zeiss software function to exclude cells that had fluorescence overexpression in the region of interest in either the eGFP channel or the mCherry channel. This was to ensure that overall FRET results were not skewed by overexpression. Normal distribution was checked using the descriptive statistics function in excel. Factors including skewness and kurtosis were used to assess normality. For experiments using RICA26S, skewness ranged from -0.48 to 0.67 and kurtosis ranged from 0.16 to 4.45. For experiments with T177S, skewness ranged between -0.05 and 0.63 across 6 conditions and kurtosis -0.25 and 2.99. Finally, for V196F, skewness ranged from -0.85 to 1.11 and kurtosis ranged from -0.13 to 5.83. has been suggested that skewness values of -2 and +2 are acceptable for normal distribution (George and Mallery, 2010; Hair et al., 2010; Byrne et al., 2010). In addition, kurtosis coefficients outside -7 and +7 have been suggested as acceptable for normality assumption (Hair et al., 2010; Byrne, 2010). Indeed,

these cut offs are more conservative than the -3 to +3 for the skewness coefficient and -10 to plus for the kurtosis coefficients proposed by Kline, 2011 and thus the former cut-offs were used for normality assumptions. To compare FRET efficiency between groups and thus establish differences between interactions between RIC3 in its variant and WT forms and α7, two sample- t tests, assuming unequal variance, were conducted in excel to test for means differences between two groups of interest at a time. This test was chosen as sample sizes differed between groups and data appeared normally distributed. In addition, T-tests have been reported to be particularly robust to normality assumptions (Lumley et al., 2002; Sullivan et al., 1992). One-tailed results were used as it was hypothesised that a positive effect of V196F and A26S on the interaction between RIC3 and α7. Results of $p<0.05$ were considered to be statistically significant. Where outliers were removed, boxplots were re-designed using R and these were used to identify the outliers. This process was repeated until no more outliers were present.

### 3.2.9 Co-localisation analyses

Co-localisation analyses were conducted on images obtained throughout the FRET experimentation process. All co-localisation analyses were conducted on the FijiImageJ processing software, using the plugin JACoP (Just another Co-localization Plugin; see figure 3.5 for workflow explanation). The JACop was used for the generation of cytofluorograms and obtaining co-localisation measures in the form of Pearson coefficients (Bolte et al., 2006), Average Pearson‘s coefficients were calculated.

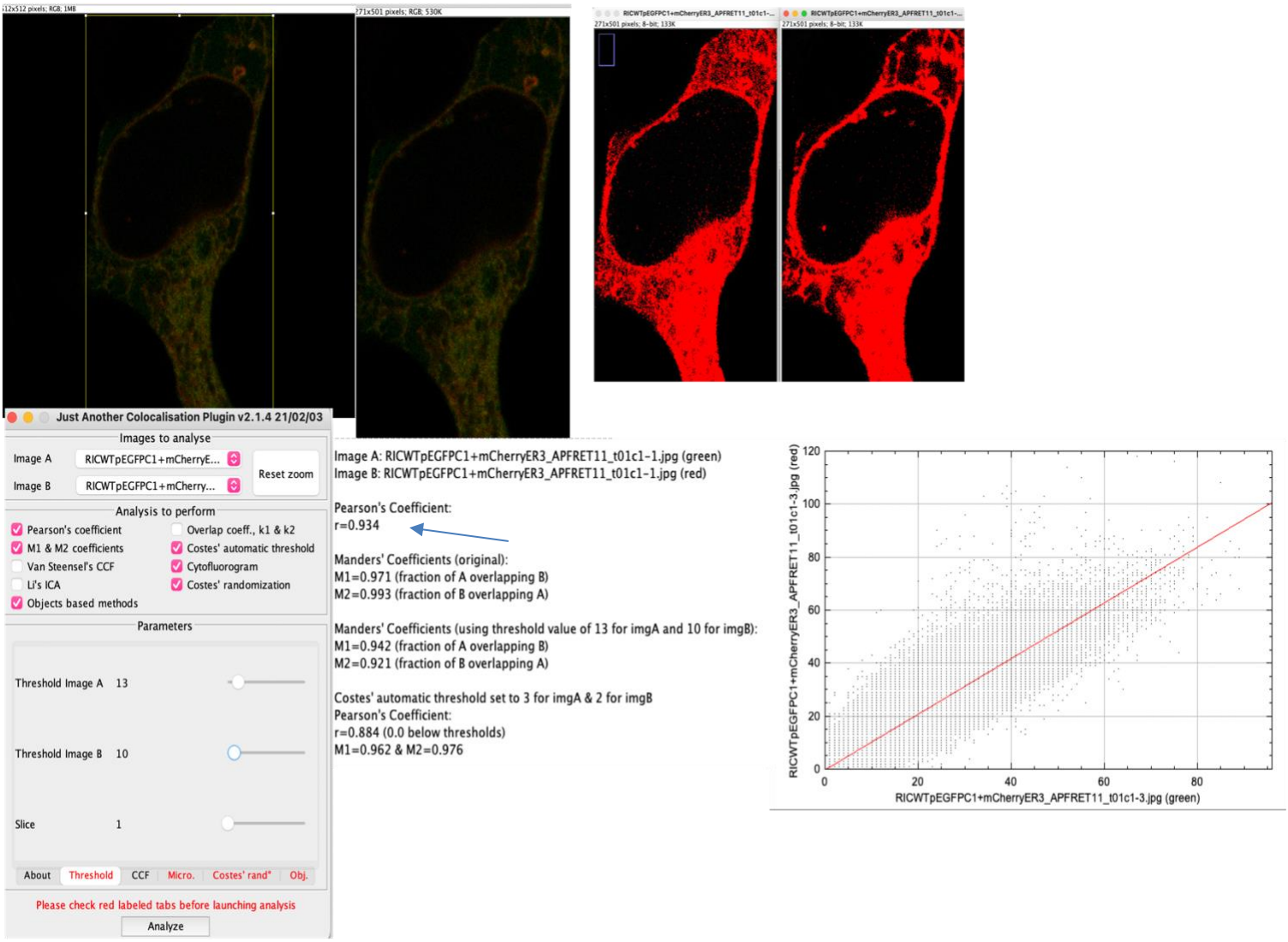



*Figure 3.5 Workflow image process using the JaCop plugin to assess the colocalization between RIC3 in its wild-type and variant forms and mCherry-er3.*

Representative Images were selected and split into channels, red and green. JACoP from "Plugins" menu opens user interface for the tool, where channels for analysis are selected, and intensity threshold for analysis set. Running analysis generates an output text log of coefficient analysis values and a cytofluorogram. Pearson‘s correlation coefficients obtained from analysis of each image (n=10) were recorded.

### 3.2.10 RIC3 effects on α4β2

In order to measure the effects of RIC3 variants and on α4β2 cell expression, first the same primers as above (see table 5) were used to introduce the variant V196F into RIC3-CFP

(cerulean; NM_001206671.4) which had been previously cloned in our lab. HEK 293T cells were transfected with FuGene, as detailed above, with 1μg total α4-mCherry and β2-eGFP per dish. For experiments involving RICWT and RICV196F, RIC3 was transfected at an amount which was 0.1:1 ratio to α4β2; 0.1μg RIC3 per dish. This specific amount was chosen as this particular ratio of RIC3 to α4β2 had shown robust effects on α4β2 fluorescence in an early study (Dau et al., 2013). Efforts were made to ensure an amount as accurate to that reported here was used for transfections, to enable for future comparisons.

### 3.2.11 Fluorescence analysis

As three different fluorophores were used, these were separated into different channels two different tracks for improved separation with eGFP in track one and CFP and mCherry in another. Detection wavelengths for each fluorophore were as follows; 526-570, 454-581, and 580-696 for eGFP, CFP, and mCherry respectively. Detector gain was kept consistent throughout each experiment Images were taken at 1054x1054 pixels using a 63x/1.4 oil immersion objective lense. Images were loaded onto imageJ, split into channels, and a region of interest was drawn around and as close to the cell of interest. This same region of interest was used to measure background fluorescence signal. Background signal was subtracted, and fluorescence intensity measurements were taken from cells not expressing saturated regions in either the α4 (red) or β2 (green) expressing image channels. Cells with overexposed regions were not included in these analyses as to not exaggerate and thus skew whole cell fluorescence measurements.

## 3.3 Results

### 3.3.1 RIC3 variants taken forward for further consideration

Searches for individuals containing RIC3 variants in the UK10K database yielded a total of 9 rare variants across 12 individuals. Overall, table 6 Shows that there is a trend towards deleterious effects of RIC3 variants on protein function with seven of the nine variants found having SIFT scores close to 0. In individuals with RIC3 variants which appeared tolerated, as highlighted by their SIFT score, were found to have overall lower cognitive scores (see table 6 and appendix table S2, which includes the scores of remaining individuals identified in this process). This led to a shift in focus towards the cognitive tests scores.

The table below (table 6) highlights that individuals 9 and 10, in which the A26S variant was found, both had consistently above average word combination scores, VIQ scores, verbal comprehension scores, and high total IQ. Indeed, individual 8, with another variant also had high scores in many of these variables, however, often, they were similar rather than higher than those of individual 10. Additionally, 2 (individuals 3 and 5) of the 3 individuals (individuals 3, 4, and 5) with variant V196F, achieved high scores in all language related variables. However, it is of note that there was wide variability when considering scores across these individuals with V196F variant, with individual 4 achieving average scores across most categories.

Table 6- UK10K individuals carrying RIC3 variants

| ID | Individual. 3 | Individual 4 | Individua l 5 | Individual 6 | Individual 9 | Individual 10 |
|---|---|---|---|---|---|---|
| Variant | **V196F** | | | **T177S** | **A26S** | |
| Position ( **NM_024557)** | C- terminus | | | C- terminus | N-terminus (signal sequence) | |
| SIFT_Score | 0.007 | 0.007 | 0.007 | 0.56 | 0 | 0 |
| PolyPhen2 | 1 | 1 | 1 | 0.313 | 0.976 | 0.976 |
| CADD_Phred | 33 | 33 | 33 | 14.82 | 28 | 28 |
| PhyloP100Way | 5.752 | 5.752 | 5.752 | 2.234 | 1.636 | 1.636 |
| WISC- Verbal IQ: F@8 | 141 | 101 | 128 | 107 | 113 | 129 |
| WISC - Categorical Total IQ: F@8 | 7 | 4 | 6 | 4 | 5 | 6 |
| WISC - Verbal Comprehension Index: F@8 | 65 | 38 | 67 | 44 | 51 | 59 |
| WISC - Freedom from Distractibility Index: F@8 | 37 | 26 | 18 | 21 | 17 | 28 |
| WISC - Similarities scaled score: F@8 | 19 | 8 | 17 | 12 | 16 | 8 |
| WISC - Arithmetic scaled score: F@8 | 19 | 13 | 6 | 12 | 10 | 15 |
| WISC - Vocabulary scaled score: F@8 | 19 | 9 | 19 | 11 | 9 | 17 |
| WISC - Digit span scaled score: F@8 | 18 | 13 | 12 | 9 | 7 | 13 |
| WISC - Total IQ: F@8 | 138 | 108 | 125 | 101 | 114 | 128 |

### 3.3.2 Variants in RIC3 do not affect the expression and localisation within the cell

For RIC3 to exert its chaperone effects, its ER localisation must be retained. Therefore, we assessed the effect of RIC3 variants on its cellular localisation using JaCop, to consider the extent to which RIC3 occurred "concurrently" with mCherry-ER3. Confocal imaging initially showed that all RIC3 clones displayed a perinuclear position and overt ER localisation (figure 3.6). Although a somewhat crude assessment of colocalisation, JaCOP localisation analyses highlighted that, overall, RIC3 both in its WT forms and variant forms, colocalised with mCherry-ER3 (table 7). Although no statistical significance analyses were conducted, average Pearson's values obtained for cells from 10 images across from 2 replicates across all constructs were relatively similar to those obtained by mCherry-EGFP and notably higher than those for α7+LCK-GFP (negative control). The latter consists of the 2 fluorophores connected by a 3 amino acid linker and thus expresses both fluorophores in tandem in the cell. As such, the similarity in colocalisation between mCherry-ER3 and the RIC constructs further reinforces its presence as an ER protein meanwhile also no overt effects of the variants introduced on the localisation of the protein within the cell.

Table 7- Colocalisation of each construct with the ER marker mCherry-ER3

| Construct (n) | Average Pearson‘s coefficient * | SD |
|---|---|---|
| RICWT + mCherry-ER3 (12) | 0.859 | 0.045 |
| RICA26S+ mCherry-ER3 (10) | 0.829 | 0.071 |
| RICV196F+ mCherry-ER3 (12) | 0.862 | 0.044 |
| RICT177S+ mCherry-ER3 (12) | 0.863 | 0.036 |
| mCherry-EGFP (20) | 0.967 | 0.024 |
| α7+LCK-GFP (13) | 0.420 | 0.080 |

*Based on analyses of cells from 10 randomly chosen images overall from 2 experimental repeats of each construct

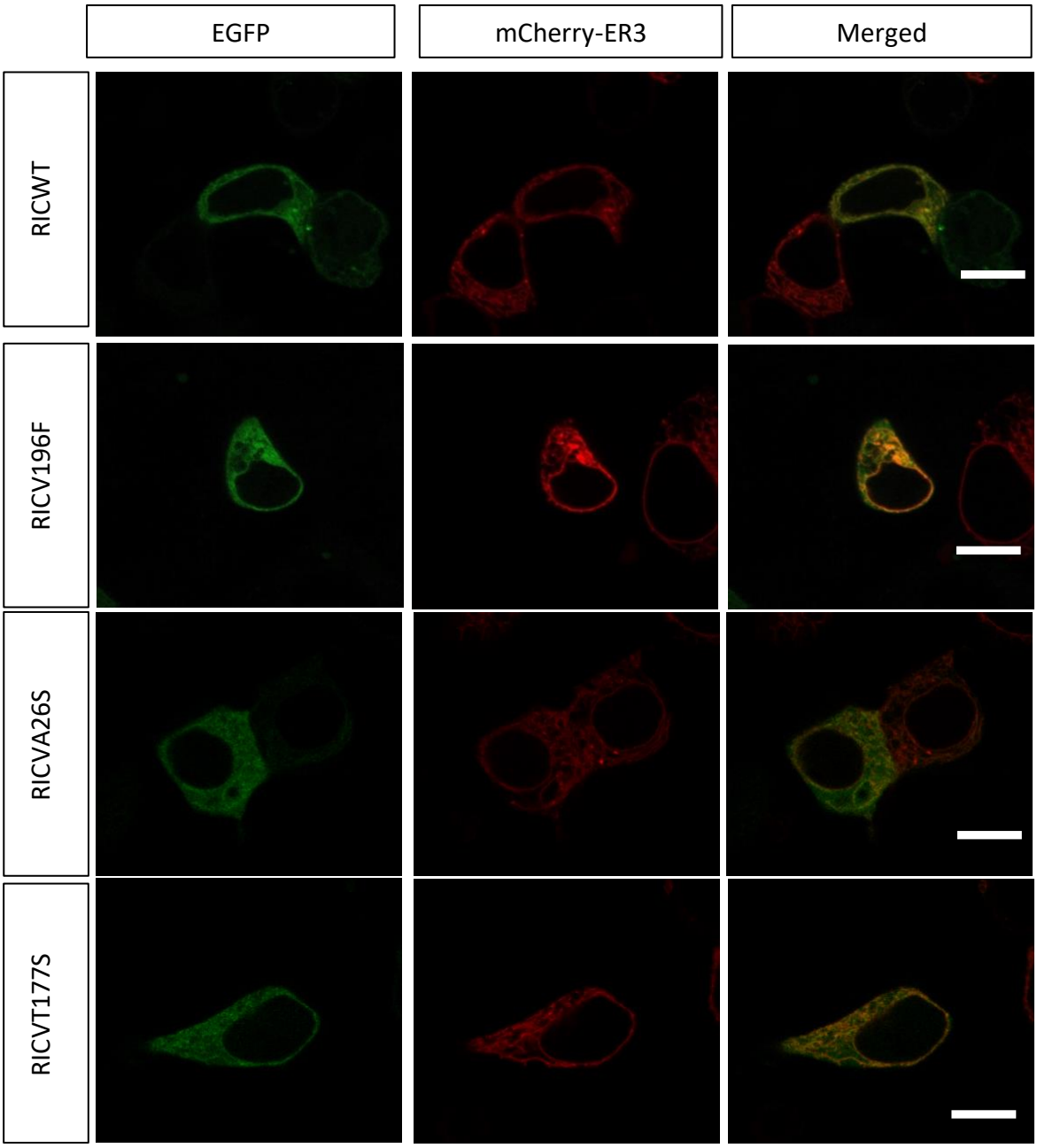



*Figure 3.6 RICWT and RIC3 variants are expressed in the ER of transfected HEK293T cells.* Representative images of HEK 293T cells which were FuGene transfected with either RICWT or RICV196F/A26S/T177S and mCherry-ER3, an ER maker, Confocal imaging shows that in the presence of the RIC variants, eGFP tagged RIC3 was still expressed in the ER. Scale bar represents 10μm.

α7-mCherry was also seen to localise to the ER (figure 3.7). This was true in the absence of RIC3, or in the presence of RIC3 in its wild-type form or any of the chosen variant forms. In addition, similar α7 distributions were seen in the presence of RIC3 and its variants, highlighting that introducing variants to RIC3 did not alter the cellular distribution of α7. In addition, no cell surface expression of α7 can be seen in the presence of RIC3 in any variation.

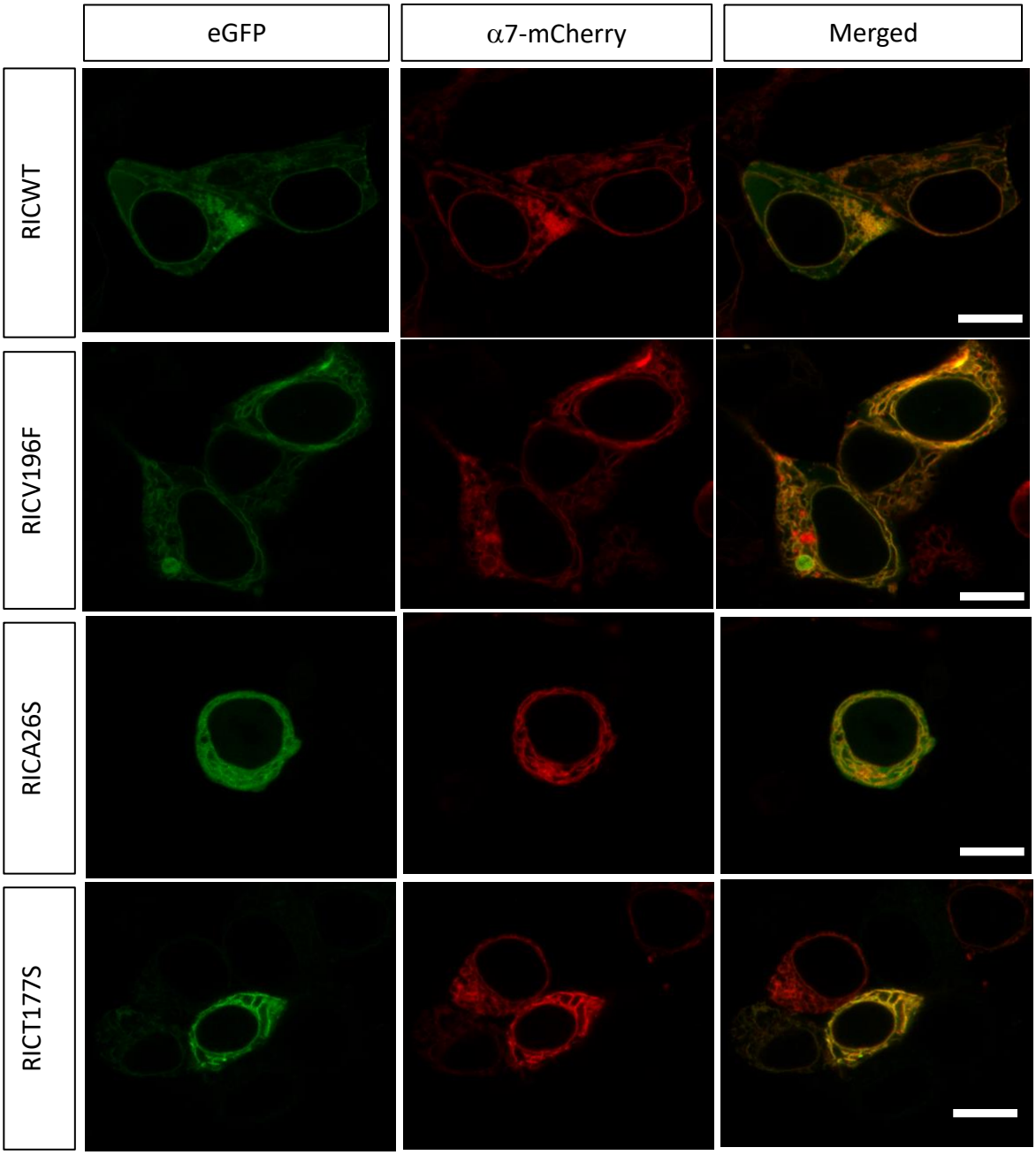


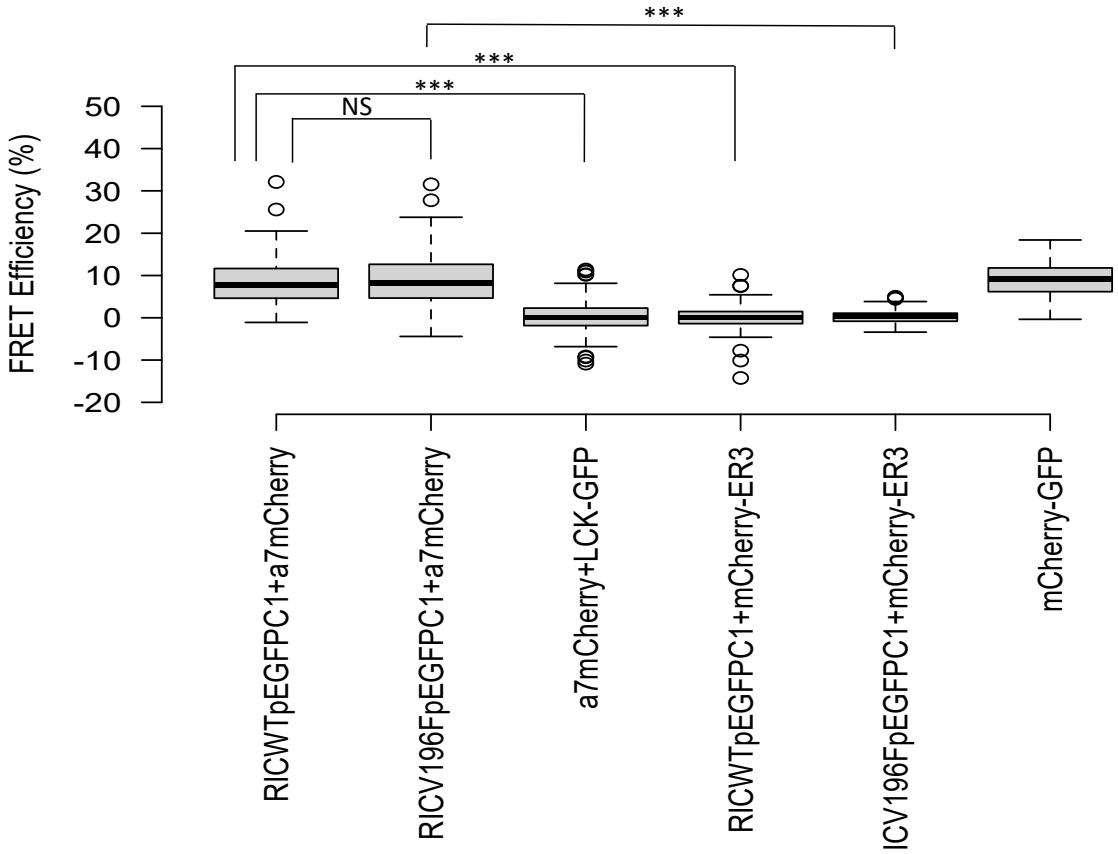

*Figure 3.7 Effect of the RIC3 variants on the cellular localisation of α7 in HEK293 cells.* Representative images of *transient expression of α7 tagged to mCherry in HEK293 cells, which demonstrated ER localisation as indicated by the presence of the ER mesh. This can be seen in the presence of RIC3 in its wild type for as well as its variant form (RICV196F, RICA26S, RICT177S). Images were adjusted using imageJ for ease of visibility. Scale bars represent 10μm.*

### 3.3.3 Variants in RIC3 do not affect its interaction with α7

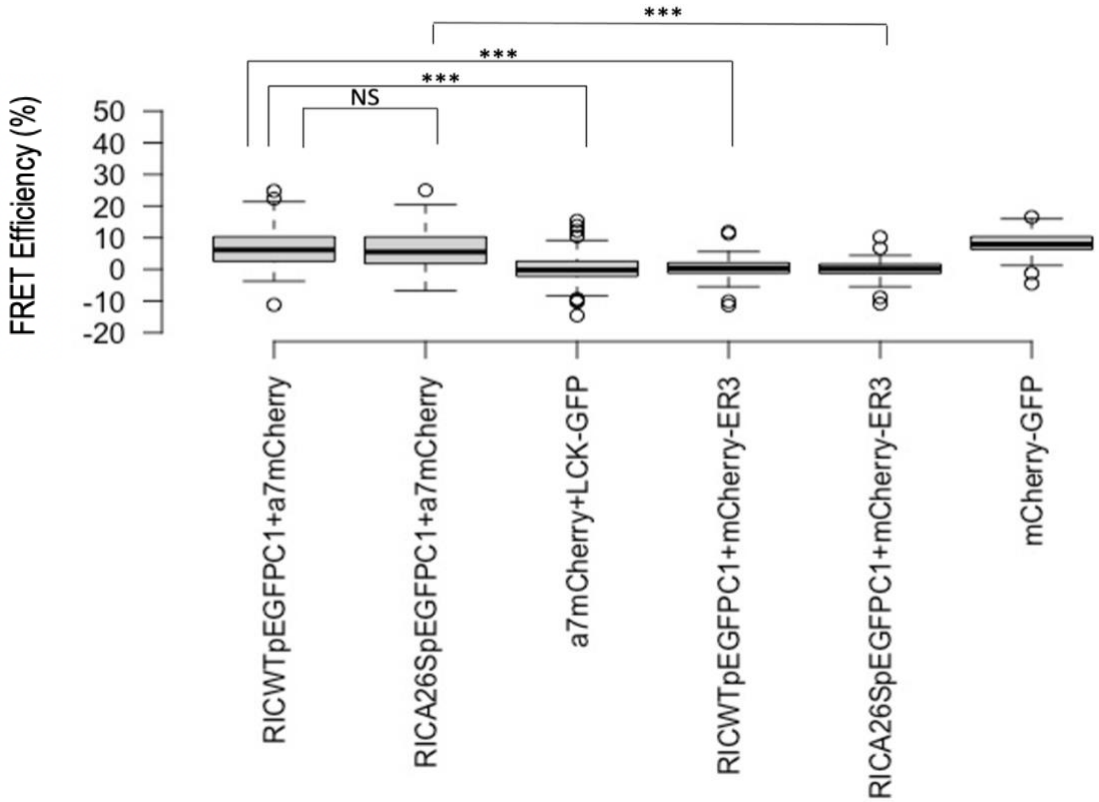
Previous studies have found that RIC3 interacts robustly with α7 and found this interaction to peak when mammalian cells are transfected with the chaperone and nAChR at a 1:1 ratio, as measured by confocal FRET experiments, (Dau et al., 2013). To obtain direct evidence of whether any of the RIC3 variants introduced affected its interaction with α7 apFRET, was employed.

There was no significant difference between the FRET efficiency between RICWT and α7 and RICA26S or RICV196F and α7 ($p>0.05$). This highlights that the interaction between RIC3 and α7 was preserved despite the introduction of variants (table 8). In addition, the negative controls were found to interact significantly less ($p<0.05$) than RIC3 in its WT or variant form and α7, indicating that interactions between α7 and RICWT or in its variant form were α7 specific in this instance. Interestingly, the T177S was found to significantly reduce interactions with α7 compared to RICWT ($p=0.046$, table 8)

A)


***
***
***
NS
FRET Efficiency (%)
50
40
30
20
10
0
-10
-20
RICWTpEGFPC1+a7mCherry
RICV196FpEGFPC1+a7mCherry
a7mCherry+LCK-GFP
RICWTpEGFPC1+mCherry-ER3
ICV196FpEGFPC1+mCherry-ER3
mCherry-GFP

B)


***
***
***
NS
FRET Efficiency (%)
50
40
30
20
10
0
-10
-20
RICWTpEGFPC1+a7mCherry
RICA26SpEGFPC1+a7mCherry
a7mCherry+LCK-GFP
RICWTpEGFPC1+mCherry-ER3
RICA26SpEGFPC1+mCherry-ER3
mCherry-GFP

C)

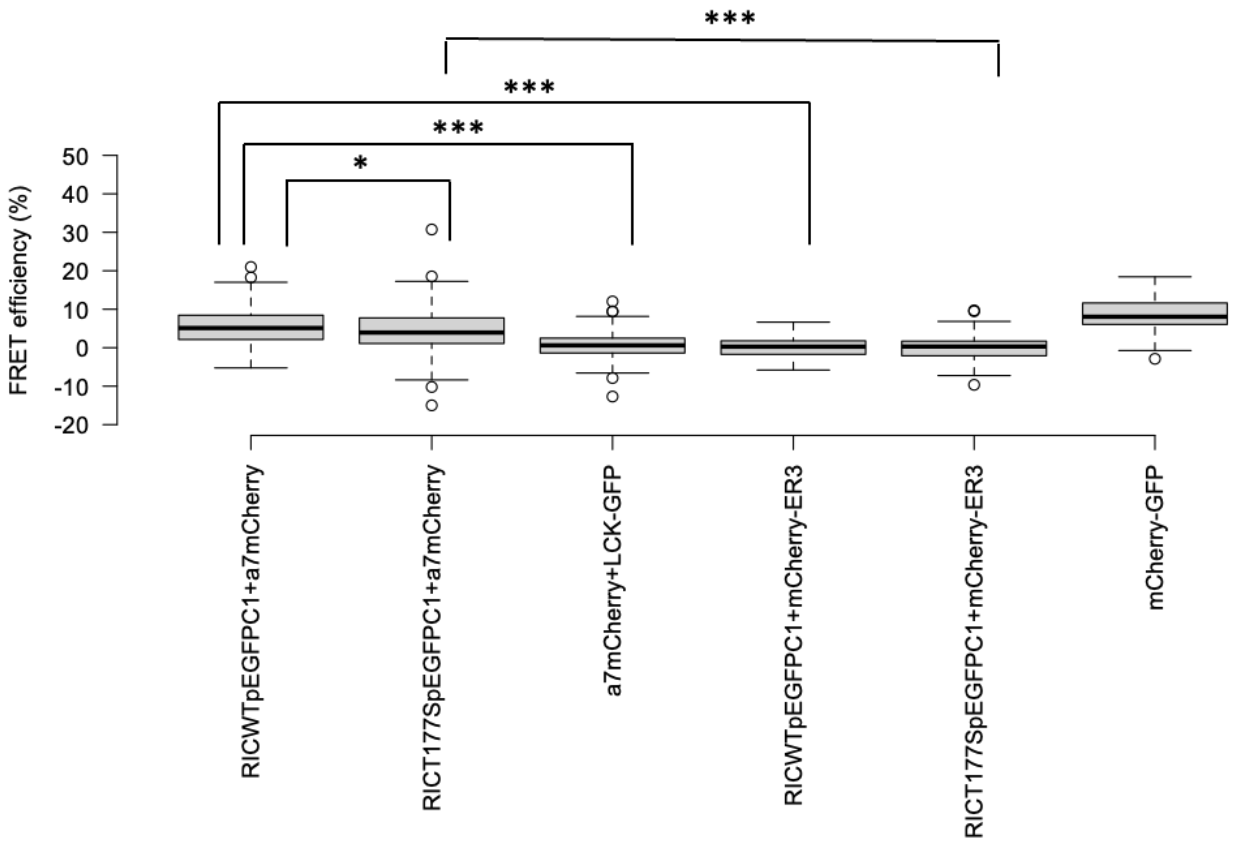



*Figure 3.8 Interaction between α7 and RIC3 variants as measured by* apFRET. HEK293T cells were transiently transfected with eGFP-RICWT and in its variant form. eGFP was used as the FRET donor and mCherry as the FRET acceptor. *pmCherry-eGFP (Addgene, plasmid#86639) was used as positive control, while LCK-GFP (Addgene, plasmid#61099) and α7-mCherry were used as negative controls. Donor and acceptor fluorescence intensity post acceptor bleaching was normalised. FRET efficiencies were masured between A) RICV196F, B) RICA26S, and C) RICT177S.* There was no statistically significant difference in the interaction between; A) RICV196F (p=0.285, n=101), RICVA26S (p=0.5, n=114), and *α7 compared to RICWT*. C) RICT177S (p=0.044, n=97) was found to have a significantly reduced interaction with *α7 compared to RIC WT. The FRET efficiency seen in negative controls was significantly lower than that of RICWT or RIC3 in its variant form and α7.*

Table 8- FRET efficiencies measured between RIC3, its variants, and α7

| Experiment | Combinations (n) | Significance of interaction between the construct P value (t-test) | Interaction between the 2 proteins of interest |
|---|---|---|---|
| V196F | RICWT+ α7 (95)<br>RICV196F+ α7 (101) | 0.285 | ✓<br>✓ |
| | RICWT+ α7 (95)<br>RICWT+ mcherry-ER3 (negative control;82) | 1.29E-24 | ✓<br>✗ |
| | RICV196F+α7 (101)<br>RICV196F+mCherry-ER3 (negative control; 60) | 2.60E-24 | ✓<br>✗ |
| | RICWT+ α7 (95)<br>α7mCherry+ LCK-GFP (negative control; 76) | 9.85E-20 | ✓<br>✗ |
| A26S | RICWT+ α7 (119)<br>RICA26S+ α7 (114) | 0.50 | ✓<br>✓ |
| | RICWT+α7 (119)<br>RICWT+mCherry-ER3 (negative control; 92) | 8.35E-19 | ✓<br>✗ |
| | RICA26S+ α7(114)<br>RICA26S+mCherry-ER3 (negative control; 91) | 2.45E-20 | ✓<br>✗ |
| | RICWT+α7 (119)<br>α7mCherry+ LCK-GFP (negative control; 94) | 4.26E-16 | ✓<br>✗ |
| T177S | RICWT+α7 (93)<br>RICT177S+α7 (97) | 0.044 | ✓<br>✓ |
| | RICWT+ α7 (93)<br>RICWT+mCherry-ER3 (negative control; 91) | 1.52E-18 | ✓<br>✗ |
| | RICT177S+ α7(97)<br>RICT177S+mCherry-ER3 (negative control; 95) | 2.85E-08 | ✓<br>✗ |
| | RICWT+α7 (93)<br>α7mCherry+LCK-GFP (negative control; 77) | 1.73E-12 | ✓<br>✗ |

Considering that T177S was chosen due to no noticeable enhanced effects on language outcomes (see table 6 and appendix table S2) the finding that T177S led to significantly reduced interaction with α7-mCherry, compared to RICWT, was somewhat surprising and, as such, reasons for this finding were considered. Firstly, the effect of the outliers on this result were considered. It was found that removing the outliers did not account for the significant finding (figure 3.9), that is to say that, after removing outliers, the significant effect. Secondly, it was considered whether this overall effect was driven by an effect on a specific language outcome. Individual 6 had the lowest scores in verbal IQ, categorical total IQ (data not shown) of all individuals with RIC3 variants (table 6 and appendix S2).

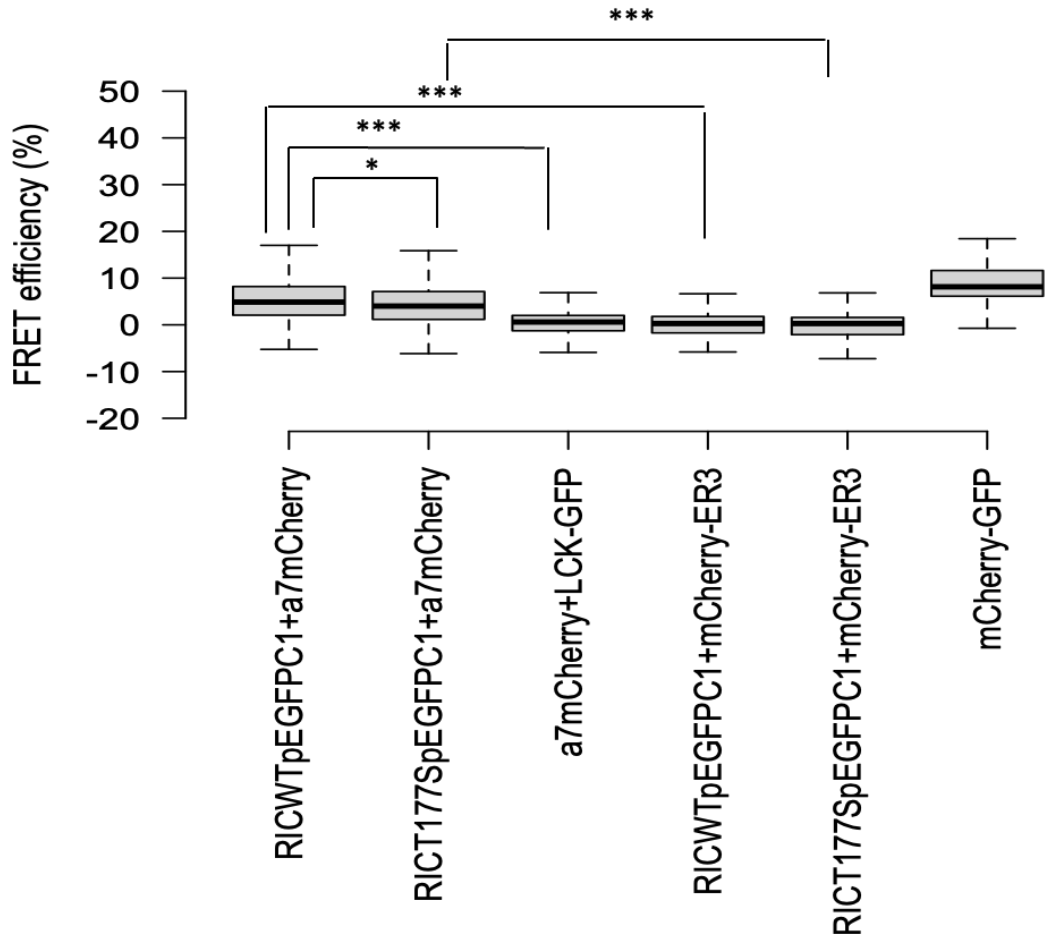
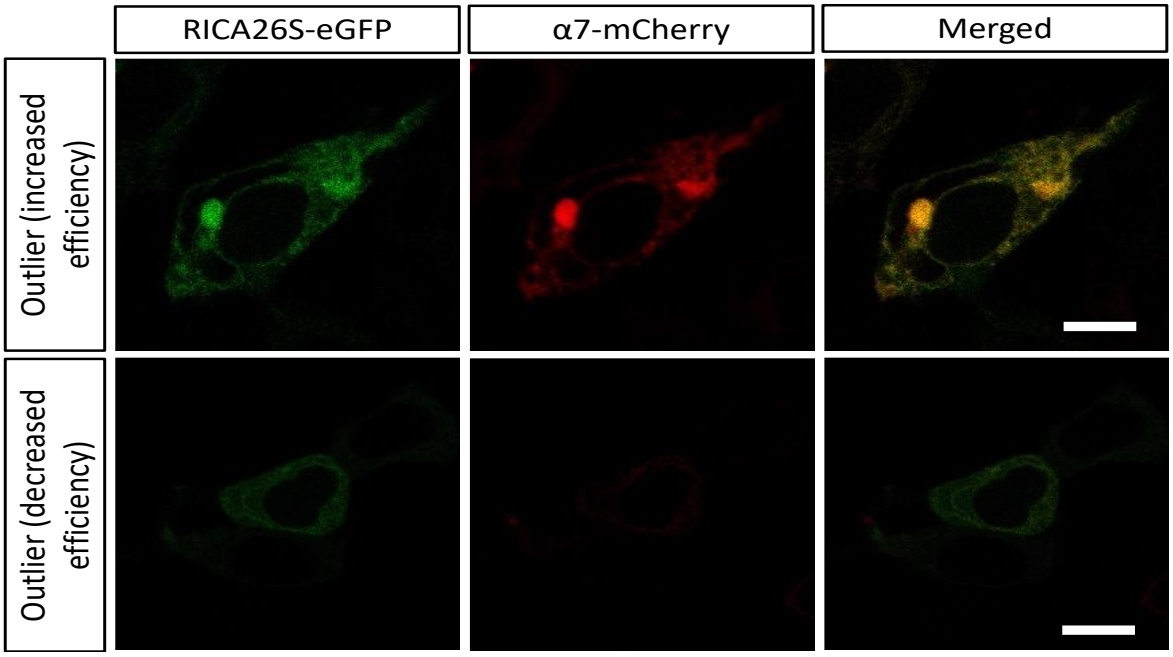



*Figure 3.9 Difference in Interaction between RICT177S and RICWT and α7-mCherry is not explained by outliers.* After removing outliers, the interaction between RICT177S and α7-mCherry remained significantly lower than that of RICWT and α7-mCherry ($p<0.05$).

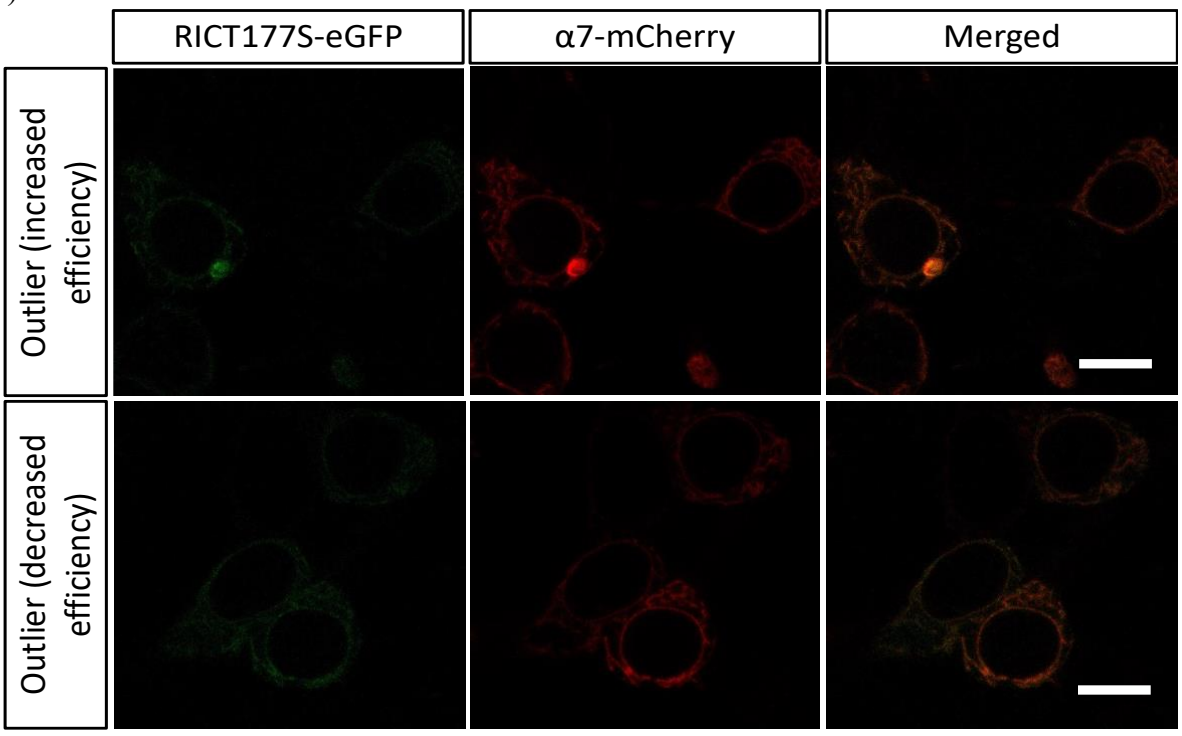
### 3.3.4 Cell-to-cell variation seen in FRET efficiency is likely due to cellular protein fluorescence differences

Confocal analyses showed high cell-to-cell variation regarding FRET efficiency across each of the variants considered. As, particularly in FRET, interaction can be linked to the protein expression by the cell, an interesting consideration was whether this was a factor contributing to the cell-to-cell variation seen in figure 3.8, which shows cells with FRET efficiencies as high as approximately 30% as well as FRET efficiencies below -10%. Figure 3.10, although a somewhat crude depiction of fluorescence, highlights a trend towards stronger eGFP and/or mCherry expression in cells with the highest FRET efficiency and lower expression of either or both these fluorophores in cells with lowest FRET efficiency. Additionally, this trend is seen in the presence of all the variants as well as in RICWT, suggesting that the trends towards FRET efficiency outliers, as seen in figure 3.8, are likely due to protein expression by the cell rather than a potential effect of the variants on the cell, although it is of note that no statistical testing was done in regards to this.

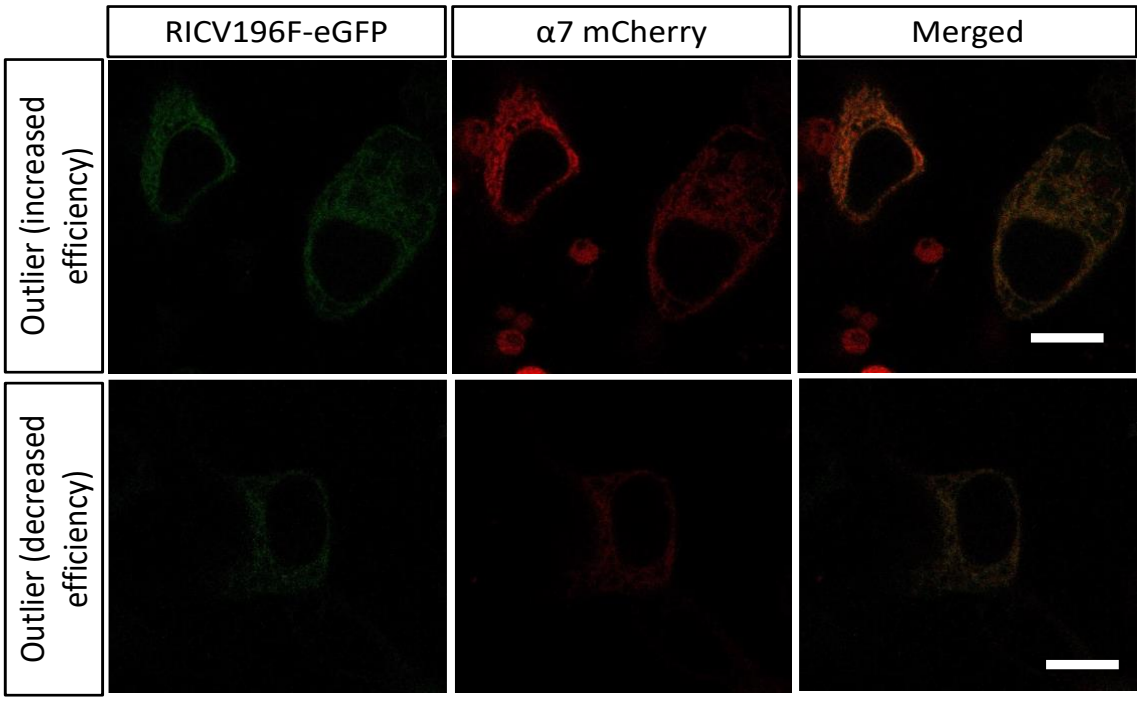
A)


RICA26S-eGFP
α7-mCherry
Merged
Outlier (increased efficiency)
Outlier (decreased efficiency)

B)


RICT177S-eGFP
α7-mCherry
Merged
Outlier (increased efficiency)
Outlier (decreased efficiency)

C)




D)

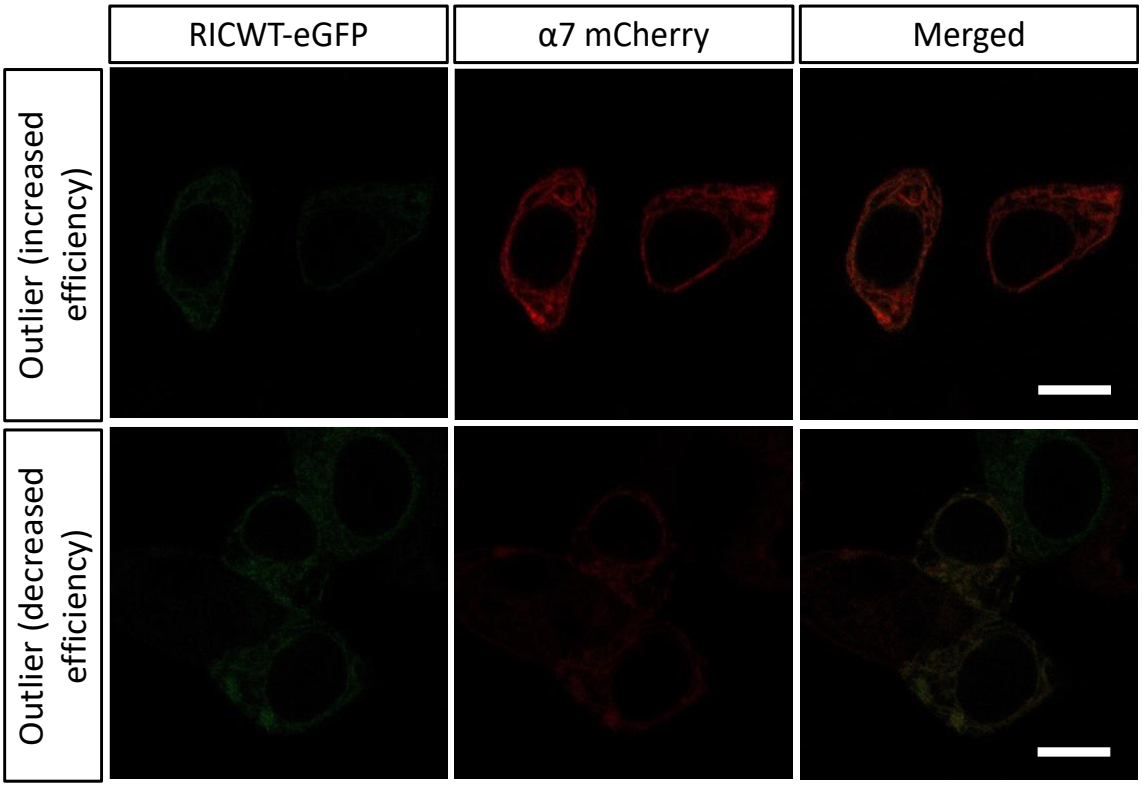



*Figure 3.10 Protein expression in cells with outlying FRET efficiency*. Highest and lowest FRET outliers were chosen for closer consideration. *Scale bars represent 10$\mu m$*

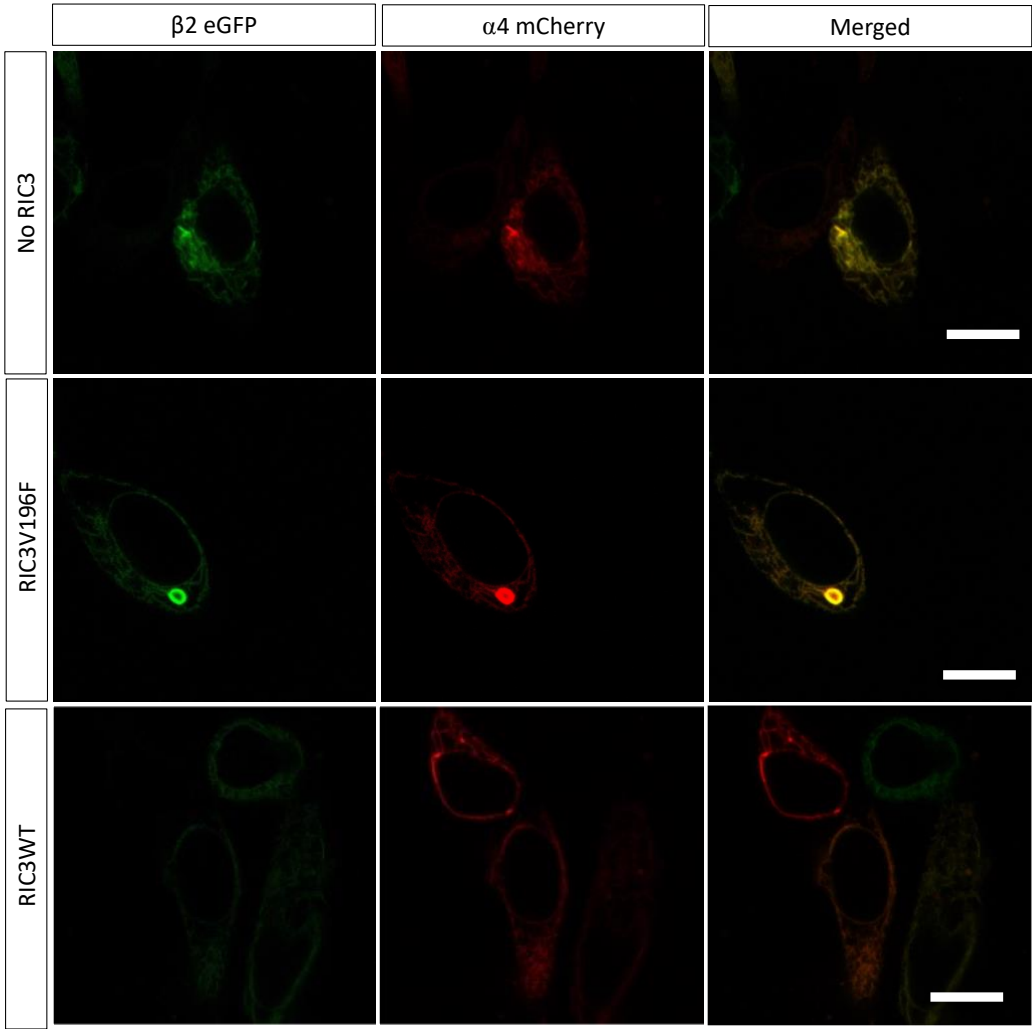
### 3.3.5 RICV196F variant increases α4 and β2 protein expression

Previous results in this study have not provided robust evidence of effects of RIC3 variants on its interaction with α7 nAChRs. RIC3 is not just capable of interacting with α7 but there is evidence that it can affect the expression of α4β2 nAChRs (Dau et al., 2013). Therefore, given the importance of α4β2 in cognition, the effects of the RIC3 variants on α4β2 expression was next examined. This part of the study focused on assessing the effects of RIC3 variants on α4β2. This approach was employed using V196F first due to the higher language attainment seen in individuals with this mutation.

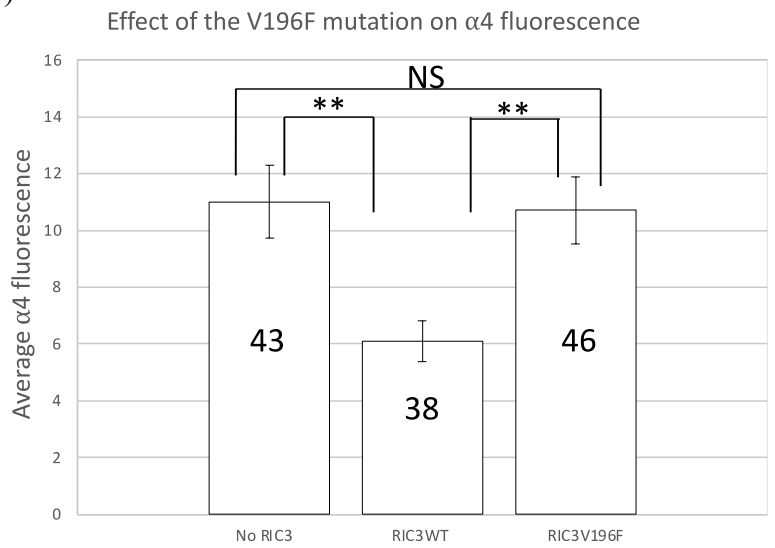
Similarly to what this study has shown regarding α7 nAChRs, RIC3, in its wild-type or variant form, did not overtly affect the cellular localisation of α4 or β2. Figure 3.11a shows that the localisation of α4 and β2 remains associated with the ER. This suggests that, similarly to α7, RIC3 does not overtly impact the cellular expression of α4β2.

RIC3 appears to exert similar effects on α4 and β2 with an initial decrease in the fluorescence levels of both fluorophores seen after addition of RICWT, compared to the absence of RIC3, although the decrease in β2 levels does not reach statistical significance (figure 3.11b and c). In contrast, V196F was found to significantly increase the level of both α4 and β2 ($p<0.01$) compared to RICWT. The presence of V196F also significantly increased β2 fluorescence levels compared to when RIC was absent ($p<0.05$), suggesting that V196F may positively affect RIC3's chaperone activity in regards to β2. This same effect was not seen in regards to α4, which was not present at increased levels in the presence of RICV196F compared to when RIC3 was absent.

A)


β2 eGFP
α4 mCherry
Merged
No RIC3
RIC3V196F
RIC3WT

B)


Effect of the V196F mutation on α4 fluorescence
Average α4 fluorescence
16
14
12
10
8
6
4
2
0
NS
**
**
43
38
46
No RIC3
RIC3WT
RIC3V196F

C)

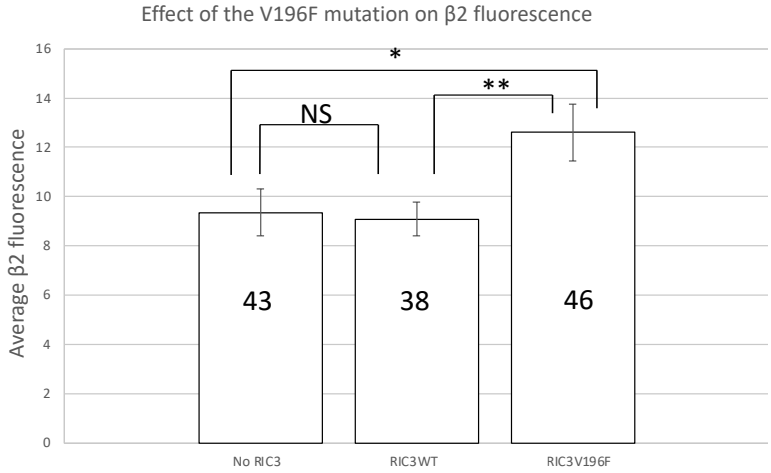

Effect of the V196F mutation on β2 fluorescence
Average β2 fluorescence
16
14
12
10
8
6
4
2
0
*
**
NS
43
38
46
No RIC3
RIC3WT
RIC3V196F

*Figure 3.11 The effect of the V196F mutation on α4-mCherry and β2-eGFP protein expression.*

Equal amounts of α4-mCherry and β2-eGFP were co-expressed with RICWT-CFP or RICV196F-CFP at a ratio of 0.1(RIC3) to 1(α4 β2).

B) Quantification of α4-mCherry mean cell fluorescence showed a significant increase in the fluorescence of RICV196F compared to RICWT ($p<0.01$). This trend was not seen when comparing α4-mCherry in the presence of RICV196F or without RIC3; the introduction of RICWT significantly decreased α4-mCherry fluorescence compared to when it was absent ($p<0.01$) and there was no significant increase in α4 fluorescence in the presence of RICV196F compared to when it was absent ($p>0.05$). C) Mean β2-eGFP also showed a significant increase in β2-eGFP fluorescence in the presence of RICV196F compared to RICWT ($p<0.01$). There was also a significant increase in in β2-eGFP fluorescence in the presence of RICV196F compared to when RIC3 was absent ($p<0.05$). There was a trend towards decreased β2-eGFP fluorescence in the presence of RICWT compared to when it is absent, but this did not reach significance. *P<0.05. **P<0.01. NS= non significant. Numbers in individual bars refer to n numbers. Scale bars represent 10 μm.

## 3.4 Discussion

Several chaperones exist that modulate the maturation and trafficking of nAChRs (Colombo et al., 2013). However, RIC3 is a highly specific chaperone, exerting significant effects on the folding and assembly of α7 receptors (Williams et al., 2005). Interestingly, a recent study has implicated a rare genetic variant of RIC3, G88R, in the unique ability to speak backwards, which in turn has been hypothesised to be. associated with better working memory (Prekovic et al., 2016). In this study we aimed to consider the functional levels of effects that other coding changes may exert on RIC3. The molecular interaction between RIC3 variants RICA26S, RICV196F, and RICT177S, and α7, with RICT177S being used as a control variant, was examined via apFRET. Here, we report that RICA26S and V196F did not significantly increased RIC3 interaction with α7 compared to RICWT, however, RICT177S led to a significantly reduced interaction with α7 compared to RICWT.

This study confirmed previous reports that RIC3 is an interacting partner of α7 (Valles and Barrantes, 2012) as each of the variants introduced produced significantly higher FRET than the negative controls used in this study (table 7), which showed only minimal interaction. Alongside this, our colocalization analyses suggest RIC3 expression in the ER, compared to mCherry-EGFP, and colocalization does not seem to be impacted by the introduction of the variants chosen, we note that this is a somewhat crude assessment of colocalization conducted with images taken for the purpose of FRET analyses.

We found that the variants we introduced to the N-terminus of RIC3 did not affect the ability of the protein to be expressed in the ER. Additionally, the maintained interaction between each of RIC3 variants and α7 suggests that these variants did not affect RIC3's chaperone activity. The latter is unsurprising when considering previous studies which have used constructs tagged to the N- terminus of RIC3 to study its chaperone activity (Dau et al., 2013; Alexander et al., 2010) and found that FRET was maintained. Additionally, the maintenance

of FRET suggests RIC3 is a type 2 transmembrane protein, as for this interaction to be maintained, the n-terminus must be positioned cytoplasmically for this interaction to occur. It is hypothesised that the M3-M4 linker of the α7 nAChR is important for the chaperone activity of α7 (Castillo et al., 2005; Castelán et al., 2008), and given that this loop is predicted to be in the cytoplasm during assembly and maturation in the ER, it would be in proximity to the tagged EGFP molecule should this be a type II transmembrane protein. In addition, despite the fact that A26S, supposedly present in the cytoplasmic SS portion of RIC3, it did not appear to overtly affect the protein's localisation or hinder its interaction with α7, further supporting the hypothesis that RIC3 is a type II transmembrane protein as if the SS was present at this site, as predicted in the type I transmembrane proposed topology, introduction of this variant would likely disrupt the signal expression in the cell thus affecting the ability to visualise the fluorescently tagged protein in the cell.

Interestingly, despite the fact that both RICWT and all the RIC3 variants interacted with α7, the RICA26S and RICV196F did not confer a statistically significant effect on this interaction; these variants did not lead to a significantly more efficient interaction with α7 nAChR, and thus likely not more efficient subunit assembly and maturation. This ultimately suggests that these variants are unlikely to result in more functional cell surface α7 nAChR expression compared to RICWT. One potential explanation for this could be the location of the variants within the protein. The C terminal domain of RIC3 contains a coiled-coil domain which has been previously implicated in protein-protein interactions (Burkhard et al., 2001). However, the coiled-coil domain has not been reported to be required for RIC3's chaperone activity. Alongside with the early finding that RIC3's coiled-coil domain was vital for its interaction with α7 nAChR, it was also proposed that interaction between RIC3 and α7 nAChR subunits occurs via dimerization of RIC3 coiled-coil domains (Wang et al., 2009). As these variants were not in the vital coiled-coil domain, it is possible that this dimerization between RIC3 and α7 nAChR subunits was not affected and thus would not occur more effectively. In addition, individual 4 carrying V196F has persistently lower language outcomes compared to the other two individuals with this variant, suggesting the presence of other factors to explain the higher attainment seen in individuals 3 and 5 with this same variant.

Conversely T177S was found to lead to significantly reduced interactions with α7 ($p<0.05$), compared to RICWT (figure 3.8). This was surprising as this is closest to V196F, which did not reveal an effect, and also does not lie in the coiled-coil domain, which we stated above is important for interaction. In addition, this was not explained by the presence of outliers as this significance remained when outliers were removed (figure 3.9). Interestingly, while the individual carrying this variant had average total IQ of 101, one contributing factor to its consideration as a control, this was the lowest score achieved of all 12 individuals (table 6 and appendix table S2), as well as low freedom from distractibility scores among all individuals. As such, it is possible that this variant does indeed negatively affect the interaction between RIC3 and α7. Further research on this variant, potentially considering its effects on functional cell surface receptor expression as measured by α-BGTx for example, could help shed light on the effects of this variant on α7 nAChR cell surface expression.

Despite the maintained interaction between α7 and RIC3WT or RIC3 in its variant form, and the lack of difference between the FRET efficiency in the presence of our RICA26S and RICV196F, our results do not shed light on the effect of these variants on the cell surface expression of functional α7. Indeed, we only considered interaction at the ER level. We found that, prior to trafficking, α7 was also present in the ER. This is in line with early expression studies by Cooper and Millar, 1997, who found a similar expression pattern of α7 in HEK293T cells to that which we have found in this study. This is also similar to the distribution seen by Dau et al., 2013. This is likely due to the fact that functional nAChR cell surface expression is better ascertained by α-BGTx, an α7 antagonist. As we did not conduct use α-BGTx assays to establish the presence of α7 nAChRs on the cell surface, we were not able to consider the effects of RIC3 variants at this level and are unable to make conclusions regarding the effect of RIC3 variants on the cell surface expression of α7 at this stage; in order to do this, future studies should conduct α-BTX assays as a means of assessing the effect of chaperone variants on cell surface expressed α7 nAChRs.

We found that there was wide cell to cell variability in FRET efficiency. This is likely due to varying amount of DNA being taken up by different cells. Indeed, there is evidence that

varying amounts of RIC3 being present in a cell leads to varying levels of FRET efficiency and with higher amounts of RIC3 leading to the formation of aggregates in the cytoplasm. One way to improve uniformity of the amount of protein uptake would be to express both of the proteins of interest in a single vector as this would lead to the consistent presence of both proteins, in the same amount, in tandem in transfected cells.

We found that RIC3V196F led to a significant increase in the expression of both α4 and β2 subunits compared to RIC3WT (figure 3.10). This is expected as previous studies have shown that the association of other chaperone proteins such as 14-3-3 and phosphorylation of α4 results in increased expression of α4β2 receptors (Wecker et al., 2010). Compared to other subunits, α4 has the largest M3-M4 cytoplasmic loop (~260 aa) with numerous post-translational modification sites that can potentially regulate protein expression and receptor turnover (Nashmi et al., 2003; Wecker et al., 2010; Exley et al., 2006) . It is possible that when RIC3 binds to α4β2 receptors RIC3 may act like 14-3-3 to upregulate receptor protein expression levels Exley et al., 2006) possibly by inhibiting signals targeting for degradation. In fact, β2 subunits have a strong ER retention motif and by binding to RIC3, this may stimulate forward trafficking of α4β2 receptors and thus, protect them from ER associated degradation (Alexander et al., 2010). Conversely, we saw a decrease in the expression of both α4 and β2 in the presence of RIC3WT compared to when it was not present. This is unexpected as the presence of RIC3 has been found increase the fluorescence and thus expression of both α4 and β2 (Dau et al., 2013). However, Dau et al., 2013, did find that the addition of RIC3 at a higher ratio such as ratio 5:1, led to a decrease in both α4 and β2 fluorescence. It is possible that triple transfected cells may be expressing a relative excess of RIC3 compared to α4 and β2 which could in turn lead to the formation of aggregates in the ER, reducing this expression. At times, we did witness the formation of these ER RIC3 aggregates (data not shown) and, when these led to overexposed areas in the cell, this cell was excluded from analyses. This was to avoid skewing the fluorescence obtained as due to the fact that all cell fluorescence was measured, overexposed areas would be included in this. The latter may cause a bias towards analysis of cells with an overall lower level of RIC3 expression leading to lower α4 and β2 expression.

Additionally, despite the fact that the variants of interest were chosen due to the trends towards higher language attainment in those carrying RICA26S and RICV196F, there was still wide variability in the scores achieved across subtests by individuals carrying the same variants, particularly V196F (table 6). In light of the findings in chapter 2, this variability could be due to effects of maternal IQ or SES, factors highlighted as major confounders of the effects of prenatal cigarette smoke exposure and child language outcomes (Peixinho et al., 2022). In addition, RIC3 is one chaperone of α7 that we have focused on here, however it is not the only one. Other chaperones, such as NACHO, have been reported to be involved in α7 nAChR assembly and trafficking (Gu et al., 2016; Kweon et al., 2020) and, as such, this could be a potential avenue of investigation of chaperone effects on nAChRs and subsequent cognitive effects.

Taken together, the results of this chapter highlight that RIC3A26S and RIC3V196F, do not confer more efficient interaction between RIC3 and α7 nAChR subunits and thus is unlikely to alter the cell surface expression of α7 nAChR subunits. It is of note that we did not confirm this using $\alpha$-BGT assays, which would have highlighted the presence of functional cell surface receptors and provide further validate the influence of the contribution of RIC3 variants on overall nAChR cell surface function. It is also important to acknowledge that α4β2 is another major neuronal nAChR which to which RIC3 is also a chaperone with well-established cognitive links. To this end, our results implying a lack of influence of these RIC3 variants on α7 nAChR function, do not suggest a lack of influence of these variants on α4β2 nAChR function. Future studies should hence consider the effect of these RIC3 variants on α4β2 nAChR as a potential mechanism to explain links between cigarette smoking on language.

## Chapter 4 - Concluding discussion and future work

Overall, the results of these studies taken together allow us to conclude that, whilst prenatal CS likely negatively affects cognition in terms of language, as was found in chapter two, however, the effects of CS on $\alpha$7 nAChR assembly and, ultimately, function do not appear to be a main mechanism involved in this, as supported by chapter 3.

Firstly, our review supports the already existing plethora of evidence that environmental insults, such as CS and drinking during pregnancy can have numerous negative effects on the fetus. However, the review takes this one step further by considering these effects, specifically in terms of language, which had not been done previously (Peixinho et al., 2022). One important consideration is that our review considers CS effects only during pregnancy. Our review did not focus on the exposure of the child to CS throughout childhood, however, it is well known that brain development is a continuous process, continuing to occur not only in the prenatal period but well throughout the early postnatal period. For example, from birth to 2 years, overall brain size increases and reaches close to 90% of adult volume (Pfefferbaum et al., 1994). Gray matter volume also reaches a lifetime maximum at around 2 years (Hüppi et al., 1998; Matsuzawa et al., 2001). The review does not specifically consider other factors during childhood, such as CS exposure throughout childhood; a child not exposed to CS prenatally but exposed postnatally could thus still have poorer language outcomes, potentially linked to early childhood exposure, which would have not been captured in the scope of our review. As such, our review cannot establish whether the trend towards poorer language outcomes is due to prenatal exposure or more starkly affected by early childhood factors. Further research should investigate effects postnatally to establish whether exposure at this time reveals the same trend regarding childhood language outcomes.

Overall, we did not find that RIC3 variants lead to more efficient interactions between RIC3 and α7. Does this mean that chaperone variants do not affect their interaction with nAChRs? Two major factors need consideration, firstly, even though we did not find an effect of RIC3 variants on α7 nAChRs, we cannot assume that these variants do not have effects on interactions with nAChRs in a general sense. RIC3 is not just specific to α7 but rather also exerts its effects on α4β2, the other major neuronal nAChR. Indeed, Dau et al., 2013, found that RIC3 at different concentrations is affects the expression of α4β2. Considering that α4β2 has also been linked to cognition, investigation of RIC3 variant effects on α4β2 may provide a different mechanism for considering links between RIC3 and cognition.

Secondly, another important consideration is that RIC3 is not the only chaperone involved in nAChR assembly and trafficking but rather a single chaperone which we have focused on throughout this project. Considering α7 nAChRs in particular, other chaperones, including Nacho have been shown to be important in its assembly and maturation. Nacho has been shown to increase ACh-evoked currents as well as the amount of the receptor at the plasma membrane (Gu et al., 2016). NACHO is thought to act at early intracellular stages of nAChR subunit assembly and then synergizes with RIC3 for receptor surface expression (Matta et al., 2017). It is possible that a disruption of the interruption in this interaction could affect the assembly and trafficking of receptors to the cell surface and thus considering Nacho‘s interactions throughout this process could be an interesting and valid avenue of future research.

In addition, as nicotine, the main and addictive component of CS, acts on nAChRs and upregulates their expression, we began this work by focusing on α7. However, one of the main effects known to be associated with CS is DNA methylation alterations. Nicotine is a well-known epigenetic modulator which has been associated with hypomethylation (Breitling et al., 2011; Wan et al., 2012; Zeilinger et al., 2013), which in turn is associated with gene expression. One of the factors affecting protein function is its expression. Even though we did not find a significant effect of RIC3 variants on its interaction with α7, it is important to note that FRET remains only one way of considering this interaction. Indeed, another way of considering CS effects is by considering methylation alterations across we did not assess the

effects of CS on methylation at on either RIC3 or $\alpha$7 and thus cannot make conclusions regarding whether CS effects the gene expression of *RIC3* or *CHRNA7*.

Further to the above, we could expand the proposed by considering how CS alters methylation in another approach which we could be relevant in shedding light on the links between language could be to look at methylation alterations in some of the chaperone genes mentioned here.

Further work

Considering the above, I propose two other mechanisms to further investigate how cigarette smoking can affect language; through its action on other neuronal nAChR subtypes, such as α4β2, or through specific effects on methylation at the genes of interest mentioned here; RIC3, α7, α4β2. The findings from these studies could pave the way towards establishing an explicit link between prenatal smoking and poorer language outcomes as well as help to reiterate the dangers of cigarette smoking during pregnancy and educate expectant mothers of dangers in a new capacity.

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# Appendix

Table S1- Systematic review inclusion and exclusion criteria

| Inclusion | Exclusion |
| --- | --- |

| | |
|---|---|
| Human study population | Paper not in English. |
| Examined prenatal exposure to smoking or nicotine | Paper was a review, systematic review, opinion piece or meta-analysis |
| Assessment of nicotine measures obtained during pregnancy and up to 6 months of age (to help ensure better memory of events). | Language outcomes were tested before 2 or after 12 years |
| Study considered specific measures of language as an outcome (this did not need to be the primary focus of the study) | Study considered only neurodevelopmental disorder or broad cognition (no specific measures of language were considered) |
| | Paper focused upon prenatal drug use or factors other than nicotine exposure |
| | Study participants were selected to have a certain disorder |
| | Paper could not be accessed |

Table S2- Key scores of remaining individuals carrying RIC3 variants

| ID | Individual 1 | Individual 2 | Individual 7 | Individual 8 | Individual 11 | Individual 12 |
|---|---|---|---|---|---|---|
| SIFT_Score | 0.021 | 0.373 | 0.01 | 0.353 | . | . |
| PolyPhen2 | 0.994 | 0.038 | 1 | 0.001 | . | . |
| CADD_Phred | 26.1 | 10.63 | 33 | 10.87 | . | . |
| PhyloP100Way | 6.356 | -2.523 | 6.734 | 3.134 | . | . |
| WISC - Verbal IQ: F@8 | 116 | 109 | 121 | 143 | 116 | 109 |

| | | | | | | |
|---|---|---|---|---|---|---|
| WISC - Verbal Comprehension Index: F@8 | 48 | 48 | 48 | 67 | 50 | 45 |
| WISC - Freedom from Distractibility Index: F@8 | 25 | 21 | 27 | 31 | 23 | 21 |
| WISC - Similarities scaled score: F@8 | 10 | 14 | 14 | 17 | 10 | 17 |
| WISC - Arithmetic scaled score: F@8 | 15 | 10 | 19 | 19 | 13 | 13 |
| WISC - Vocabulary scaled score: F@8 | 9 | 8 | 12 | 19 | 14 | 9 |
| WISC - Digit span scaled score: F@8 | 10 | 11 | 8 | 12 | 10 | 8 |
| WISC - Total IQ: F@8 | 112 | 105 | 114 | 128 | 114 | 115 |

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REVIEW ARTICLE

WILEY

# The effects of prenatal smoke exposure on language development - a systematic review

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## Abstract

The negative health effects of cigarette smoking during pregnancy (SDP) on the foetus are well known. Despite previous reports of poor cognitive performance in offspring exposed to SDP, few studies specifically consider language outcomes according to maternal smoking. In this study, we systematically review the literature to assess the relationships between SDP and child language. Of the 14 studies reviewed, 13 (93%) reported significant associations between maternal smoking or exposure and language outcomes. Despite this consistent association, only 8 of the 13 studies reporting associations (62%) concluded direct relationships between exposure and outcome. The remaining studies suggested that the relationship between smoking and language could be explained by factors such as maternal IQ, socioeconomic status (SES) and parental age. Future studies should apply careful study designs allowing for confounding factors across child, parental, environmental and genetic influences. Our review suggests that smoking cessation is likely to positively affect child language outcomes.

## Highlights

- Does maternal smoking during or exposure to smoking during pregnancy affect the language outcomes in exposed offspring?

- A systematic review of the literature highlighted consistent negative effects of smoking or smoke exposure during pregnancy on language outcomes.
- Exposure to SDP is associated with language. Mothers must be educated regarding the effects of tobacco smoking on language outcomes.

**KEYWORDS**
exposure, language, maternal, prenatal, smoke

## 1 | INTRODUCTION

A variety of evidence shows that smoking while pregnant can lead to adverse effects on the mother and foetus (Nuffield Trust, 2019). This evidence has led to widespread medical and societal sanctions against tobacco smoking during pregnancy (SDP) that correlate with a reduction in women who smoke at the time of delivery; in England in 2019–2020, 10.4% of pregnant women smoked at the point of delivery compared to 14.2% ten years earlier (2009–1010) (Nuffield Trust, 2019). More recently, the emergence of new tobacco products, including electronic cigarettes and hookah, has become common among youth and women of reproductive age women with the potential to increase rates of infants born exposed to nicotine or tobacco (Bowker et al., 2021).

A recent study predicted that the prevalence of smoking in England is projected to decrease to 10.8% by 2022, down from 14.4% in 2018 (Song, Elwell-Sutton, & Naughton, 2020). However, significant differences have been reported across socioeconomic groups (Song, Elwell-Sutton, Naughton, & Gentry, 2020) whereby individuals from lower socioeconomic backgrounds are more likely to smoke.

Nicotine is the major reinforcing component of tobacco smoke. Nicotine is an alkaloid naturally found in the nightshade family of plants, including the tobacco plant and primary exposure to this chemical is through active or passive smoking (Fagerström, 2014). In the brain, nicotine binds nicotinic acetylcholine receptors (nAChR) activating the reward system and exerts its action in the brain through $\alpha4\beta2^*$ nAChR (*denotes possible assembly with other nicotinic subunits) (Tapper et al., 2004).

The various negative effects of smoking have been long established and well reported. Smoking not only impacts the individual but also others in proximity. Maternal smoking and exposure to cigarette smoke during pregnancy remain a substantial health concern. It is estimated that 1.7% of pregnant women worldwide and 8.1% of pregnant women in Europe smoke (Lange et al., 2018). The effects of maternal smoking on new-born children are also of concern as studies have shown that infants nursed by smoking mothers have detectable amounts of nicotine and cotinine (the primary metabolite of nicotine) in their serum and urine (Luck & Nau, 1985). In addition, longer breastfeeding duration has been linked to more favourable outcomes on cognitive development (Kim & Choi, 2020) which may hence encourage more mothers, despite their smoking status, to breastfeed.

Maternal SDP has well-established and direct negative effects on birth outcomes including low birth weight and preterm birth (Salihu & Wilson, 2007), both of which are markers of foetal health and are associated with neurological and psychiatric outcomes (Hack et al., 2005). Prenatal and early nicotine exposure is further associated with negative perinatal health including respiratory and ear infections, asthma, reduced cognitive function and behavioural difficulties (DiFranza et al., 2004) which may have serious health implications. In terms of neurodevelopmental disorders, in utero smoke exposure is primarily associated with an increased risk of Attention Deficit Hyperactivity Disorder (ADHD) (odds ratio [OR] = 2.39) (Langley et al., 2005), (pooled risk ratio [RR] = 1.58) (He et al., 2020) and Conduct Disorder (OR = 2.06) (Ruisch et al., 2018) and has been reported to increase the risk of schizophrenia by

29% (Hunter et al., 2020). Smoking is associated with some subtypes of Autistic Spectrum Disorders (ASD) such as Pervasive Developmental Disorder (PDD) (Tran et al., 2013) and ASD-not otherwise specified, (Kalkbrenner et al., 2012), although these findings were not supported by meta-analyses (Jung et al., 2017; Rosen et al., 2015; Tang et al., 2015). Interestingly, a single study has reported that the genetic risk of dyslexia modulates the performance of memory in interaction with maternal SDP, although this was only true for variation within a single candidate gene of five studied by this group, *DYX1C1* (Mascheretti et al., 2013; Mascheretti et al., 2015). Furthermore, smoking was not identified as an independent risk factor in another study of environmental contributors to dyslexia by the same group (Mascheretti et al., 2013) or by systematic reviews of risk factors in dyslexia (Becker et al., 2017; Mascheretti et al., 2018).

There are many mechanisms by which exposure to tobacco, containing many thousands of chemicals, may influence cognitive development. Smoking reduces foetal blood flow and oxygen levels and nicotine has been found to be a teratogen in animals in whom it crosses the placenta and stimulates foetal cholinergic neurons affecting neuronal migration, synaptogenesis and apoptosis (Dwyer et al., 2008). Additionally, nicotine and other chemicals present in tobacco smoke can affect critical cellular processes such as protein synthesis and enzyme activity (Dempsey & Benowitz, 2001; Jauniaux et al., 2001). Nonetheless, it has been highlighted that the ascertainment of a direct causal relationship between SDP and offspring outcomes, such as ADHD or conduct problems requires careful study design (Rice et al., 2018) in terms of phenotype (Clifford et al., 2012) and exposure (Jung et al., 2017) measurement as well as the avoidance of confounding factors that are associated with both exposure and outcome. In particular, inherited factors, maternal IQ and socioeconomic status have all been shown to increase the likelihood of starting to smoke (Agrawal et al., 2008; Hiscock et al., 2012; Reid et al., 2010) and may act as confounders as these factors are also associated with an increased risk of neurodevelopmental disorder (Batty et al., 2006; Özmert et al., 2005; Thapar et al., 2009). Fifty years ago, the smoking prevalence for all education groups was consistent, with nearly 40% of degree level educated individuals and approximately 45% of individuals in all other education groups smoking. Recently, this has decreased to 6.5% of degree level and 23.1% of individuals with a high school education (secondary school) or less in the US (Drope et al., 2018). The latter pinpoints the importance of education and SES as a confounder when considering smoking but many other confounders exist and, importantly, can have bidirectional effects. As such studies that do not adjust for confounder factors can overestimate the association between smoking and cognition (Batty et al., 2006). In particular, shared genes, environments and behaviours can all influence language and SDP.

There are well-established ages by which most linguistic developmental milestones are expected to be achieved. Active vocabulary begins to develop in the second year. Indeed, after the first year of life, word comprehension increases rapidly and a child's ability to understand language largely surpasses their ability to produce it (Fenson et al., 2000). The time when children begin school, at around 6 years, is considered vital for their cognitive development. Introduction to teaching alters the linguistic input to which a child is exposed (Riva et al., 2000) and by the age of 6 children have a well-developed vocabulary that is vast and have complete phonological production ability (Hoff, 2009). In addition from early childhood (6 years) to puberty (around 12 years), strategies for generating and integrating information emerge, including more sophisticated use of language through the use of more complex sentences and grammar (Rosselli et al., 2014) Considering the above, the ages of 2 to 12 years appears particularly relevant regarding language trajectories.

In the current study, we use a systematic review design to investigate the relationship between maternal smoking or smoke exposure and childhood language development. In a previous study of 1102 children, Tomblin and colleagues reported that maternal and paternal SDP were associated with an increased risk of developmental language disorder (DLD) (Tomblin et al., 1997; Tomblin et al., 1998). However, in line with the studies described above, this association disappeared when parental education was included in their model. This leads to the conclusion that parental smoking is not independently associated with DLD (Tomblin et al., 1998). Eicher and colleagues found that children exposed to prenatal nicotine performed 4.8%–5.4% worse on language tasks (Nonword repetition and verbal comprehension) at age 8 than children without smoke exposure (Eicher et al., 2013). They further reported that language performance was dosage-sensitive with regard to the level of prenatal exposure, as was the risk of language

impairment (LI) (exposure ≤17 mg/day nicotine LI OR = 1.25, exposure >17 mg/day, LI OR = 3.84). However they did not compare the number of cigarettes smoked or tar: nicotine ratio (Eicher et al., 2013). Social class and sex were included as covariates in these analyses but neither maternal education or IQ were directly controlled for (Eicher et al., 2013). Another study considering overall risk factors for LI has highlighted the importance of various risk factors such as very low birth weight (OR = 2.2), low 5 min Apgar score (OR = 2.0), lower maternal education (OR = 1.3–1.6), being an unmarried mother (OR = 1.4), and later stage of commencement of prenatal care (OR = 1.2–1.3) in the risk of the development of LI (Stanton-Chapman et al., 2002) This study, however, did not conclude tobacco use to be a major risk factor for development of LI (OR = 1.0) (Stanton-Chapman et al., 2002).

Although other studies may include language and communication as part of their consideration of cognition or neurodevelopment, there are few which focus primarily on language development or disorder in relation to smoking. Studies have demonstrated discrepancies in language development compared to other cognitive abilities and indeed other communication abilities. A study of early communication development in toddlers highlighted differing developmental patterns in their levels of social, speech, and symbolic skills (Maatta et al., 2012). Considering smoking's links to cognition and the fact that smoking has been shown to impair synaptic maturation in the auditory brainstem which in turn may affect auditory processing (Baumann & Koch, 2017) it is important to consider language in a smoking context. In addition, the critical role of language in the overall development of the child highlights it is vital to robustly examine the association between smoking and language development. In this systematic review, we aim to examine published studies, which consider language outcomes after prenatal exposure to nicotine.

## 2 | METHODS

### 2.1 | Sources

A systematic review of journal articles published between the years 2000 and 2020 was conducted. Web Of Science (https://clarivate.com/webofsciencegroup/solutions/web-of-science/) and Pubmed (https://pubmed.ncbi.nlm.nih.gov/) were searched using comprehensive search strategies as detailed below. The reference lists of identified articles were also searched to identify additional relevant references. Data collection was completed between February and March 2021.

### 2.2 | Search strategy

(((((([Child*]) AND ((((((((((((((([Develop*]) OR ([Language])) OR ([languages*])) OR ([Language*])) OR ([Language[MeSH Terms]])) OR ([Neuro*])) OR ([Vocab*])) OR ([Grammar])) OR ([Attention deficit hyperactivity disorder])) OR ([Attention deficit hyperactivity disorder[MeSH Terms]])) OR ([Autis*])) OR ([Dyslex*])) OR ([Dysprax*])) OR ([Speech])) OR ([Speech[MeSH Terms]]))))) AND ((((((((([Nicotin*]) OR ([Cigar*])) OR ([tobacco products])) OR ([Tobacco products[MeSH Terms]])) OR ([tobacco])) OR ([tobacco[MeSH Terms]])))))) AND ((((((((((([Parent*]) AND ([Smok*]))) OR ((([Matern*]) AND ([Smok*])))) OR ((([Passive]) AND ([Smok*])))) OR ((([secondhand]) AND ([Smok*])))) OR (((secondhand) AND (smok*)))) OR ((([household]) AND ([Smok*])))) OR ((([household*]) AND ([Smok*]))))))) AND ((((((((((([prenatal*]) OR ([prenatal])) OR ([pregnan*])) OR ([uterus])) OR ([utero])) OR ([uterus[MeSH Terms]])) OR ([Mother])) OR ([mothers])) OR ([mum])) OR ([Mothers[MeSH Terms]])))) AND ([Expos*]).

The same search terms were used in the Web Of Science, with the exception of the inclusion of MeSH terms as this is not available on this platform. Filters applied to both were that these studies must have been conducted in the last 20 years, the study must be in English, and outcomes should be articles or letters. In Web Of Science, no measures were included at the search stage to exclude animal studies as there was no clear option in its search engine but any animal studies were excluded at further stages.

The search was limited to studies conducted from the year 2000 to 2020 to make the search more manageable. The search terms included neurodevelopmental disorders such as ADHD and ASD due to the fact that language development is usually relevant to these conditions. In order to draw conclusions and make comparisons between papers, only studies that specifically discussed language outcomes were included in the final analyses.

Papers yielded from these searches were examined in two stages. The first considered only information in the title and abstract and acted as a broad screen to exclude non-relevant results. A second stage considered more detailed information from the full text and screened for in-depth details of the study design. The same inclusion and exclusion criteria were employed across both stages, as detailed below.

Inclusion criteria:

1. Human study population.
2. Examined prenatal exposure to smoking or nicotine.
3. Study includes an assessment of nicotine measures obtained during pregnancy and up to 6 months of age (to help ensure better memory of events).
4. Study considered specific measures of language as an outcome (this did not need to be the primary focus of the study).

Exclusion criteria:

1. Paper not in English.
2. Paper was a review, systematic review, opinion piece or meta-analysis.
3. Language outcomes were tested before 2 or after 12 years.
4. Study considered only an neurodevelopmental disorder or broad cognition (no specific measures of language were considered).
5. Paper focused upon prenatal drug use or factors other than nicotine exposure.
6. Study participants were selected to have a certain disorder.
7. Paper could not be accessed.

### 2.3 | Study selection and data extraction

Two authors independently screened titles and abstracts for all search results. Discordant decisions were resolved by further assessment of paper content and discussion between the authors. All papers which met the inclusion criteria above were catalogued in detail noting the size of the study population, ascertainment criteria, how nicotine was measured, age of children considered in the analyses, outcome measurements, and the confounders identified. Quality assessments of the studies were conducted according to the Kuyper, (1991) checklist (Table S1). At this stage, further studies were excluded if the language outcome was not verbal, if the age of the child's language assessment did not fall into the range above, if the exposure did not specifically consider maternal SDP or exposure or if they did not meet the quality criteria. Where multiple studies in the final list used the same cohort, one study was selected on the basis of the relevance of the outcomes studied and the sample size.

## 3 | RESULTS

Initial literature searches yielded 1376 studies from the Web Of Science and 911 articles from PubMed (Figure 1). After title and abstract review, 420 studies were taken forward for a full review. Of these, 134 were found to be

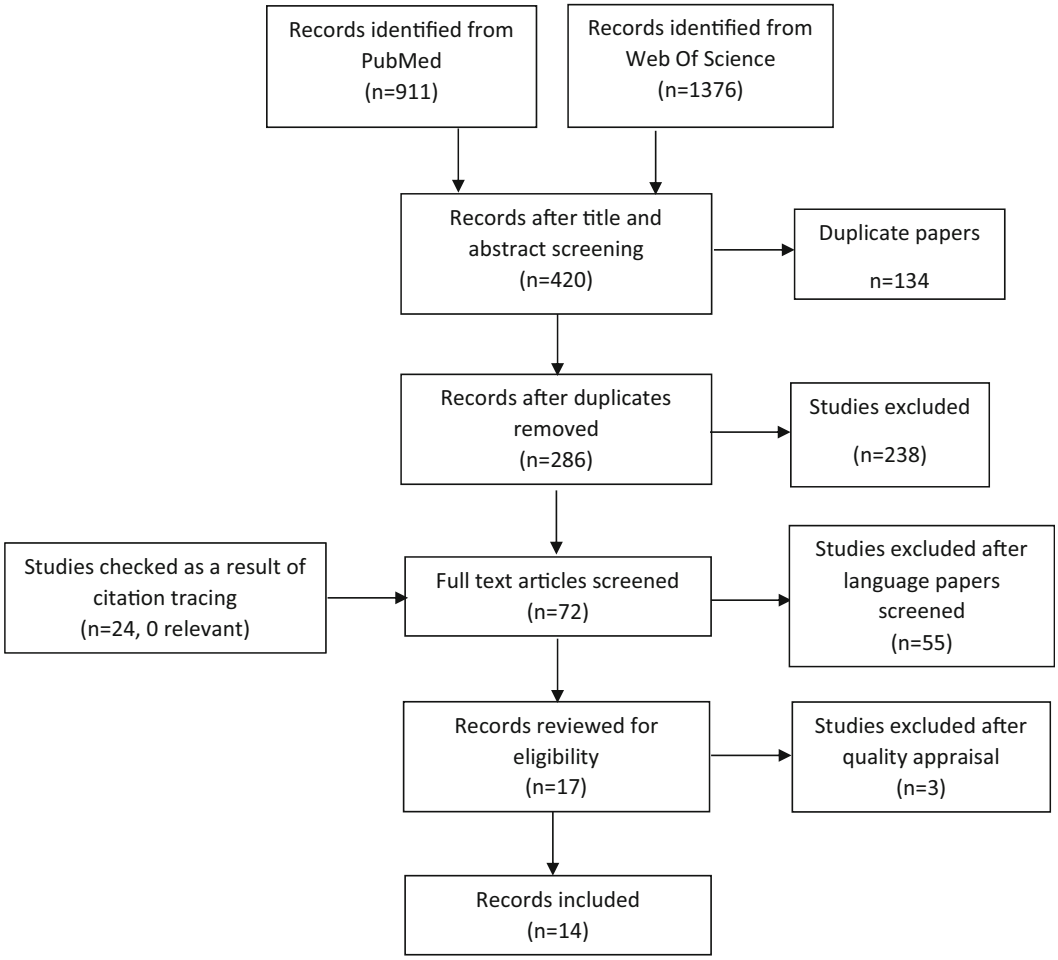


**FIGURE 1** Flow chart of the study screening process. See text for details of inclusion and exclusion criteria at each stage.

duplicate studies that were deposited in both PubMed and Web Of Science. After removing duplicate studies, 286 papers were examined in greater detail. The study exposure and outcome measurements were evaluated for their relevance to the aims of this systematic review (consideration of child language and nicotine exposure during pregnancy). This screening led to the exclusion of a further 238 studies and the addition of 24 further papers identified from citation tracing, leaving 72 papers that were then taken forward for an in-depth full-text review. At this stage, full study design and outcomes were recorded and studies, which met our full inclusionary criteria (as detailed in methods; primarily a measurement of a verbal language outcome between the ages of 2 and 12 years, relation of this outcome to maternal tobacco SDP or exposure) were retained leaving 17 articles. Additional reviews were performed to ensure that the studies met high-quality research (as defined by Kuyper, 1991) and to confirm that no one cohort was represented twice. These additional screens led to the exclusion of three further studies, leaving 14 papers, which were then taken forward to the systematic review (Tables 1 and S1). All papers were independently screened by two authors at each screening stage. Classification concordance was 85% across all stages. A summary of study findings is shown in Figure 2.

**TABLE 1** Fourteen studies are included in the systematic review (method details).

| Author (year) | Title (PMID) | Sample ascertainment and size | How was smoking assessed (classification groups)? | Outcome variables |
|---|---|---|---|---|
| MacArthur et al. (2001) | Effects at age 9 of maternal smoking in pregnancy: experimental & observational findings (11213007). | $n = 1218$. American population cohort ascertained for RCT of smoking. Recruited from antenatal clinics. | Maternal questionnaire post-delivery. (non-smokers, stopped by 6 weeks, stopped 7–16 weeks, stopped >17 weeks, persistent smokers). | Clinical assessment of VIQ* at 9–11 years (mean = 9.4 years) - British Ability Scales (BAS). |
| Huijbregts et al. (2006) | Interrelations between maternal smoking during pregnancy, birth weight & sociodemographic factors in the prediction of early cognitive abilities (28360824). | $n = 1544$. Canadian prospective birth cohort (Québec Longitudinal Study of Children's Development). | Maternal questionnaire, 5 months after birth (non-smokers, 1–9, ≥10 cigarettes/day). | Clinical assessment of vocabulary at 3.5 years - Peabody Picture Vocabulary Test (PPVT). |
| Julvez et al. (2007) | Maternal smoking habits & cognitive development of children at age 4 years in a population-based cohort (17550944). | $n = 420$. Spanish prospective birth cohort. Recruited all women presenting for antenatal care over a 12-month period. | The maternal repeated questionnaire, 3rd trimester to 4 years. (non-smoker, pregnancy smoker (>1 cigarette in pregnancy), post-natal (but not pregnancy) smoker). | Clinical assessment of VIQ at 4 years - McCarthy Scales of Children's Abilities (MCSA, Spanish-adaptation). |
| Alati et al. (2008) | Intrauterine exposure to alcohol & tobacco use and childhood IQ: Findings from a parental-offspring comparisonwithin ALSPAC (18670372). | $n = 4332$. UK prospective birth cohort (Avon Longitudinal Study of Parents & Children - ALSPAC). | Mother & partner repeated questionnaire, 1st trimester (non-smoker, 1–9 cigarettes/day, 10–19 cigarettes/day, 20+ cigarettes/day) | Clinical assessment of VIQ at 8 years - Wechsler Intelligence Scale for Children (WISC), abbreviated. |
| Gilman et al. (2008) | Maternal smoking during pregnancy & children's cognitive & physical development: a causal risk factor? (18653646). | $n = 35{,}566$. American prospective birth cohort study (the Collaborative Perinatal Project - CPP). | Maternal repeated questionnaire, 1st trimester to birth (non-smoker, 1–9 cigarettes/day, 10–19 cigarettes/day, 20+ cigarettes/day). | Clinical assessment of VIQ at 7 years - Wechsler Intelligence Scale for Children (WISC), abbreviated. |
| Hsieh et al. (2008) | *CYP1A1* Ile462Val & *GSTT1* modify the effect of cord blood cotinine on neurodevelopment at 2 years of age (18577398). | $n = 145$. Taiwanese prospective birth cohort (Taiwan Birth Panel Study). | Cord blood cotinine levels at delivery. (unexposed (<0.16 ng/mL), exposed (0.16–14 ng/mL). | Clinical assessment of developmental indices at 2 years - Comprehensive Developmental Inventory for Infants & Toddlers (CDIIT), language scale. |
| Heinonen et al. (2011) | Longitudinal study of smoking cessation before pregnancy & children's cognitive abilities at 56 months of age (21397413). | $n = 973$. Finnish prospective birth cohort (Arvo Ylppö Longitudinal Study - AYLS). | Mother & partner questionnaire at birth (non-smokers, pre-pregnancy (1–10 cigarettes/day), pre-pregnancy (>10 cigarettes/day), persistent smoker). | Clinical assessment of vocabulary & receptive comprehension at 56 months - Verbal competence test (Finnish) & Logopädischer Sprachverständnis Test (LSVT). |

(Continues)

**TABLE 1** (Continued)

| Author (year) | Title (PMID) | Sample ascertainment and size | How was smoking assessed (classification groups)? | Outcome variables |
|---|---|---|---|---|
| Eriksen et al. (2012) | Effects of tobacco smoking in pregnancy on offspring intelligence at the Age of 5 (23316364) | $n = 1782$. Danish prospective birth cohort (Lifestyle During Pregnancy Study, subset of Danish National Birth Cohort). | Maternal questionnaire, 17 gestational weeks (non-smokers, 1–9 cigarettes/day, ≥10 cigarettes/day). | Clinical assessment of VIQ at 5-years - Danish abbreviated version of the Wechsler Primary & Preschool Scales of Intelligence Revised (WPPSI-R). |
| Hernandez-Martinez et al. (2017) | Effects of prenatal nicotine exposure on infant language development: A cohort follow up study (27465062). | $n = 92$. Prenatal sample recruited for a single-site study of the effects of prenatal smoke exposure on cognition at Sant Joan University Hospital in Reus (Spain). Women recruited at <11 weeks of singleton pregnancy with no complications. | Maternal repeated questionnaire, trimesters 1,2,3 (non-smokers, smokers, exposed to second-hand smoke). | Clinical assessment of language & vocabulary at 30 months - Bayley Scales of Infant Development (BSID-II), MacArthur Bates Communicative Development Inventory & Peabody Picture Vocabulary Test (Spanish adaptation, PPVT-III). |
| Mohamed et al. (2018) | Early life second-hand smoke exposure assessed by hair nicotine biomarker may reduce children's neurodevelopment at 2 years of age (28803192). | $n = 107$. Malaysian prenatal sample from a single hospital site for a study on maternal–infant adiposity. Women were recruited in the second trimester with no complications. | Direct measure of nicotine levels in maternal hair samples 1–5 days after delivery (quantitative). | Parental questionnaire of language at 2 years - communication scale of Ages & Stages Questionnaire (ASQ-3, translated Malay version). |
| Polanska et al. (2017) | Environmental tobacco smoke exposure duringpregnancy & child neurodevelopment (28714930). | $n = 292$. Polish prospective birth cohort (REPRO-PL). | Direct measure of maternal cotinine from saliva, 1st, 2nd & 3rd trimesters. The maternal questionnaire of home smoke exposure. | Clinical assessment of language at 2 years - Bayley Scales of Infant & Toddler Development (BSD-III, language scale). |
| Lee et al. (2019) | Exposure to prenatal second-hand smoke and early neurodevelopment: MOCEH study (30894196) | $n = 352$. South Korean prospective cohort (Korean multicentre birth cohort study, Mothers & Children Environmental Health, MOCEH). | Direct measure of maternal cotinine from urine 12–20 gestational weeks (cotinine levels below the median (≤1.9 ng/mL) or above median (>1.9 ng/mL)). | Clinical assessment of language at 2 years - Mental Development Index (MDI) of Korean version of Bayley Scale of Infant Development II (K-BSID-II). |
| Neumann et al. (2019) | A longitudinal study of antenatal & perinatal risk factors in early childhood cognition: Evidence from Growing Up in New Zealand (30974313). | $n = 4587$. New Zealand prospective birth cohort (Growing Up in New Zealand Study). | Maternal questionnaire, before pregnancy & 3rd trimester (smoker, non-smoker pre-pregnancy). | Clinical assessment of vocabulary at age 4.5 years - Peabody Picture Vocabulary Test (PPVT-III). |
| Moore et al. (2020) | Prenatal exposure to tobacco & offspring neurocognitive development in the healthy start study (31759580) | $n = 246$. American prenatal sample from a single hospital site for a study on neonatal adiposity (Healthy Start Study). Women were recruited at <24 weeks of pregnancy with no complications. | Direct measure of maternal cotinine from urine, 27 gestational weeks (non-smoker (below limit of detection), smoker (cotinine detected)). | Parental questionnaire of language at 4.5-years - communication scale of Ages & Stages (ASQ-3). Clinical assessment of receptive vocabulary at 4.5 years - picture vocabulary (NIH Toolbox). |

| Author (Year) | PMID | Sample size<br>92-999<br>1000-9999<br>>10000 | Smoking measurement<br>questionnaire<br>direct measurement | Outcome measure | Significance<br>P<0.05<br>P<0.001<br>P<0.0001<br>NS | Effect<br>direct<br>indirect<br>no effect |
|---|---|---|---|---|---|---|
| MacArthur et al (2001) | 11213007 | | | VIQ 9-11yrs | | |
| Huijbregts et al (2006) | 28360824 | | | Vocab 3-5yrs | | |
| Julvez et al (2007) | 17550944 | | | VIQ 4yrs | | |
| Alati et al (2008) | 18670372 | | | VIQ 8yrs | | |
| Gilman et al (2008) | 18653646 | | | VIQ 7yrs | | |
| Hsieh et al (2008) | 18577398 | | | Language 2yrs | | |
| Heinonen et al (2011) | 21397413 | | | Vocab & comprehension 4-5yrs | | |
| Eriksen et al (2012) | 23316364 | | | VIQ 5yrs | | |
| Hernandez-Martinez et al (2016) | 27465062 | | | Language & vocab 2-3yrs | | |
| Mohamed et al (2017) | 28803192 | | | Language 2 yrs | | |
| Polanska et al (2017) | 28714930 | | | Language 2 yrs | | |
| Lee et al (2019) | 30894196 | | | Language 2 yrs | | |
| Neumann et al (2019) | 30974313 | | | Vocab 4-5yrs | | |
| Moore et al (2020) | 31759580 | | | Language & vocab 4-5yrs | | |

**FIGURE 2** Summary of broad study findings.

## 3.1 | Study findings

The 14 studies included in this review considered 11 different population nationalities: American, Spanish, Taiwanese, UK, Finnish, Canadian, Polish, Malaysian, South Korean, New Zealand, and Danish populations (Table 1). The combined number of participants across the 14 studies was 51,656, with the smallest study comprising 92 participants and the largest comprising 35,566.

Of the 14 studies included in this review, 13 (93%) reported negative associations between maternal prepregnancy smoking, smoking during pregnancy or exposure to smoke and childhood language outcomes (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017) (Figure 2, Table 2). Differences in study design and reporting methods make it difficult to directly compare effects between studies but six of the fourteen studies reviewed (43%) report highly significant effects (Alati et al., 2008; Gilman et al., 2008; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; MacArthur et al., 2001) ($p \leq 0.001$) and seven found marginal effects ($0.001 < p < 0.05$) (Eriksen et al., 2012; Heinonen et al., 2011; Julvez et al., 2007; Lee et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017) (Figure 2, Table 2). Although most studies found consistent associations, their conclusions differed; five concluded that the effects of smoking on child language could be explained by indirect effects, primarily socioeconomic in nature (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Huijbregts et al., 2006; MacArthur et al., 2001) (Figure 2, Table 2). The other eight studies reported a direct effect of nicotine levels on child language (Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh

**TABLE 2** Fourteen studies were included in a systematic review (results summary).

| Author (year) | Title (PMID) | *p*-value (effect size, where given)[a] | Study conclusion | Confounders and effects |
|---|---|---|---|---|
| MacArthur et al. (2001) | Effects at age 9 of maternal smoking in pregnancy: experimental & observational findings (11213007). | $p < 0.001$ (Max VIQ change $= -3.7$) | Significant association between maternal smoking and VIQ (persistent vs. stopped-smokers). The effect was indirect. | Parental factors (Mother's educational level and age), birth factors (birthweight, birth-length, head circumference, breastfeeding) and family/home factors (parity, home location, maternal employment) were independent predictors of VIQ. Association to smoking was accounted for by these variables. |
| Huijbregts et al. (2006) | Interrelations between maternal smoking during pregnancy, birth weight & sociodemographic factors in the prediction of early cognitive abilities (28360824). | $p < 0.001$ ($\beta \pm SE = -0.17 \pm 0.034$) | Significant association between maternal smoking and vocabulary. Effect was indirect | Parental factors (maternal education), birth factors (birthweight, gestation, sex) and family/home factors (family income) were independent predictors of vocabulary. Association to smoking was accounted for by maternal education and birth weight. |
| Julvez et al. (2007) | Maternal smoking habits & cognitive development of children at age 4 years in a population-based cohort (17550944). | $p = 0.03$ ($\beta = -0.59$, 95% CI $= -1.11$ to $-0.07$) | Marginal association between maternal smoking and VIQ. The effect was direct. | Parental factors (maternal education) and family/home factors (social class) were independent predictors of VIQ. Association to smoking remained after adjusting for these effects. |
| Alati et al. (2008) | Intrauterine exposure to alcohol & tobacco use and childhood IQ: Findings from a parental-offspring comparison within ALSPAC (18670372). | $p < 0.001$ (Mean VIQ change $= -2.63$, 95% CI $= -3.42$ to $-1.84$) | Significant association between maternal and paternal smoking and VIQ Effect was indirect. | Parental factors (Parental education), child factors (sex) and family/home factors (social class, parity, home ownership and house crowding) were independent predictors of VIQ. Association to smoking was accounted for by parental education. |
| Gilman et al. (2008) | Maternal smoking during pregnancy & children's cognitive & physical development: a causal risk factor? (18653646). | $p < 0.001$ (Max VIQ change $= -0.77$, 95% CI $= -1.12$ to $-0.41$, adjusted model) | Significant association between maternal smoking and VIQ Effect was indirect. | Parental factors (Mother's educational level, parental age, marital status, parental mental health) and family/home factors (social class, parity, maternal employment) were independent predictors of VIQ. Association to smoking remained after adjusting for these variables. |

**TABLE 2** (Continued)

| Author (year) | Title (PMID) | *p*-value (effect size, where given)[a] | Study conclusion | Confounders and effects |
|---|---|---|---|---|
| Hsieh et al. (2008) | *CYP1A1* Ile462Val & *GSTT1* modify the effect of cord blood cotinine on neurodevelopment at 2 years of age (18577398). | $p < 0.0001$ ($\beta \pm SE = -10.15 \pm 2.24$) | Significant association between maternal cotinine levels and language. Effect was direct | Parental factors (maternal education and ethnicity) and family/home factors (income) were independent predictors of language. Association to smoking remained after adjusting for these effects. |
| Heinonen et al. (2011) | Longitudinal study of smoking cessation before pregnancy & children's cognitive abilities at 56 months of age (21397413). | $p < 0.05$ ($\beta = -12.83$, 95% CI $= -21.30$ to $-4.35$, pre-pregnancy smoking) | Marginal association between smoking >10 cigarettes/day before pregnancy and language comprehension. Effect was direct. | Parental factors (Parental education), birth factors (sex) and family/home factors (social class, parity, home ownership and house crowding) were independent predictors of comprehension. Association to smoking remained after accounting for these variables. |
| Eriksen et al. (2012) | Effects of tobacco smoking in pregnancy on offspring intelligence at the age of 5 (23316364) | $p < 0.05$ (max VIQ change $= -2.5$, 95% CI $= -4.7$ to $-0.4$) | Significant association between smoking >10 cigarettes/day and VIQ Effect was indirect. | Parental factors (parental education, maternal IQ, maternal age, maternal BMI), family factors (parity, smoke in house, parental marital status, home environment) were associated with child outcomes. Association to smoking was accounted for by these variables. |
| Hernandez-Martinez et al. (2017) | Effects of prenatal nicotine exposure on infant languagedevelopment: A cohort follow up study (27465062). | $p = 0.001$ (mean Language Development Age change $= -1.24$) | Significant association between smoking and language development. Effect was direct | Parental factors (maternal age) and family/home factors (social class) were independent predictors of language. Association to smoking remained after accounting for these variables. |
| Mohamed et al. (2018) | Early life second-hand smoke exposure assessed by hair nicotine biomarker may reduce children's neurodevelopment at 2 years of age (28803192). | $p = 0.025$ ($\beta = -1.920$) | Marginal association between hair cotinine level and communication. Effect was direct. | Parental factors (parental education), child factors (sex) and family factors (household income) were independently associated with the communication. Association to smoking remained after adjusting for these variables. |
| Polanska et al. (2017) | Environmental tobacco smoke exposure duringpregnancy & child neurodevelopment (28714930). | $p = 0.009$ ($\beta = -5.19$, adjusted model) | Marginal association between maternal cotinine levels in 1st and 2nd trimester and language development. Effect was direct. | Models were adjusted for parental factors (maternal IQ, maternal age, alcohol consumption), family factors (SES, parental marital status and parity) and birth factors (gestation, pregnancy complications, breastfeeding). Association to smoking remained after accounting for these variables. |

(Continues)

**TABLE 2** (Continued)

| Author (year) | Title (PMID) | *p*-value (effect size, where given)[a] | Study conclusion | Confounders and effects |
|---|---|---|---|---|
| Lee et al. (2019) | Exposure to prenatal second-hand smoke and early neurodevelopment: MOCEH study (30894196) | $p = 0.04$ ($\beta = -2.73$, 95% CI $= -5.32$ to $-0.15$, adjusted model) | Association between urinary cotinine and language development. Effect was direct. | Parental factors (maternal education, maternal age), birth factors (birthweight, breastfeeding), family factors (home location) and genetic factors (polymorphisms in *GSTM1/GSTT1* genes) were associated with development. Association to smoking remained even after accounting for these variables. |
| Neumann et al. (2019) | A longitudinal study of antenatal & perinatal risk factors in early childhood cognition: Evidence from Growing Up in New Zealand (30974313). | $p < 0.05$ (OR language below expected $= 1.28$ (95% CI $= 1.04$–$1.57$, adjusted model, pre-pregnancy smoking). | Marginal association between smoking pre-pregnancy and receptive language. Effect was direct. | Parental factors (maternal anxiety/depression and maternal diet) were independently associated with vocabulary outcomes. Association to smoking remained even after accounting for these variables. |
| Moore et al. (2020) | Prenatal exposure to tobacco & offspring neurocognitive development in the healthy start study (31759580) | $p = 0.83$, OR $= 1.8$ (95% CI $= -3.0$ to 6.6, adjusted model) | No association between smoking and receptive vocabulary or communication difficulties. | Parental factors (maternal education, maternal age, maternal ethnicity), birth factors (birthweight and breastfeeding) and family factors (family income) were associated with language outcomes. |

[a]Effect sizes are reported with non-smokers as the baseline. In many papers, multiple comparison groups (e.g., different smoking levels) and different outcomes were considered. In these studies, the maximum effect is reported. Effect sizes will not be comparable across studies. All effects reported are for unadjusted baseline models unless stated. See each individual paper for details of measures, models and effects.

et al., 2008; Julvez et al., 2007; Lee et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017) which remained significant even after correcting for possible socioeconomic confounders (Figure 2, Table 2).

Seven (50%) investigations (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017) explored the relationship between nicotine dosage and language and six of these reported stronger effects in groups who smoked heavily during pregnancy (Alati et al., 2008; Eriksen et al., 2012; Heinonen et al., 2011; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017). Similarly, six studies (43%) categorized language outcomes in relation to the point of nicotine exposure (Heinonen et al., 2011; Huijbregts et al., 2006; Julvez et al., 2007; MacArthur et al., 2001; Mohamed et al., 2018; Polanska et al., 2017) and four of these (67%) found that smoking before or during early pregnancy had the biggest effects on the outcome (Heinonen et al., 2011; Julvez et al., 2007; Mohamed et al., 2018; Polanska et al., 2017).

Only one study (7.1%) failed to find significant association between prenatal smoke exposure and language (Moore et al., 2020). This investigation included 246 individuals and considered prenatal cotinine levels (no exposure, $n = 181$ vs. exposure $n = 65$) in relation to dichotomised communication scores and a continuous measure of receptive vocabulary. Analyses were adjusted for possible confounders including maternal age, sex, race, annual household income, non-specified maternal psychiatric disorder and maternal daily caloric intake during pregnancy. They reported that children who were exposed to nicotine prenatally had a decreased inhibitory control and poor fine motor skills, however, no significant differences were found in terms of the language-specific outcomes mentioned above.

## 3.2 | Study design

The majority of the studies included in this review (11 of 14, 79%) were prospective birth cohort investigations, where mothers were recruited during pregnancy from multiple sites (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Neumann et al., 2019; Polanska et al., 2017) (Table 1). These population studies did not apply ascertainment criteria regarding maternal smoking and, instead, these data were collected as part of a broad investigative battery. Only one sample set was specifically ascertained to investigate the effects of smoking on cognition (Hernandez-Martinez et al., 2017). Two additional studies (Mohamed et al., 2018; Moore et al., 2020) also ascertained targeted sample sets, focussing on the effects of prenatal smoking on infant adiposity, although they also collected information regarding language development. These three targeted studies tended to have smaller sample sizes (mean $n = 148$, range 92–246) than the population-based studies (mean $n = 4655$, range = 145–35,566) but did not differ in their analytical approaches, which primarily relied upon regression modelling and included covariates for possible confounder effects.

## 3.3 | Nicotine exposure

The selection criteria applied within this systematic review specified that information regarding nicotine exposure had to be collected from mothers within 6 months of birth (see methods). However, the exact time-point of data acquisition differed between studies (Figure 2, Table 1). Five studies (36%) collected exposure data at a single time-point during pregnancy, three (21%) in the second trimester (14–26 weeks gestation) (Alati et al., 2008; Eriksen et al., 2012; Lee et al., 2019) and two (14%) in the third trimester (27–40 weeks gestation) (Moore et al., 2020; Neumann et al., 2019). Five further studies (36%) collected this information post-delivery; four within a week of delivery (Heinonen et al., 2011; Hsieh et al., 2008; MacArthur et al., 2001; Mohamed et al., 2018) and one study five months after birth (Huijbregts et al., 2006). The remaining four studies (29%) took repeated measures throughout

pregnancy in the first, second, and third trimesters (Gilman et al., 2008; Hernandez-Martinez et al., 2017; Polanska et al., 2017) and every year up to 4 years postnatally (Julvez et al., 2007).

Nine of the 14 studies (64%) used parental questionnaires to assess nicotine exposure (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Julvez et al., 2007; MacArthur et al., 2001; Neumann et al., 2019) while 5 (36%) used direct measurement of cotinine; a metabolite of nicotine (Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Moore et al., 2020; Polanska et al., 2017) (Figure 2, Table 1). Direct measures can provide a more accurate measurement of exposure and allow exposure to be treated as a continuous variable enabling the investigation of possible dosage effects. Although in reality, only three studies (21%) performed a continuous regression (Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017). Direct measurements were made using urine (Lee et al., 2019; Moore et al., 2020) or saliva samples at prenatal visits (Polanska et al., 2017), cord blood (Hsieh et al., 2008) or hair samples (Mohamed et al., 2018). Direct measurement is more expensive and time-consuming and this is therefore reflected in the sample sizes; studies which employed questionnaires tended to be larger than those with cotinine measurement (mean $n = 5612$, range = 92–35,566, compared to mean $n = 228$, range = 107–352 respectively). With the exception of Moore et al. (2020), all studies that employed cotinine measurements concluded that there was a direct effect between nicotine exposure and language outcomes, although these conclusions were always based upon results of marginal significance ($p > 0.001$) (Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017). In contrast, studies that employed questionnaires reported both significant and marginal results with direct and indirect effects, regardless of the time point collected.

Six studies (43%) sub-categorized smokers in terms of the number of cigarettes smoked daily (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011) or by quantitative cotinine levels (Lee et al., 2019; Polanska et al., 2017). Five studies (36%) also considered the time point of exposure (prenatal, postnatal, or persistent) (Heinonen et al., 2011; Julvez et al., 2007; MacArthur et al., 2001; Mohamed et al., 2018; Polanska et al., 2017). The remaining five studies (36%) employed a binary consideration (smokers vs. non-smokers) (Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Moore et al., 2020; Neumann et al., 2019). No obvious differences were observed in the findings across these studies in terms of the direction of effects or significance levels.

One difficulty in considering nicotine exposure is the challenge of distinguishing between direct exposure and environmental passive exposure (Jung et al., 2017). The use of maternal questionnaires considers only self-declared cigarette consumption, that is, active exposure. Whilst the direct measurement of cotinine quantifies both active and passive exposure levels, questionnaires were used to assess nicotine exposure in ten of the fourteen studies (71%) included in this review (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Julvez et al., 2007; MacArthur et al., 2001; Neumann et al., 2019; Polanska et al., 2017). Six studies which employed questionnaires did attempt to address passive exposure through the use of paternal or home environment data (Alati et al., 2008; Eriksen et al., 2012; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Julvez et al., 2007; Polanska et al., 2017). One study (Hernandez-Martinez et al., 2017) reported non-significant effects of these environmental exposures while three studies reported significant effects (Alati et al., 2008; Eriksen et al., 2012; Huijbregts et al., 2006). The other two studies included these covariates in their models but did not report their significance.

## 3.4 | Language outcomes

Although all the studies in this review were screened and selected to consider child language development, the methods of ascertaining language ability varied between studies, as did the age of child assessment (Table 1). Five of the fourteen studies (36%) included in this review considered verbal IQ (VIQ) as a measure of language ability (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Julvez et al., 2007; MacArthur et al., 2001). The exact IQ test

varied between studies but each has overlapping subtests and represents direct clinical measures of language ability across a range of developmental domains. MacArthur et al. (2001) employed the British Ability Scales (Elliot et al., 1983) which includes subtests of word definitions and verbal similarities when children were 9–11 years (mean age = 9.4 years), Julvez et al. (2007) used McCarthy's Scales of Children's Abilities which includes the assessment of vocabulary, verbal memory, verbal fluency, and verbal similarities when children were 4 years (McCarthy, 1972). The remaining three studies used an abbreviated version of the Wechsler Intelligence Scales for Children (Wechsler, 1992, 2006), which considers verbal comprehension verbal reasoning, verbal memory, verbal fluency, vocabulary, and verbal similarities. These measurements were taken at 5 years (Eriksen et al., 2012), 7 years (Gilman et al., 2008) or 8 years of age (Alati et al., 2008).

Six other studies (43%) used broad assessment measures of language development at early ages, between 2 and 5 years of age (Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Moore et al., 2020; Polanska et al., 2017). Mohamed et al., (2018), and Moore et al., (2020) used the Ages and Stages Questionnaire (ASQ-3) which combines direct testing with parental questionnaires to assess language development and considers both language production and understanding. Mohamed et al., (2018) applied this test to assess early communication at 2 years of age, while Moore et al. (2020) used it to assess later communication at 4–5 years of age. Polanska et al. (2017), Hernandez-Martinez et al., (2017), and Lee et al. (2019) used the Bayley Scales of Infant Development (BSID) (Bayley, 1993; Park & Cho, 2006), a clinical assessment that can be used to capture development across mental and motor scales in young children (0–42 months). The Mental Development Index (MDI) of the Bayley Scales includes a specific scale of language development. While Hernandez-Martinez et al. (2017) and Polanska et al. (2017) employed the more focused language scale at the age of 1 and 2 years (of which we used the latter information). Lee et al. (2019) used the broader Mental Development Index at 2 years of age. Finally, Hsieh et al. (2008), used the Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) (Wang et al., 1998) at 2 years of age. This is a broad developmental battery, which consists of direct assessment across cognitive, emotional and motor domains and includes a language subscale.

Vocabulary forms a subtest of many of the batteries used above and has long been considered as a proxy for early language development. Five studies (36%) included in this review considered specific tasks of receptive vocabulary as an outcome measure (Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Moore et al., 2020; Neumann et al., 2019). In two studies (Huijbregts et al., 2006; Neumann et al., 2019) vocabulary was the sole language outcome and was assessed with the Peabody Picture Vocabulary Test (PPVT) (Dunn et al., 1997; Dunn & Dunn, 1981) at 42 months (Huijbregts et al., 2006) or 54 months (Neumann et al., 2019). Three further studies (23%) considered receptive vocabulary alongside additional language measures. Hernandez-Martinez et al., (2017) combined the PPVT (Campbell et al., 2001) with the MacArthur-Bates Communicative Development Inventor (López Ornat et al., 2005), which focuses on vocabulary production and comprehension as well as a gesture. Alongside these two vocabulary tests, they also completed the BSID-II as described above. Each of these tasks was completed at different times across the ages of 6–30 months. Heinonen et al. (2011) included an alternative picture naming test verbal competence test alongside a language comprehension task (following instructions) at 56 months. Moore et al. (2020) used the picture vocabulary task from the NIH toolbox and combined this with the ASQ-3 index described above at 48, 54 and 60 months of age.

Studies that employed VIQ as a language outcome measure (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Julvez et al., 2007; MacArthur et al., 2001) generally considered an older age group (mean age 79 months, range 4 years to 11 years). In addition, these were more likely to report $p$-values ≤0.001 (Alati et al., 2008; Gilman et al., 2008; MacArthur et al., 2001) and indirect effects (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; MacArthur et al., 2001) than studies of developmental language indices (Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Moore et al., 2020; Polanska et al., 2017) or vocabulary (Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Huijbregts et al., 2006; Moore et al., 2020; Neumann et al., 2019); these generally involved testing at younger ages (mean age 34 months, range 2 to 5 years) and were more likely to report marginal $p$-values ($0.001 \geq p \leq 0.05$) (Heinonen et al., 2011; Hsieh et al., 2008; Lee

et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017) and direct effects (Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017).

### 3.5 | Confounding effects

As outlined in the introduction, it has previously been argued that confounder effects, particularly maternal education/IQ may lead to the inflation of association between smoking and child development (Batty et al., 2006; Tomblin et al., 1998). Indeed, Stanton-Chapman (Stanton-Chapman et al., 2002) and colleagues have identified maternal education as a significant risk factor for LI. All of the 14 studies in this systematic review included some consideration of confounder effects by the inclusion of covariates within their models (Table 2). Some included covariates in their baseline model, other tested specifically for the effects of possible confounders. Common confounder effects can be split into child factors (including sex, ethnicity, health), birth factors (including prenatal and perinatal effects), family factors (such as SES, diet and parity), parental factors (such as education, age, alcohol consumption and environmental smoke exposure) and test factors (such as assessment point or evaluator).

In line with previous research, the most commonly identified significant confounder effects were SES and maternal education/IQ. Twelve studies (86%) included maternal education/IQ in their analyses (Alati et al., 2008; Eriksen et al., 2012; Heinonen et al., 2011; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Moore et al., 2020; Neumann et al., 2019; Polanska et al., 2017) and eight of these (67%) (Alati et al., 2008; Eriksen et al., 2012; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Moore et al., 2020) explicitly reported this to be a significant confounder, although it did not explain all of the variance in all of these studies. Two studies (14%) (Heinonen et al., 2011; Mohamed et al., 2018) reported this factor to be non-significant in their models. Twelve studies (86%) included indicators of SES such as home location, ownership, income and employment, in their analyses (Alati et al., 2008; Gilman et al., 2008; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Moore et al., 2020; Neumann et al., 2019; Polanska et al., 2017) and nine of these (75%) reported it to be a significant confounder effect (Alati et al., 2008; Gilman et al., 2008; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Moore et al., 2020). Only one study (8%) (Huijbregts et al., 2006) reported SES to have no effect.

Other commonly identified confounders included parental age (maternal and/or paternal) birth weight, breastfeeding and environmental smoke exposure. Each of these factors was investigated in at least 8 of the 14 (57%) studies reviewed and was found to be significant by the majority.

Child sex is often considered as a confounding factor in studies of language development but was not reported to act as such in this instance. Thirteen studies (93%) included sex as a covariate and only two of these reported it as a significant confounder (Alati et al., 2008; Mohamed et al., 2018).

Other factors which were largely reported as non-significant were pregnancy complications (such as preeclampsia and gestational diabetes), maternal alcohol consumption, maternal body mass index (BMI) and study-related factors. These factors were consistently reported as non-significant in terms of confounder effects, although most were only included across a few of the studies reviewed (5 or less).

## 4 | CONCLUSIONS

Despite the vast literature regarding the effects of nicotine exposure on foetal health and child cognition, there is little research regarding direct effects on language development. In this systematic review, we screened over 1000 papers focused on 14 papers that specifically considered language outcomes in relation to *in-utero* nicotine exposure.

Thirteen of the 14 papers examined (93%) reported a negative association between maternal smoking or exposure and language outcomes (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017).

As with previous reports, there was some inconsistency regarding the nature of the relationship between maternal smoking or exposure and language development; eight studies concluded that smoking directly impaired early language (Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Julvez et al., 2007; Lee et al., 2019; Mohamed et al., 2018; Neumann et al., 2019; Polanska et al., 2017) while five concluded that the observed effects could be explained by confounding factors (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Huijbregts et al., 2006; MacArthur et al., 2001). These confounding factors varied between study designs making it hard to make a conclusion about the direction of effects.

Various studies have highlighted a strong correlation between IQ and education (Barber, 2005; Matarazzo & Herman, 1984; Ritchie et al., 2013). Commonly identified confounders included maternal IQ/education (significant confounder in eight of twelve studies that considered this factor; (Alati et al., 2008; Eriksen et al., 2012; Hsieh et al., 2008; Huijbregts et al., 2006; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Moore et al., 2020), SES (significant confounder in nine of twelve studies that considered this factor; (Alati et al., 2008; Gilman et al., 2008; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Julvez et al., 2007; Lee et al., 2019; MacArthur et al., 2001; Mohamed et al., 2018; Moore et al., 2020) and parental age (significant confounder in six of ten studies that considered this factor; (Eriksen et al., 2012; Gilman et al., 2008; Hernandez-Martinez et al., 2017; Lee et al., 2019; MacArthur et al., 2001; Moore et al., 2020). Educational differences in smoking, with less-educated individuals being more likely to smoke, have been well documented in the literature (Cutler & Lleras-Muney, 2010; de Walque, 2007; Drope et al., 2018; Jürges et al., 2011; Kenkel et al., 2006; Maralani, 2013). Education is widely regarded as a driver of social progression and SES is often used as a proxy for education (Reilly et al., 2010). It is widely established that education and IQ, whilst different, are highly correlated at the behavioural level. An early study found that those who completed 16 years or more of education had a higher mean IQ (FSIQ = 115.3) than those who completed 12 years (FSIQ = 100.1) (Matarazzo & Herman, 1984). Another study reported that IQ was higher in countries, which extended education as indexed by secondary school enrolment and conversely that IQ was lower in countries with high levels of illiteracy (Barber, 2005). A more recent review has found that years of education were positively associated with IQ and that these associations continued into later life (Ritchie et al., 2013). Nonetheless, as previously discussed, it can be difficult to disentangle cause and effect within models that consider directly measured behaviours (as happened to be the case for all studies included in our systematic review). Correlations do not indicate causation and questions remain as to the direction of any causal effects, especially when those effects are transgenerational in nature. The recent application of Mendelian randomisation methods in large population cohorts has shown that the effects of cognitive ability upon smoking behaviour attenuate when educational attainment is introduced into the model. This finding indicates that the effects of educational attainment drive the relationship between cognition and smoking (Sanderson et al., 2019; Wells & Ostberg, 2021). However, an important limitation is noted for these findings in as much as they do not allow for transgenerational effects where parental education may have an effect on child smoking status that is not explained by the education level of the child (Sanderson et al., 2019). Importantly, the same dynastic effects could be applied to language and smoking where individual genetics directly affects parental language ability which then has an effect upon child language irrespective of smoking. Such complexities underline the need for careful study designs and well-powered cohorts when considering these effects (D'Onofrio et al., 2014).

Individuals living in low SES areas often have a higher level of tobacco use (Laveist et al., 2007; Reid et al., 2010; Zhang et al., 2013). SES, in turn, has been linked to reduced cognition (Özmert et al., 2005; Sarsour et al., 2011; Turkheimer et al., 2003) and lower academic achievement (Crosnoe et al., 2010; Marks, 2006). Similarly, maternal education level is associated with the academic and language abilities of children (Hanscombe et al., 2012; Reilly et al., 2010). None of these effects are linear and each involves many interacting factors making the complex relationships difficult to disentangle at the behavioural level (Batty et al., 2006; Puglisi et al., 2017).

Birthweight and breastfeeding were also commonly identified as confounders across the studies in this review. These two factors have also been previously related to child language and cognition (Hack et al., 1995; Kim & Choi, 2020). Extremely low birth weight has negative impacts that span both childhood and adulthood and has been described as a marker of the child's later neurological and psychiatric outcomes (Hack et al., 2005). Although smoking during breast-feeding has not been directly linked to cognition (Gibson & Porter, 2020), nicotine has been shown to transfer through breastmilk to the baby and also changes the composition and taste of milk (Napierala et al., 2016) which can lead to earlier weaning and lower weight (Horta et al., 2001) both of which, in turn, are associated with reductions in cognitive outcomes. These findings again highlight the complexity of these interacting effects and suggest that further studies will be required to disentangle these relationships at the behavioural level.

Existing studies have consistently suggested a small effect of biological sex on early language in favour of girls but this is reported to be dependent on age as well as the language component assessed (Bouchard et al., 2009; Simonsen et al., 2014; Thal et al., 2004). Conversely, it has been argued that there are more similarities than differences between genders regarding their language ability (Rhoda Kesler Unger, 2001). In this review, the biological sex of the child was not found to be a significant confounder by the majority of the 13 studies that included it in their adjustments. Our review identified a clear consensus that there is a dose-response effect of smoking on general health. All seven studies that considered differing doses of smoking found a negative dose–response relationship between prenatal smoking and language outcomes (Alati et al., 2008; Eriksen et al., 2012; Gilman et al., 2008; Heinonen et al., 2011; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017). These findings reflect those in the overall literature and are in line with those from animal and epidemiological studies (Hellstrom-Lindahl et al., 1998; Huizink & Mulder, 2006; Levin & Simon, 1998; Linnet et al., 2003; Weitzman et al., 2002). Animal studies similarly show that the neuronal effects of smoking are more pronounced at earlier gestational periods (Slotkin et al., 2015). The latter is reflected in the studies used in this systematic review in which four studies reported that smoking before or during early pregnancy had the biggest effects on the outcome (Heinonen et al., 2011; Julvez et al., 2007; Mohamed et al., 2018; Polanska et al., 2017).

Study design and sample size did not seem to affect the trends observed; one of the smallest studies in this review was the only one that failed to find an association (Moore et al., 2020). It should be noted however, that sample sizes can affect the relative effect sizes associated with any given *p*-value. Where reported, we include both effect size and *p*-value in Table 2. Perhaps unexpectedly, studies that employed direct measures of cotinine as a proxy of nicotine exposure (Hsieh et al., 2008; Lee et al., 2019; Mohamed et al., 2018; Polanska et al., 2017) generally had less significant findings than those which relied upon questionnaires. Direct measurement of nicotine by measurement of its major metabolite, cotinine, present in saliva, urine, or hair is often considered the "gold standard" for smoking detection as inconsistencies have been reported between self-report and cotinine concentrations (Britton et al., 2006). However, direct measurement methods also have limitations as cotinine only has a half-life of approximately 19–24 hours (Benowitz et al., 1983), and can be produced by nicotine replacement therapies such as nicotine patches, leading to false positives as the nicotine present in these is metabolized the same way. The reduced association in studies that employed direct measurements may reflect shared confounder factors between questionnaire data, smoking and language which would act to conflate the association between the two latter factors falsely increasing the association signal in studies, which rely upon questionnaire data. Conversely, it should also be noted that we restricted our review to include only studies that assessed smoking within 6 months of birth. This restriction was applied to maximize the reliability of smoking measures and hence the validity of our conclusions. Nonetheless, it is not necessarily true that retrospective reports are less reliable than contemporaneous measurements. In particular, since many women try to give up smoking during pregnancy, their memory of smoking habits during this period may show increased accuracy (Pickett et al., 2005). Studies show that the correlation between cotinine and contemporaneous reports is 70% (Petitti et al., 1981) and that retrospective reports are usually within 1%–3% of contemporaneous reports (Kenkel et al., 2003).

Just as exposure measurement may affect results, so may the choice of outcome measurement. In this review, we observed that studies, which employ measures of verbal IQ at later developmental stages (Alati et al., 2008;

Eriksen et al., 2012; Gilman et al., 2008; Julvez et al., 2007; MacArthur et al., 2001) reported stronger associations than investigations that employed early language indices or vocabulary measures (Heinonen et al., 2011; Hernandez-Martinez et al., 2017; Hsieh et al., 2008; Huijbregts et al., 2006; Lee et al., 2019; Mohamed et al., 2018; Moore et al., 2020; Neumann et al., 2019; Polanska et al., 2017). These studies also tended to report indirect associations that could be explained by confounder effects. There is debate in the literature as to the exact construct measured by each of the tests employed. Tests of VIQ assess the ability to access and apply acquired knowledge of words, including verbal concept formation, reasoning and expression rather than a specific construct of language itself (Lange et al., 2018). The age at which these tests are performed will also affect performance with different strategies typically applied to different age groups. Studies show that the heritability of intelligence increases over the life span reflecting a "genetic amplification" by which children select differential environments which act to compound genetic propensities (Plomin & Deary, 2015). This effect is also described in relation to language, where environmental factors account for a greater proportion of language variability earlier in development (Hayiou-Thomas et al., 2012; Tosto et al., 2017). Thus it could be argued that the strengthened association in older children again represents a falsely inflated association due to shared genetic confounders. This hypothesis is supported by research on ADHD where it has been suggested that genetically sensitive study designs, such as Mendelian randomisation, should be employed in the testing of causal hypotheses about prenatal exposure and offspring outcome (Rice et al., 2018; Thapar et al., 2009).

Finally, it should be noted that any systematic review is limited by its choice of search terms and papers included in the final review stages. Whilst our search terms were optimized to return relevant papers, they do not reflect the entire field. For example, we note that none of the 14 studies included in the final review stage employed a quasi-experimental design. This point is of particular relevance when considering confounder effects, which were noted as a primary influencing factor in our findings. All of the studies explored here employed a *post-hoc* adjustment to allow for specific measured confounder effects. In contrast, quasi-experimental methods allow for unmeasured confounders. Such studies indicate that associations between smoking during pregnancy and child cognition and behaviour may be explained by confounding factors rather than the direct effects of smoking (D'Onofrio et al., 2013). In particular, sibling comparison studies have shown that the association between smoking and reading outcomes can be explained by shared genetic and environmental factors (Ellingson et al., 2014; Micalizzi et al., 2021).

In conclusion, our systematic review finds consistent evidence for an association between maternal SDP or exposure and reduced language performance at early ages. However, the review also highlighted the complexities of the relationships within this process. Potential confounder factors include maternal IQ/education, SES, parental age, birth weight and breastfeeding and future studies should be carefully designed to account for these confounder effects. We observed strengthened relationships between smoking and language at points, which suggest inflation by study design rather than a true increase in association, again highlighting the need for careful study design supporting previous conclusions in this area (Thapar et al., 2009) and the findings of more sensitive approaches (D'Onofrio et al., 2013).

Despite systematic reviews upholding more robustly than other reviews, there are still limitations to be considered. Only studies in English and those with full text available were included meaning that potentially relevant studies may have been omitted. Additionally, despite the fact that efforts were made to carry out a broad and complete search, the possibility remains that some may have been overlooked. Only two databases were searched in this review and more could have been searched.

All of the studies included in our review used language measurements in population cohorts. Many of the studies we included looked at language as a corollary of cognition rather than focusing upon language itself meaning that outcomes differed between studies and none included clinical cohorts of language disorder. While it is possible that the findings here may be relevant to language disorder, it is also possible that risk effects differ between typical language development and language disorder. Previous studies (Eicher et al., 2013; Tomblin et al., 1997; Tomblin

et al., 1998) have suggested a link between smoking and language disorder but our review does not allow us to comment on the findings from this literature.

Future studies should aim to address weaknesses by considering careful study design which allows for confounding factors across child, parental, environmental and genetic influences. The network of effects underlying the associations identified here is so complex that more detailed studies of interactions between factors will be required. Such studies should extend beyond behavioural measurements and, if possible, include consideration of inherited effects (Thapar et al., 2009). Genetic and epigenetic effects were not considered in any of the papers we reviewed but, nonetheless can confer considerable risk for smoking, cognition and language and may interact with environmental factors to mediate outcomes (Agrawal et al., 2008; Newbury et al., 2009).

To conclude, this systematic review suggests a specific association between exposure to SDP pregnancy and language development. This may be used for the education of expectant mothers regarding the little-understood effects of tobacco smoking, including nicotine exposure specifically on language outcomes. Smoking cessation may help to optimize child outcomes in terms of language and would have positive effects on other aspects of child development bearing in mind that the most nicotine replacement drug strategies are nicotine mimetics.

## AUTHOR CONTRIBUTIONS

**Jessica Peixinho:** Formal analysis; methodology; writing – original draft; writing – review and editing. **Umar Toseeb:** Conceptualization; methodology; supervision; writing – review and editing. **Hayley S. Mountford:** Methodology; supervision; writing – review and editing. **Isabel Bermudez:** Methodology; supervision; writing – review and editing. **Dianne F. Newbury:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; writing – original draft; writing – review and editing.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## PEER REVIEW

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## DATA AVAILABILITY STATEMENT

NA

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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ORIGINAL ARTICLE

# Unraveling the molecular interactions between α7 nicotinic receptor and a RIC3 variant associated with backward speech

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## Abstract

Recent work putatively linked a rare genetic variant of the chaperone Resistant to Inhibitors of acetylcholinesterase (*RIC3*) (NM_024557.4:c.262G > A, NP_078833.3:p.G88R) to a unique ability to speak backwards, a language skill that is associated with exceptional working memory capacity. RIC3 is important for the folding, maturation, and functional expression of α7 nicotinic acetylcholine receptors (nAChR). We compared and contrasted the effects of RIC3G88R on assembly, cell surface expression, and function of human α7 receptors using fluorescent protein tagged α7 nAChR and Förster resonance energy transfer (FRET) microscopy imaging in combination with functional assays and $^{125}$I-α-bungarotoxin binding. As expected, the wild-type RIC3 protein was found to increase both cell surface and functional expression of α7 receptors. In contrast, the variant form of RIC3 decreased both. FRET analysis showed that RICG88R increased the interactions between RIC3 and α7 protein in the endoplasmic reticulum. These results provide interesting and novel data to show that a RIC3 variant alters the interaction of RIC3 and α7, which translates to decreased cell surface and functional expression of α7 nAChR.

**Keywords** RIC3 · Nicotinic acetylcholine receptors · Backward speech

## Abbreviations

| | |
|---|---|
| apFRET | Acceptor photobleaching fluorescence resonance energy transfer |
| eGFP | Enhanced green fluorescent protein |
| ER | Endoplasmic reticulum |
| FRET | Förster resonance energy transfer |
| nAChR | Nicotinic acetylcholine receptor |
| MCS | Multiple cloning site |
| mCherry | Monomeric cherry fluorescent protein |

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## Introduction

The homomeric α7 nicotinic acetylcholine receptor (nAChR) is one of the most abundant nAChRs in the brain. It is highly expressed in the hippocampus, thalamus, and cortex and contributes to cognition, attention, and working memory [1–3]. Our understanding of the exact links between α7 nAChR and cognitive functions are limited but validated links exist between α7 nAChRs and cognitive deficits associated with schizophrenia [3–5] and with Alzheimer's disease, in which α7 is proposed to exert a neuroprotective effect [6, 7]. Furthermore, single nucleotide polymorphisms (SNPs) in the *CHRNA7* gene (which encodes the α7 subunit) have been associated with dementia [8], Alzheimer's disease [9, 10] and schizophrenia [11]. Although α7 usually forms homopentamers, in basal forebrain neurones, it can also assemble with β2 nAChR subunits to form heteromeric α7β2 nAChRs, thus increasing its functional range [12].

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The diversity of α7 signaling is further enhanced by the ability of α7 nAChR to link with G-proteins, diverse intracellular signal pathways, and modulate intracellular calcium release from the endoplasmic reticulum (ER) [13].

To exert its signaling functions, α7 nAChR must be present on the cell surface, which largely depends on the correct folding and assembly of the receptor subunits in the ER and subsequent trafficking of the assembled receptor to the cell surface [14]. Robust experimental evidence indicates that the ER-resident chaperone RIC3 enhances α7 subunit folding and oligomerization in the ER leading to "mature" assemblies that are then trafficked to the cell surface [15–18]. In host cells that do not express RIC3, heterologous expression of the chaperone cDNA enables functional expression of α7 nAChR [15, 19, 20]. RIC3 also interacts with other nAChRs and the closely related 5-HT3 serotonin receptor but its effects, which can be positive or negative, depend on the identity of the receptor subunits, the host cell [16, 19] and the ratio of receptor to RIC3 [21, 22]. Although the role of RIC3 on the expression of α7 nAChR in vivo is not fully understood [23], *RIC3* expression has been linked to cognitive maintenance [24] and, crucially, there is a good correspondence between *RIC3* and α7 nAChR expression in the rat hippocampus [25]. Furthermore, recent autoradiographic analysis of the brain of a *Ric3* knockout mouse show a decrease in $^{125}$I-α-bungarotoxin binding in the hippocampus and the cortex [23], brain regions that contribute to working memory and language. In addition, the expression of *RIC3* shows a high level of correlation with α7 nAChR in postmortem brain tissues from population and disease cohorts [26].

Our interest in RIC3 stems from its potential role in language. Several studies have linked copy number changes of chromosome 15q13.3 (the location of the *CHRNA7* gene) with an increased risk of speech and language disorders, usually alongside more global developmental delays and neuropsychiatric phenotypes [27–31]. Although these chromosome rearrangements typically include 1.5–2 Mb of DNA and seven genes, smaller deletions affecting only *CHRNA7* result in similar developmental profiles, leading some to suggest that haploinsufficiency of *CHRNA7* underlies some of the features seen in this syndrome [32–34]. *RIC3* expression is specifically upregulated in both patients with schizophrenia and bipolar disorder [26], both of which include language dysfunction [35–37]. Furthermore, a recent study investigated a case family with the unique ability to speak backwards, a language skill that they postulated was made possible by exceptional working memory capacity [38]. This study identified three potential contributory variants including a rare polymorphism in *RIC3* which confers a coding change (NM_024557.4:c.262G > A, NP_078833.3:p.G88R) [38]. The genetic and behavioral bases for this skill remain unknown but the putative implication of RIC3 provides an intriguing link that we aim to substantiate on a functional level in this paper.

Given that cell surface expression is a pre-requisite for α7 nAChR signaling, the identification of the structural domains involved in the chaperone activities of RIC3 has been a long-standing research goal. RIC3 is a disordered protein with little homology between species [17]. Its structural domains comprise an N-terminal region that contains two hydrophobic segments linked by a proline-rich linker and a long C-terminal region that contains either one (human, mouse, *Drosophila*) or two (*C. elegans*) coiled-coil motif [19] (Fig. 1). The G88R variant identified in the backward speech study occurs within a poly-glycine stretch found inside the proline-rich linker domain (Fig. 1). For invertebrate species, the two hydrophobic segments are predicted to be transmembrane domains. In contrast, for mammalian species, the location of the segments is controversial. Wang et al. working with mouse RIC3 identified a cleavable signal peptide in the N-terminus, which led to the suggestion that RIC3 is a single-pass type I transmembrane protein with its N-terminus located in the lumen of the ER and the C-terminus with its coiled-coil domain in the cytoplasm [17]. In the human RIC3, Cheng et al. [39] reported a cleavable sequence in the N-terminus of human RIC3 [39], but others found no evidence the N-terminus is cleaved during translation [25, 40]. Our findings suggest that human RIC3 is a type II transmembrane protein with the N- and C-termini facing the cytoplasm (Fig. 1). The latter topology is consistent with the findings that the complete N-terminus is crucial for efficient cell surface expression of invertebrate and mammalian α7 nAChR [17, 18, 22, 25, 40].

The exact mechanism by which RIC3 promotes α7 nAChR assembly is unknown [41] although a direct interaction is expected as α7 co-precipitates with RIC3 [20, 42]. Wang et al. suggested that each RIC3 protein associates with a single folded α7 subunit [17]. The receptor is then built through RIC3 dimerization at the C-terminal coiled-coil motif, pulling subunits together to form the pentamer [17]. However, others note that the coiled-coil domain is not required for RIC3 function [40, 43] and that some isoforms of RIC3 lack the coiled-coil domain but are still able to promote α7 assembly [18, 22]. Ben-David et al. further showed that the shorter isoform, which lacks the coiled-coil domain, has different functional properties from the full protein and acts as an inhibitor of AChR assembly and function [22]. Kweon et al. later suggested that the α7 assembly process involves a host of chaperone proteins, including NACHO, OST, RPN1/2, and calnexin, as well as RIC3 passing α7 through the secretory pathway [44]. Each of these chaperones is thought to bind a distinct region of α7 [44] and it has been proposed that RIC3 binds between the M3 and M4 transmembrane domains [44]. This loop is

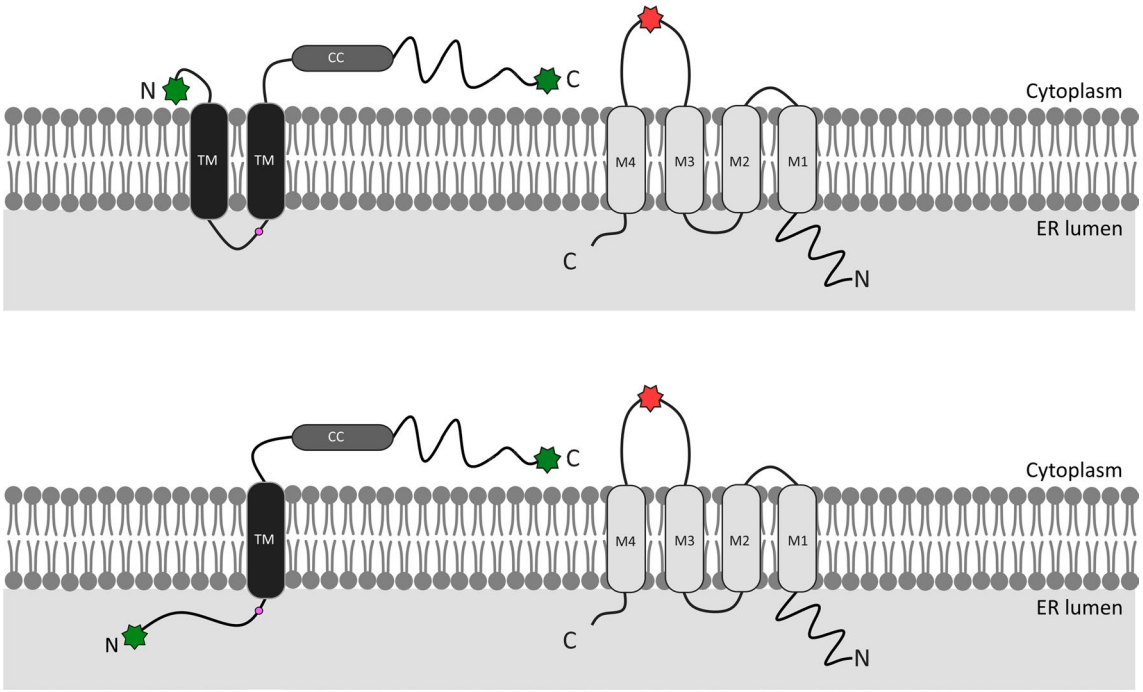


**Fig. 1** Schematic showing proposed topology and tagging of α7 and RIC3. **A**: Two transmembrane (TM) domains result in cytoplasmic C- and N-termini. **B**: A single transmembrane domain results in a cytoplasmic C-terminal domain and a lumenal N-terminal domain. Red star denotes tagging of α7 subunit between the TM3 and TM4 domains. Green stars represent tagging of RIC3, Pink dot denotes position of G88R variant. CC denotes coiled-coil domain

largely disorganized but includes an MX helix and an MA helix, that runs into the M4 transmembrane domain [41]. Although studies have shown that this region is necessary for the effects of RIC3 [44] and that substitution of residues in the MA helix ablates RIC3 enhancement of assembly [25], structural modeling suggests that interactions in this region would block pentameric assembly [41].

In the present study, we examined the consequences of G88R variant on interactions between RIC3 and α7 nAChR subunits in HEK293 mammalian cells, using RIC3 and α7 nAChR tagged with fluorescent proteins. Led by previous research, we examined three levels of function; the cellular localization of the RIC3, interactions between RIC3 and α7, and the surface expression of mature α7 receptors. Using acceptor photobleaching (ap) FRET, we found that G88R increases interaction between RIC3 and α7 in the ER. Interestingly, we found that the enhanced interaction results in decreased functional expression of α7 nAChR in *Xenopus* oocytes and reduced $^{125}$I-α-bungarotoxin binding in HEK293 cells. We suggest that the G to R variant exerts a functional effect through increased interaction between α7 nAChR and RIC3 in the ER, ultimately leading to reduced functional expression.

This investigation not only establishes a functional effect for the G88R variant but provides additional evidence on the structure of RIC3 and the mechanism of interaction between RIC3 and α7.

## Methods

### Reagents

$^{125}$I-α-Bungarotoxin (NEX126H050UC) was obtained from PerkinElmer, UK. Fugene was obtained from Promega (E5911). Acetylcholine (A2661), polyethylenimine, 25,000 MW (4008727), and Triton (648466) were obtained from Merck.

### Cell culture and cell transfections

Human Embryonic Kidney 293 cells (HEK293, supplied by ATCC, UK) were cultured in DMEM (1X) with high glucose (Life Technologies, UK) supplemented with 10% fetal calf serum (FCS; Life Technologies, UK). Cells were used for experimentation once they reached 60–70% confluency. Cells were plated on poly-d-lysine (0.1 mg/ml, Sigma)-coated glass-bottomed μ-dish 35 mm Ibidi dishes (Thistle Scientific), UK at a density of 120,000 cells/ml. All cultures were maintained at 37 °C and 5% $CO_2$.

For confocal microscopy, HEK293 cells were transfected (0.5 µg of α7, RIC3, LCK, and ER3 plasmids) using FuGene HD (Promega) following manufacturer's instructions.

## Constructs

### α7 clone

Wild-type human α7 nAChR subunits were synthesized by GeneArt (ThermoFisher, UK). The sequence of the cDNA was optimized for expression in mammalian cells. Fluorescently tagged α7 nAChR subunits were produced by inserting mCherry cDNA into the M3-M4 cytoplasmic loop of α7 at amino acid 391 (α7-mCherry).The positioning of the tag has previously been demonstrated to retain the functional properties of the receptor [45] and sits 74 amino acids away from the MX helix, which is proposed to be the site of interaction between RIC3 and α7 [46, 47]. Both wild-type and fluorescent α7 nAChR subunit cDNAs were subcloned into the pCI expression vector (Promega, UK).

### RIC3 clones

Wild-type RIC3, henceforward termed RIC3WT, cDNA (NM_024557) was amplified using primers containing EcoRI (5' TATTCGAATTCGCGTACTCCACAGTGC AGAGAGTCGCTCTGG 3') and KpnI (5' AATAAGGTA CCTCACTCTAAACCCTGGGGGTTACGCTTCCTCAG 3') restriction sites. Site-directed mutagenesis (F-primer 5' AGGTGGAGGTGCTGGACGTGGAGGTAGTGGAAG AGG 3', R-primer 5' CCTCTTCCACTACCTCCACGT CCAGCACCTCCACCT 3') was used to introduce the G88R variant (NM_024557.4:c.262G > A, NP_078833.3:p.G88R) into RIC3 (RIC3G88R).

RIC3WT and RIC3G88R cDNAs were subsequently cloned into the MCS of pEGFP-N1 (NovoPro Bioscience, Shanghai, China) to fuse the fluorescent eGFP tag to the C-terminus of RIC3WT (RIC3WT-eGFP) or RIC3G88R (RIC3G88R-eGFP) or pEGFP-C1 (NovoPro Bioscience, Shanghai, China) to fuse the eGFP tag to the N-terminus of the RIC3 protein (eGFP-RIC3WT and eGFP-RIC3G88R).

## Western blots

HEK293T cells were seeded at $3.5 \times 10^6$ cells per 10 cm plate and transfected with 17.5 µg plasmid (eGFP-RIC3WT or eGFP-RIC3G88R). After 48 h, protein lysates were extracted and quantified using a BCA assay. Proteins were separated on a 10% SDS-PAGE gel for 30 min before transfer to a nitrocellulose membrane using a semi-dry protocol for high MW proteins. Membranes were blocked in 5% milk powder in 1 × TBS Tween before detection with primary rabbit polyclonal antibodies for GFP (AbCam; ab290) and secondary goat anti-rabbit IgG (Licor.com; IRDye 680RD) for RIC3 detection. A primary mouse monoclonal antibody for α-tubulin (Merck; T5168) with secondary anti-mouse IgG (Licor.com; IRDye 800CW) was used as a positive control. All antibodies were used at a 1 in 1000 dilution. Membranes were washed six times with 1 × TBS Tween and visualized on a Typhoon biomolecular imager (Cytiva) against a marker precision plus ladder (Biorad; 161-0374).

## Confocal microscopy and acceptor photobleaching FRET

Acceptor photobleaching fluorescence resonance energy transfer (apFRET) [48, 49] was used to detect interactions between the tagged α7 and RIC3 proteins using a Zeiss LSM880 confocal microscope 2 days after transfection.

eGFP was used as the FRET donor and mCherry as the FRET acceptor. pmCherry-eGFP (Addgene, plasmid#86639) was used as positive control, while mCherry-ER3 (Addgene, plasmid#55041) and LCK-GFP (Addgene, plasmid#61099) were used as negative controls for RIC3-eGFP and α7-mCherry, respectively. FRET between donor and acceptor was confirmed by bleaching of mCherry which lasted 5 s and monitoring the concomitant increase in eGFP fluorescence across five successive 0.47 s windows. mCherry was excited with 561 nm light and eGFP with 488 nm light. The mCherry and eGFP laser transmission was kept at 2% and 1.5%, respectively, during scanning to avoid photobleaching but mCherry was set at 100% during bleaching. HEK293 cells expressing either eGFP or mCherry alone were imaged with the apFRET settings to confirm that fluorophore crosstalk was minimized, and that the bleaching step did not reduce eGFP fluorescence. Five pre-bleach and five post-bleach scans of the eGFP and mCherry fluorescence were carried out at 0.47 s intervals in a constant sized region of interest (ROI) which was manually selected to represent an ER location with comparable levels of red and green fluorescence. Fluorescence intensity was monitored in the ROI and analyzed using Microsoft Excel. For data analysis, the eGFP fluorescence intensity was normalized onto a percentage scale as described previously [48, 49]. To calculate the FRET efficiency $E_F$, the following equation was used, as described by Graumann et al. [49]:

$$E_F = \text{eGFPpost} - \text{eGFPpre}$$

where eGFPpost is the fluorescence intensity immediately after the photobleaching (scan 6) and eGFPpre is the average fluorescence intensity across all five scans before the photobleaching. Note that this calculation assumes 100% photobleaching of the acceptor [50]. All confocal work was performed at the Oxford Brookes Centre for Bioimaging. For each experimental and control sample, approximately 100

live cells (in DMEM) were imaged with a 63 × oil immersion objective (Plan-Apochromat 63×/1.4 Oil DIC M27) at 37 °C and 5% $CO_2$. Each experiment was repeated three times. After excluding outliers (> ± 1.5(IQR)), the number of intensity measurements included in the FRET calculation for each condition were $N = 246$ (α7-mCherry + eGFP-RIC3WT), $N = 257$ (α7-mCherry + eGFP-RIC3G88R), $N = 233$ (α7-mCherry + LCK-GFP, negative control), $N = 239$ (eGFP-RIC3WT + mCherry-ER3, negative control), $N = 231$ (eGFP-RIC3G88R + mCherry-ER3, negative control), $N = 244$ (pmCherry-ER3-eGFP, positive control).

## $^{125}$I-α-Bungarotoxin binding

RIC3WT or mutant RIC3G88R cDNA, in combination with α7 cDNA (at a ratio of 1:5 or 1:1), were transfected into HEK293 cells using polyethylenimine. Surface α7 expression was determined 48 h after transfection by overlaying the cells in phosphate-buffered saline (PBS) containing 10 nM $^{125}$I-α-bungarotoxin and 1 mg/mL bovine serum albumin for 60 min. Cells were washed four times with PBS and removed from the plate in 10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM ethylenediaminetetraacetate, and 1% Triton X-100. $^{125}$I-α-Bungarotoxin binding was determined by gamma counter.

## Functional expression of nAChR in Xenopus oocytes

Electrophysiological experiments were carried out on oocytes nuclearly injected with either α7 cDNA, RIC3WT, RIC3G88R, α7 + RIC3WT or α7 + RIC3G88R. We also tested the effects of wild-type and variant RIC3 on the functional expression of human α4β2 nAChRs. For these experiments, oocytes were nuclearly injected with equal amounts of α4 and β2 cDNA. For both types of injections (α7and α4β2 nAChR subunit cDNAs), the total amount of cDNA injected was kept at 5 ng for nAChR subunit cDNA and 1 nG for RIC cDNAs. Oocytes were harvested from mature *Xenopus laevis* females and used for electrophysiological experiments 2 days after injection, as described previously [51].

Acetylcholine-induced currents in Xenopus oocytes expressing heterologously α7 nAChR were recorded using an automated platform equipped with standard two electrode voltage-clamp configuration (HiClamp; Multi Channel Systems, Reutlingen, Germany). The electrodes were filled with 3 M KCl and the recordings were carried at a holding potential of − 60 mV throughout the experiment. All recordings were performed at 18 °C, and cells were perfused with a solution containing 82 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES at pH 7.4. Data were filtered at 10 Hz, captured at 100 Hz, and analyzed using proprietary data acquisition and analysis software running under Matlab (Mathworks Inc., Natick, MA). Maximal functional expression was determined using 1 mM acetylcholine, a concentration that produces maximal current responses at oocytes expressing α7 nAChRs [51]. The concentration–response curve for acetylcholine at α7 nAChR was also determined to establish whether the variant affected the function of α7 nAChR. For these experiments, we used a protocol of 7–8 concentrations of acetylcholine with a reference response (1 mM ACh, a maximal ACh concentration in wild-type human α7 nAChR). Acetylcholine was applied for 10 s and the washing period between applications was 5 min to allow for full recovery from receptor desensitization [51]. The concentration–response data were fit with the Hill equation to estimate the acetylcholine potency (ACh $EC_{50}$), as previously described [51]. For α4β2 nAChR assays, functional expression of α4β2 nAChR in oocytes injected with α4 and β2 cDNAs ± WT RIC3 or RIC3G88R cDNA was assessed by measuring the amplitude of current responses elicited by application of a maximal ACh (1 mM) to the impaled oocytes. For these assays, currents were recorded using an oocyte clamp OC-725Camplifier (Warner Instruments). For all receptor subtypes assayed and examined, the current responses to ACh were recorded 2 days after injection and all experimental conditions (RIC3WT or RIC3G88R) were done on the same day.

## Image processing and statistical analysis

Images were analyzed within Fiji [52] to assess co-localization of proteins. Images were imported as raw. czi files and a single timepoint was extracted for the red and green channels. All images were subject to background subtraction using sliding paraboloid method with a rolling ball of radius 50 pixels. Co-localization analyses were performed on a region of interest that included the whole cell using a Coloc2 plugin (https://imagej.net/plugins/coloc-2). 2D intensity histograms for the representative images shown in Figs. 2 and 3 are provided as Supplementary data. Co-localization is reported as Pearson correlation coefficients (PCC) throughout.

Unadjusted representative images were exported as montages of raw files in which red was replaced with magenta. Brightness and contrast were adjusted for all channels simultaneously in PowerPoint.

Data are expressed as means ± SEM from 100 experiments carried out using 12–14 batches of transfected cell batches or ten *Xenopus* donors. Data are reported as mean ± SEM. To compare significant differences (at $p < 0.05$) between more than two groups of data meeting assumptions of normality and equal variance, a one-way ANOVA was performed followed by a Tukey test for all pair-wise comparisons.

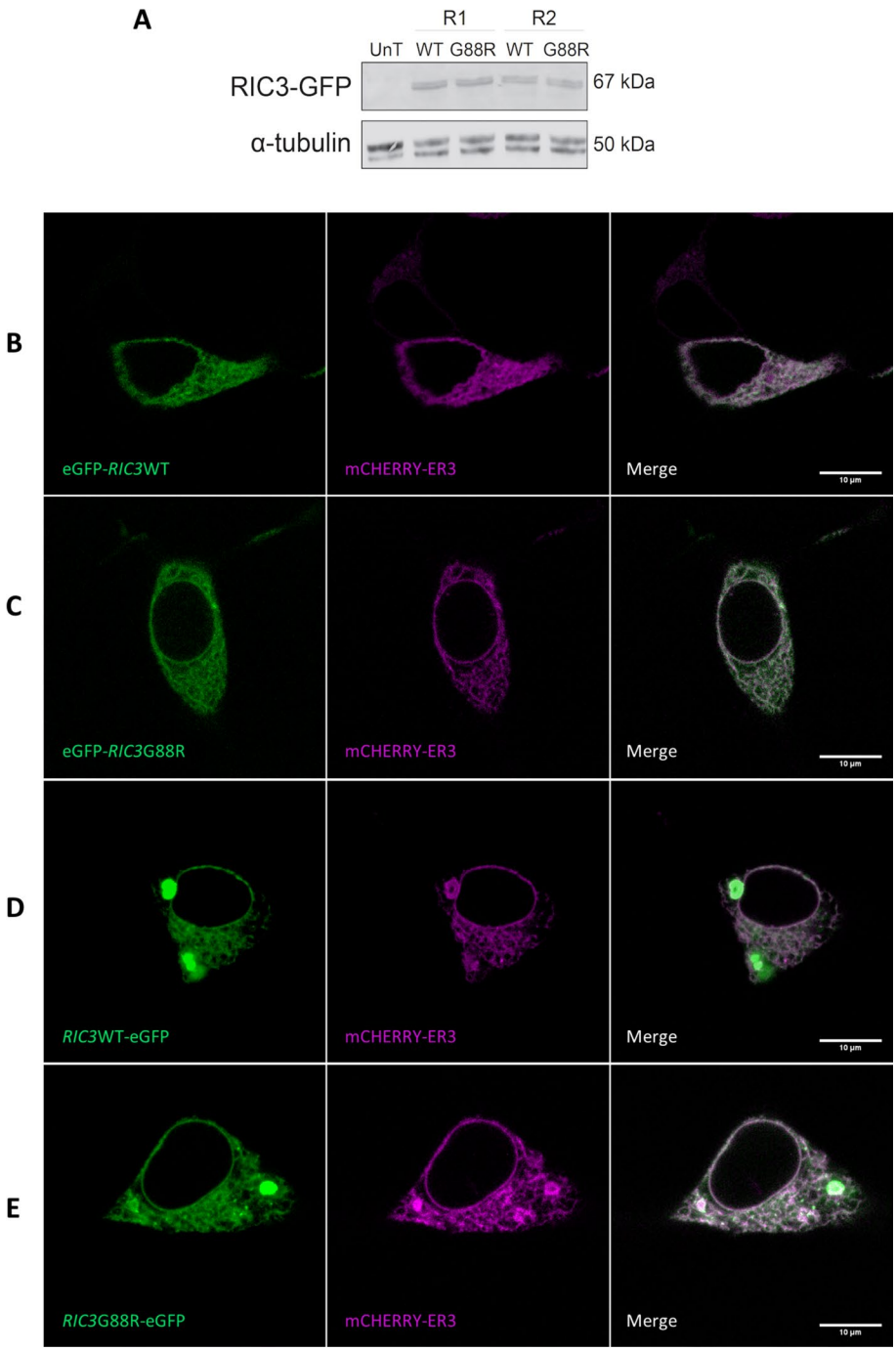


**Fig. 2** Cellular localization of RIC3WT and RIC3G88R. **A**: Western blot of transfected cells showed the presence of an eGFP-RIC3 protein at the expected 67KDa size (UnT—untransfected HEK293 cells). Two biological replicates were performed (R1 and R2). No observable differences were present between wild-type (WT) and variant (G88R) cell-lines. **B** and **C**: N-terminal fusion of eGFP on wild-type (eGFP-RIC3WT) and G88R (eGFP-RIC3G88R). **D** and **E**: C-terminal fusion of eGFP on wild-type (RIC3WT-eGFP) and G88R (RIC3G88R-eGFP) Formation of RIC3 bright oval structures was observed in the ER. RIC3 demonstrated a strong overlap with the ER marker (mCherry-ER3) in both the wild-type (WT) and variant (G88R) forms and for both N-terminal and C-terminal fusions. The average Pearson correlation coefficient (PCC) across five representative images for eGFP-RIC3WT + mCherry-ER3 (Panel **B**) was 0.73 (SD = 0.12), for eGFP-RIC3G88R + mCherry-ER3 (Panel **C**) was 0.67 (SD = 0.08), for RIC3WT-eGFP + mCherry-ER3 (Panel **D**) was 0.61 (SD = 0.14) and for RIC3G88R-eGFP + mCherry-ER3 (Panel **E**) was 0.78 (SD = 0.11). Size bars = 10 µm. Raw Western blot images are provided in supplementary data

## Results

### RIC3G88R localization

Previous studies consistently report that RIC3 localizes to the ER, where it binds unassembled α7 subunits promoting receptor assembly [17, 21, 53]. Therefore, we first sought to assess whether the G88R variant affected RIC3 cellular localization. Both RIC3WT and RIC3G88R were fused to eGFP at the N-(eGFP-RIC3WT and eGFP-RIC3G88R) or C-(RIC3WT-eGFP and RIC3G88R-eGFP) terminus and transiently expressed in HEK293 cells.

All four RIC3 clones co-localized with mCherry-ER3, an ER-resident protein marker (Fig. 2b–e, see Figure legends for Pearson Correlation Coefficients) confirming an ER localization for RIC3 and indicating that the G88R variant does not overtly alter cellular localization (Fig. 2b–e). In addition to the ER localization, both RIC3WT-eGFP and RIC3G88R-eGFP also resulted in bright ring structures (Fig. 2d and e). Similar bodies were observed by Wang

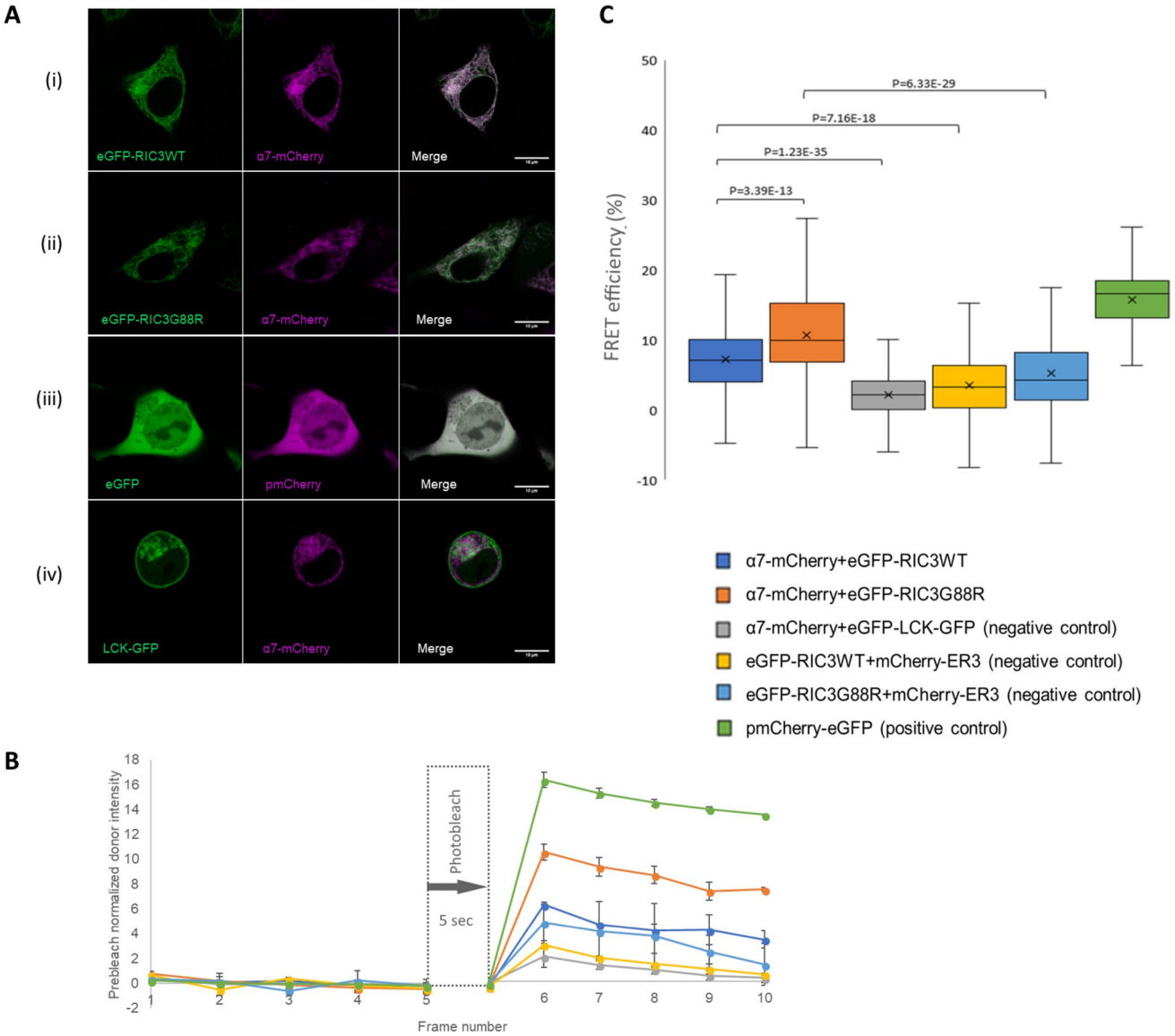


**Fig. 3** Interaction between α7 and eGFP-RIC3WT or eGFP-RIC3G88R measured by apFRET. **A**: Representative confocal images of the proteins of interest (α7 and RIC3WT (Panel i), α7 and RIC3G88R (Panel ii)). eGFP was used as the FRET donor and mCherry as the FRET acceptor. pmCherry-eGFP (Addgene, plasmid#86639) was used as positive control (Panel iii), while LCK-GFP (Addgene, plasmid#61099) and α7-mCherry were used as negative controls (Panel iv). **B**: Normalized donor and acceptor fluorescence intensity post-acceptor photobleaching. Each interval is 0.47 s. Photobleaching occurred across 5 s at time interval 5. Error bars represent SD across all measurements within each of the experimental conditions. See methods for details of normalization and N. **C**: FRET efficiencies measured for α7 and eGFP-RIC3WT or eGFP-RIC3G88R and controls, as described in methods. Boxes represent interquartile range of FRET intensity (see Methods for *N*), with lines at the median and crosses denoting mean of distribution. RIC3 demonstrated a strong overlap with α7 in both the wild-type (WT) and variant (G88R) forms. The average Pearson correlation coefficient (PCC) across five representative images for eGFP-RIC3WT + α7-mCherry (Panel A(i)) was 0.70 (SD = 0.08) and for eGFP-RIC3G88R + α7-mCherry (Panel A(ii)), the average PCC was 0.72 (SD = 0.04). The positive controls (pmCherry-eGFP) also showed a strong co-localization (Panel A(iii))—PCC across five representative images was 0.82 (SD = 0.09), while the negative controls (LCK-GFP + mCherry) had minimal overlap (Panel A(iv))—PCC across five representative images was 0.26 (SD = 0.11). Size bars = 10 µm

et al. using mouse *Ric3*, who suggested that this pattern may arise from over-expression leading to homotypic interactions between *Ric3* [17]. In addition, it was observed that both RIC3WT-eGFP and RIC3G88R-eGFP transfections led to a distorted ER structure (Fig. 2d and e), further indicating disruption of the secretory pathway. eGFP-RIC3WT (Fig. 2b) and eGFP-RIC3G88R (Fig. 2c) were, therefore, used for the remainder of experiments in this paper. Western

blots confirmed that the full-length eGFP-RIC3 protein was present at 67KDa as expected (Fig. 2a).

### Interaction between α7 and RIC3

To obtain direct evidence whether the RIC3 variant affected interaction with α7, acceptor photobleaching fluorescence resonance energy transfer (apFRET) was employed (Fig. 3). This method is based on the fact that when energy transfer occurs, the fluorescence emission by the donor fluorochrome is quenched because of the direct transfer of excitation energy to the acceptor fluorochrome. If the acceptor fluorochrome is fully bleached by a laser, FRET is dampened and the donor signal is de-quenched, thus resulting in an enhanced fluorescence emission by the donor fluorophore [21, 54]

α7 was labeled with mCherry and was observed to co-localize with eGFP-RIC3 (Fig. 3, see Figure legend for Pearson correlation coefficients). FRET efficiency between α7 and eGFP-RIC3G88R, $E_F = 10.73\% \pm 7.06$, $N = 100$, was significantly higher ($p < 0.05$; ANOVA plus Tukey test) than that observed for eGFP-RIC3WT ($E_F = 7.24\% \pm 5.97$, $N = 100$) (Fig. 3). Furthermore, the fluorescent signal emitted by the donor (eGFP) fluorochrome as a result of dequenching was 1.5-fold higher in cells carrying the variant compared to wild type ($N = 30$; $p < 0.05$; ANOVA plus Tukey test) (Fig. 3b and c) and this change persisted over the duration of the experiment (Fig. 3b). These results collectively suggest that the G88R SNP enhances interactions between RIC3 and α7 nAChR subunits.

### Cell surface expression

To evaluate the effect of the enhanced interaction between α7 and RICG88R on cell surface expression, we performed $^{125}$I-α-bungarotoxin binding on intact HEK293 cells co-transfected with mCherry α7 and eGFP-RIC3WT or eGFP-RIC3G88R. The cDNAs were transfected at either 1:1 or 5:1 α7:RIC3 ratios. α-Bungarotoxin is a neurotoxin that binds competitively to the agonist binding site of α7 nAChR subunits. The agonist binding site in nAChR is located between two adjacent subunits; hence, $^{125}$I-α-bungarotoxin binding to cells transfected with α7 cDNA can be used to probe receptor assembly. Cells transfected with α7 nAChR cDNA did not bind $^{125}$I-α-bungarotoxin (data not shown). In contrast, in the presence of RIC3, robust specific $^{125}$I-α-bungarotoxin binding was observed (Fig. 4). As shown in Fig. 4a, regardless of the transfection ratio, RIC3G88R significantly decreased the binding of $^{125}$I-α-bungarotoxin ($N = 6$; $p < 0.05$; ANOVA) by almost 50%, suggesting that enhanced RIC3-α7 interactions decrease cell surface expression of α7 nAChR. The reduction of $^{125}$I-α-bungarotoxin binding observed with transfection ratios of 1:1 or 5:1 was not statistically different to each other, in accordance with the findings of Dau et al., who reported that RIC3 significantly enhanced cell surface α-bungarotoxin binding above control levels at both 1:1 and 5:1 α7:RIC3 ratios [21].

Next, we examined whether the reduced cell surface expression of α7 nAChR affected α7 function. To examine function, α7 was expressed in the absence or presence of untagged RIC3WT or RIC3G88R in *Xenopus* oocytes, a well-established expression system ideally suited for electrophysiological recordings of recombinant ion channels. The nucleus of oocytes was injected with α7:RIC3 cDNA at a ratio of 1:1 or 5:1, and the amplitude of currents generated by 1 mM acetylcholine was recorded. At this concentration, acetylcholine stimulates maximal current responses in α7 nAChR, which are indicative of the level of functional receptors present. As shown in Fig. 4b, functional expression of α7 nAChR in the absence or presence of RIC3 does not impact the potency of Ach, as previously reported [55]. Regardless of the α7:RIC3 cDNA ratio, in the presence of RIC3WT, the expression of functional α7 nAChR increased by approximately twofold (Fig. 4c, $N = 15$, $p < 0.001$), further supporting the ability of this chaperone to promote surface expression in *Xenopus* oocytes. Consistent with the findings of the binding studies, the levels of functional α7 nAChR decreased by about 1.5-fold in the presence of RIC3G88R (Fig. 4c, $p < 0.05$). Tagged α7 and RIC3WT or RIC3G88R produced the same pattern as the non-tagged constructs (data not shown). In addition, we also examined the effect of RIC3WT or RIC3G88R on the functional expression of human α4β2 nAChR. As shown in Fig. 4c, under our experimental conditions (5 ng of α4 + β2 cDNA mixture ± 1 ng RIC cDNA), both RIC3WT and RIC3G88R increased the expression of α4β2 nAChR similarly; however, none of these effects were statistically significant compared to control (α4β2) ($N = 10$ recordings from two oocyte donors). Previous studies have shown that the effect of RIC3 on functional expression of α4β2 nAChR is not consistent, suggesting that, in oocytes, the effects may depend on other elements expressed in oocytes [18, 19, 21]. Thus, RIC3G88R appears to selectively impact the functional expression of α7 nAChR.

## Discussion

While several general protein chaperones modulate the maturation and trafficking of nAChRs [56], RIC3 is relatively specific in its chaperone activity exerting significant effect upon the folding and assembly of α7 receptors [20]. Interestingly, a rare genetic variant of RIC3 (NP_078833.3:p.G88R) was potentially implicated in a unique ability to speak backwards that is associated with

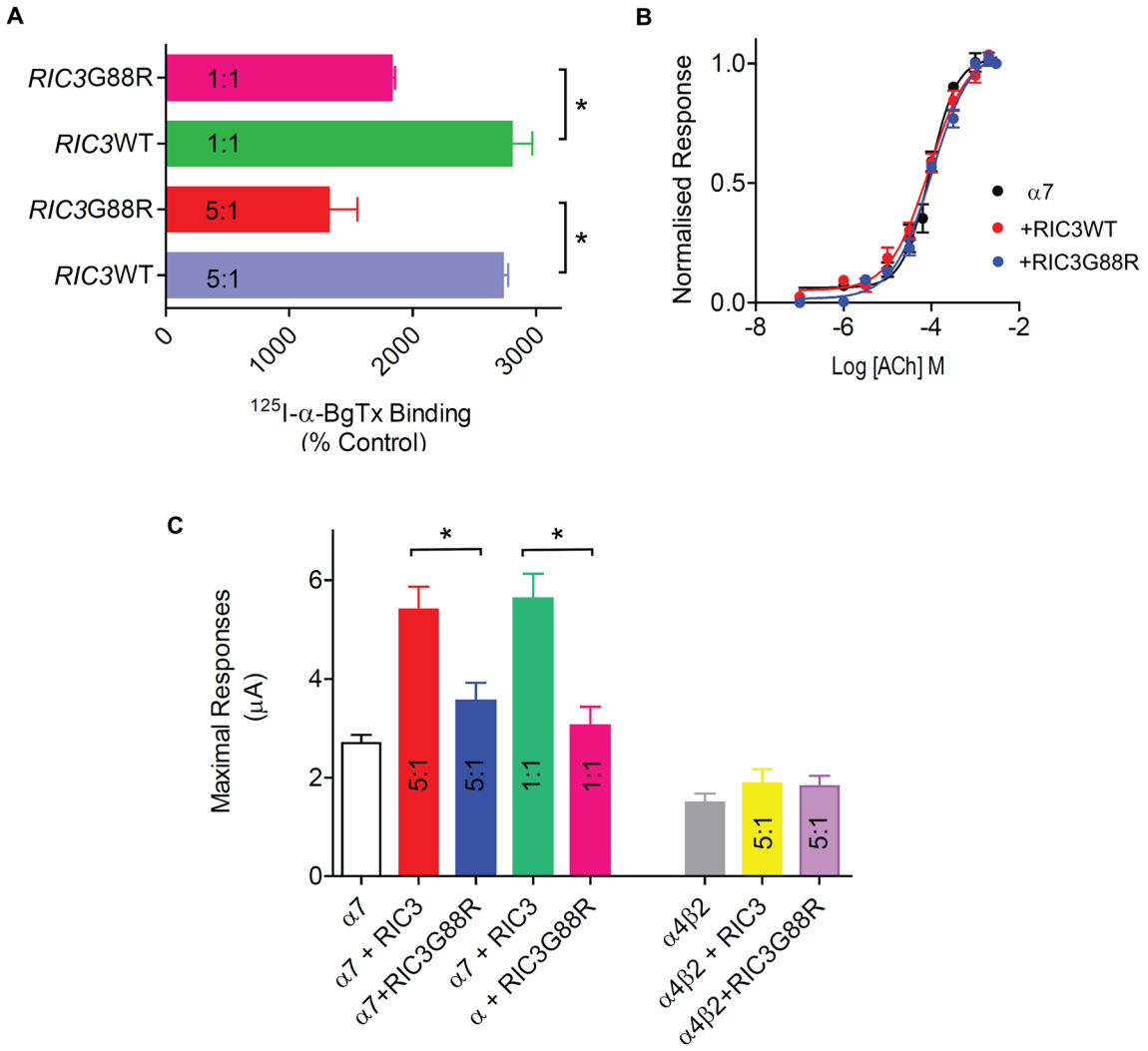


**Fig. 4** RIC3G88R variant decreases cell surface expression of α7 nAChR. **A** Cell surface expression of α7 nAChRs was assayed by $^{125}$I-α-bungarotoxin binding to intact HEK293 cells transfected with mCherry α7 and either RIC3WT or RIC3G88R at 5:1 or 1:1 cDNA ratio. **B**: The potency with which acetylcholine activates responses in α7 nAChRs is not affected by RIC3WT or the variant RIC3G88R ($N=5$). **C**: Histograms of the maximal currents activated by 1 mM Ach in α7 ($N=15$) and α4β2 ($N=10$)

higher working memory capacity [38]. This variant was one of three novel coding changes that co-segregated with the trait in the discovery family but the authors particularly highlighted the *RIC3* polymorphism, hypothesizing that this may exert a function upon cholinergic systems [38]. In this investigation, we, therefore, sought to establish the functional level of effects mediated by this coding change in *RIC3*. We find that RIC3G88R significantly increased interaction with α7 compared to the wild-type RIC3. Subsequent $^{125}$I-α-bungarotoxin binding to α7 and functional assays showed that RIC3G88R decreased cell surface binding and functional expression, suggesting that enhanced RIC3-α7 interactions in the ER reduce cell surface and functional expression of α7 nAChR. This finding indicates that the polymorphism RIC3G88R modifies RIC3-α7 interactions and that this change substantially affects α7 nAChR surface expression. The exact relationship between this functional pathway and backwards speech is still to be elucidated. Many questions remain regarding the way in which RIC3 moderates receptor assembly and function and whether these effects are specific to certain receptor types. Understanding these mechanisms will be critical to the functional characterization of this variant, which may act at many different levels.

Our investigations show that eGFP-RIC3WT produced a significantly higher FRET signal/efficiency compared to the negative controls. A previous study also used FRET to demonstrate increased assembly and cell-surfacing trafficking of α7 in the presence of RIC3 [21]. However, this investigation used a different FRET method (sensitized

emission) and measured interaction between α7 subunits [21]. Others have shown co-immunoprecipitation of RIC3 and α7 [16]. Our findings add to this baseline to further suggest a direct interaction between RIC3 and α7 within the ER. Interestingly, the FRET signal produced by eGFP-RIC3G88R was almost 1.5-fold greater than that seen with eGFP-RIC3WT. This strongly indicates that there is a direct interaction between RIC3 and α7 and that the p.G88R polymorphism implicated in backwards speech strengthens this interaction, resulting in a reduced surface expression, as shown by $^{125}$I-α-bungarotoxin binding and the decrease in the amplitude of the maximal currents elicited by Ach in oocytes expressing heterologously α7 nAChR and RIC3G88R. Although the role of RIC3 in α7-signaling dysfunction has not been explored, α7 nAChR expression is reduced in the brain of schizophrenic patients [5, 57] and the levels of RIC3 mRNA in the brains of schizophrenia patients, postmortem, are greater than in typical brains [26]. Chaperones of nAChR have been previously linked to cholinergic dysfunction. A variant of rapsyn, a muscle nAChR chaperone that concentrates and anchors muscle nAChR in the postsynaptic membrane of the neuromuscular junction, causes congenital myasthenic syndrome by altering interactions with the receptor muscle specific tyrosine kinase (MuSK) [58].

Variant G88R occurs within a poly-glycine stretch found within the proline-rich linker that joins the hydrophobic domains of RIC3. Deletion of the entire proline-rich linker in human RIC3 [25] attenuates α7 surface expression, indicating the importance of this region for the chaperone activity of RIC3, although specific singular residues are unlikely to account for this effect. The poly-glycine segment is not thought to adopt a specific folding pattern but we propose that the G-R change creates a positively charge that may alter the configuration of the proline region, thus affecting chaperone activity of RIC3.

We found that N-terminal fusion of wild-type RIC3 to eGFP (eGFP-RIC3) does not impair the expression of RIC3 in the ER or the chaperone activity of this protein. These findings are in accord with previous studies of human RIC3 that have used N-terminal fusion RIC3 constructs to examine the chaperone activity of this protein [21, 53]. The observation of FRET activity between eGFP-RIC3 and α7 directly indicates that RIC3 is a type II transmembrane protein, since the N-terminus of RIC3 must have a cytoplasmic location to allow this interaction (Fig. 1a). This supposition opposes the findings of Wang et al. who working with a C-terminal tagged mouse RIC3 construct suggested that the mouse Ric3 N-terminus is cleaved during translation [17].

In contrast, the expression of the C-terminal fusion, RIC3-eGFP, led to rings of bright fluorescence and a disordered ER, reminiscent of ER-phagy [59]. These observations suggest that tagging of the C-terminus disrupts RIC3 function leading to misfolded polypeptides within the ER and subsequent removal of damaged ER sections. If the misfolded proteins are α7, then this implicates a direct role for RIC3 in α7 folding as suggested by [17]. Given the disordered nature of RIC3, the localization of the exact interaction domain has proven to be challenging [41].

In summary, our investigations shed light upon the interaction between RIC3 and α7 in the assembly and trafficking of this important neuronal receptor. Specifically, we demonstrate that the RIC3G88R variant has a functional effect by increasing RIC3 interaction with α7 subunits in the ER and that this ultimately leads to a reduction in the cell surface and functional expression of α7 nAChR. How may a decrease in α7 receptor expression influence the ability to speak backwards? Backward speech relies on a strong working memory capacity [38] and memory is influenced by α7 nAChR signaling [60]. The role of α7 nAChR in cognition is linked to its modulation of glutamatergic and GABAergic signaling but the mechanisms driving this effect are not well-understood. This is largely due to the complexity of α7-signaling, which is affected by diverse elements including cell type, location, and complex relationship between timing of activation relative to associated glutamatergic and GABAergic pathways involved in cognition and memory (for a review, see [61]). Thus, decreased functional expression of α7 nAChR could potentially upset the balance between the modulation of excitatory/inhibitory pathways. Alternatively, decreased expression of α7 nAChR may alter neuronal development, when the foundations for the cognitive and language functions of the brain are first laid. $^{125}$I-α-Bungarotoxin binding sites are present in the human fetal brain [62], and the α7 nAChR has been implicated in neuronal migration [63] and early post-natal synapse formation [64, 65]. Thus, RIC3 can potentially affect the ability to speak backwardly by affecting the establishment of the signaling circuitries involved in speech. Further investigations will be required to link this functional finding to the reported language phenotype, providing important evidence about the function of both RIC3 and α7 nAChR in neurodevelopment.

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**Author contribution** DN, IB, SP, HM, ER, SM, DB designed the experiments. AP, ER, JP carried out the confocal studies and analyzed the data. SP, EE, JSS, and JC performed the Western blot assays. IB designed and carried out functional assays in *Xenopus* oocytes. SM

carried out α-bungarotoxin binding assays. IB, DM, DB, RW, YD, DN, HM provided supervision. AP, IB, DN wrote manuscript and prepared the figures. All authors contributed to and agreed manuscript content.

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**Data availability** The datasets generated during and/or analyzed during the current study are available in the Oxford Brookes RADAR repository [https://doi.org/10.24384/mp84-n694].

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.



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