Evaluation of Cell-Penetrating Peptides as Tools to Improve Nose-Brain Delivery of Insulin

Alexander Brown

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Dr Madeleine V. King

Dr James Dixon

Abstract

Changes in brain insulin signalling are implicated in many neurodegenerative and psychiatric disorders, including Alzheimer's disease (AD). There is therefore great interest in the therapeutic value of insulin-based therapies that have potential to overcome cognitive and emotional deficits. However, efficient delivery of large molecules, to the brain is hampered by the blood-brain barrier (BBB). Nasal administration represents an opportunity to bypass the BBB and exploit direct olfactory and trigeminal nerve nose-to-brain delivery routes. But uptake is relatively inefficient, so as little as 0.5% of an administered dose reaches its intended target. This MRes thesis involved *in vitro* studies to assess the ability of a novel cell-penetrating peptide (CPP) delivery system, termed the glycosaminoglycan (GAG)-binding enhanced transduction (GET) system, to enhance insulin delivery. Insulin-GET nanocomplexes (NCs) can be generated via electrostatic interaction. Application of these NCs to cultured RPMI 2650 nasal epithelial cells increased insulin uptake by as much as 10.5-fold compared to insulin alone without affecting cell viability. In a transwell permeation assay, insulin-GET NCs demonstrated a 12-fold increase in apical to basal accumulation compared to insulin alone, without affecting barrier integrity or metabolic activity. These encouraging *in vitro* findings support the further evaluation of whether GET can improve delivery of insulin to the brain *in vivo*. This is more complex than the *in vitro* setting as it requires perineuronal and/or perivascular transport after epithelial penetration.

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Publications

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Abbreviations

ABC	ATP-binding cassette
ACI	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
ALI	Air-liquid interface
APOE	Apolipoprotein E
ATP	5'-triphosphatase
AUC	Area under the ROC curve
Aβ	Amyloid β-protein
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BLQ	Bolinaquinone
Cmax	Maximum serum concentration
CME	Clathrin-mediated endocytosis
CNS	Central nervous system
CPP	Cell-penetrating peptide
CSF	Cerebrospinal fluid
CvME	Caveolae-mediated endocytosis
DAPI	4',6-diamidino-2-phenylindole
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
Egfp	Enhanced green fluorescent protein
EIPA	5-N-ethyl-isopropyl amiloride
Em	Emission
EMEM	Eagle' minimum essential medium
ESC	Embryonic stem cells
Ex	Excitation
FITC	Fluorescein iso-thiocyanate
FLR	FGF2B-LK15-8R
GET	Glycosaminoglycan-binding-enhanced-transduction
GI	Gastrointestinal
HBSS	Hanks buffered salt solution
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HS	Heparan sulphate
IR	Insulin resistance
LCC	Liquid-culture conditions
LDH	Lactate dehydrogenase
L-DOPA	Levodopa
LENK	Leucine-enkephalin hydrochloride
MAP	Model amphipathic peptide
MBCD	Methyl-b-Cyclodextrin

MCI	Mild cognitive impairment
MDD	Major depressive disorder
MFI	Mean fluorescent intensity
MRP	Multidrug resistance associated protein
NC	Nanocomplex
NMDA	N-methyl-D-aspartic acid
NP	Nanoparticle
OT	Oxytocin
Papp	Apparent permeability coefficient
PBS	Phosphate buffer saline
PEG	Poly(ethylene glycol)
PET	Positron emission tomography
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PLGA	Poly-lactic-co-glycolic acid
PLR	P21-LK15-8R
PNA	Peptide nucleic acid
PTSD	Post-traumatic stress disorder
ROS	Reactive oxygen species
RPMI 2650	Human nasal septum carcinoma cell line
SERT	Serotonin transporter
IgA	Immunoglobulin A
SPECT	Single photon emission tomography
SSRI	Selective serotonin reuptake inhibitor
TAT	Trans-activator of transcription
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TRD	Treatment-resistant depression

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1 Introduction

1.1 Overview

Many neurodegenerative and psychiatric disorders are poorly managed by current treatments and so there is a large gap in the drug market for novel therapeutic approaches that provide a more effective treatment. Alzheimers disease (AD) is a neurodegenerative disorder and the most common cause of dementia (Alzheimer's association, 2022), AD is characterised by confusion, poor memory, and general cognitive impairment. Major Depressive Disorder (MDD) is a psychiatric condition, distinguished by persistent low mood and motivation, lack of self-care, suicidal ideation and not as commonly, mild cognitive impairment and poor memory (Liu et al., 2020 & Culpepper et al., 2017). Both AD and MDD lack causal treatments that address the origin of these disorders. The primary treatment options available for patients of either disorder aim to alleviate symptoms and slow disease progression. Acetylcholinesterase inhibitors (ACI) such as donepezil are a first line treatment for AD patients and are approved for use at mild, moderate, and severe disease stages. Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine are the most common medications prescribed to MDD patients and similarly to ACIs, are often criticised for their limited efficacy and wide range of unwanted side effects (Joshi et al., 2018, Sharma et al., 2019). Drugs that target the central nervous system (CNS) have a significantly lower approval rate compared to non-CNS targeting drugs. Issues with limited bioavailability make disorders of the CNS particularly hard to treat. Bioavailability is severely limited in the CNS due to the presence of biological barriers, primarily the blood-brain barrier (BBB). The BBB is a highly impermeable endothelial barrier that protects the brain via a vast system of drug transporter and tight junction proteins which significantly limit drug entry through conventional delivery means (Dickens et al., 2016). Due to recent advances in our understanding of neurodegenerative and psychiatric disorders, more potential treatment options are being investigated, but the issue of bioavailability persists.

There is currently a great interest in the therapeutic use of oxytocin to alleviate social deficits in conditions such as autism and schizophrenia. Numerous studies have attempted to overcome issues with limited bioavailability by nasal administration to achieve brain delivery along olfactory and trigeminal pathways, thereby bypassing biological barriers such as gastrointestinal metabolism and the BBB (Sikich et al., 2021, Yamasue et al., 2020). However, nasally administered psychoactive compounds still struggle to achieve efficient brain bioavailability (typically 1%<) (Frey & Thorne., 2001 & Illum., 2004). Nanoparticle (NP) strategies offer a novel approach to improve delivery of multiple therapeutic agents to the brain. For example, encapsulation by polymer NPs of leucine – enkephalin hydrochloride (LENK), a peptide opioid neurotransmitter, improved delivery to olfactory bulb and deeper brain regions of the thalamus and cortex compared to LENK alone, without any peripheral exposure, and produced a strong anti-nociceptive response (Godfrey et al., 2018).

Insulin dysregulation in the brain has been associated with Alzheimer's Disease and psychiatric disorders such as depression and autism, directing research towards insulin brain delivery as a treatment option (Ghasemi, R et al., 2013). Intranasal delivery of insulin has become an ideal treatment option to bypass this barrier and research into this has highlighted an issue in the limited absorption of insulin by nasal epithelium (Tashima, T., 2020 & Khafagy, E-S et al., 2009). This thesis aims to provide early proof-of-concept data relevant to nose-brain delivery, by assessing the ability of our novel NP-based cell-penetrating peptide (CPP) to improve insulin uptake and transcytosis using cultured human nasal epithelial cells, thereby supporting progression to *in vivo* studies. This introductory chapter will cover NP delivery strategies in general and our CPP in more detail, as well as focusing on the central effects of insulin.

1.1.1 Prevalence of neurodegenerative & psychiatric disorders

As is the brain itself, neurodegenerative & psychiatric disorders are very complex, and thus it's only in the past few decades that significant progress has been made in understanding the risk factors and neurological changes associated with the development of these disorders. According to the 2021 report from the Alzheimer's Association, with 121,499 deaths caused by Alzheimer's in 2019 alone, this disease is the sixth most common cause of death in the USA. Whilst deaths caused by stroke and heart disease between 2000 and 2019 (ranked fifth and first-leading causes of death in USA respectively, according to data from 2020) have been reduced due to the development of medical treatment, the reported deaths caused by AD increased by over 145% (Alzheimer's association., 2021). From 2019 to 2020, the total recorded deaths from AD had increased by 10.5%, likely due to the COVID-19 pandemic, considering the age groups most affected by both AD and COVID-19 (Alzheimer's association., 2022). Additionally, Data from the Global Burden of Disease has revealed that between 1990 and 2017, the global prevalence of depressive disorder had increased by 49.86% from 172 million to 258 million (Liu et al., 2020). Depressive disorder prevalence has grown at a concerning rate since, coinciding with the COVID-19 pandemic which one study, using data from January 2020 to January 2021, attributes an additional 53.2 million cases of Major Depressive Disorder to (Santomauro et al., 2021). As it stands, dealing with these disorders is a great burden to healthcare systems worldwide.

1.2 Cell-penetrating peptides

The delivery of endogenously synthesised peptides (and proteins) such as insulin as therapeutic agents has several benefits over chemically synthesised drugs, they are mostly highly specialised in function and are metabolised well by the body. However, pharmacokinetic issues restrict the bioavailability and thereby the therapeutic value of insulin. For instance, when intranasal or oral administration is opted for, systemic drug circulation is limited by the presence of biological barriers such as the epithelial membranes and tight junctions between cells of the nasal cavity or the intestinal wall, which peptides and proteins such as insulin poorly permeate through due to their hydrophilic nature (Goldberg & Gomez-Orellana., 2003, Kamei et al., 2013, Wang et al., 2022). In addition to insulin's limited mucosal membrane permeability, the portion of drug that survives transcellular delivery across the nasal epithelium is subject to enzymatic degradation and clearance by cilia (hair-like structures) that line the nasal mucosa (Erdő et al., 2018 & Gupta et al., 2017). However, as research into optimising drug delivery progresses and new ideas circulate, potential solutions to these major limitations have been identified and expanded upon. The potential of cell-penetrating peptides (CPPs) to provide a method of targeted therapeutic delivery to inaccessible intracellular sites has been demonstrated with promising efficiency both *in vitro* and *in vivo* (Guidotti et al., 2017 & Suhorutsenko ., 2011).

The discovery of CPPs dates back to the late 80s, when researchers in '88 demonstrated *in vitro* cellular internalisation of the TAT (trans-activator of transcription) protein of the HIV-1 virus (Frankel & Pabo., 1988). This idea was expanded upon in '91 when the peptide penetratin was derived from *Drosophila* antennapedia homeoprotein after the homeoprotein displayed cell-penetrating capabilities (Joliot et al., 1991). This spurred the discovery of many more short peptides capable of translocation, now commonly referred to as CPPs. This peptide family is distinguished by consisting of 5 – 30 amino acid residues that are capable of efficient translocation across bio-membranes without depending on energy-dependent processes, allowing targeted delivery of biologically active conjugates or 'cargoes' into target cells (Kamei et al., 2015). Conjugation of CPPs and cargoes occurs via the formation of a covalent or non-covalent complex, these bonds can be formed with conjugates such as DNAs, siRNAs, NPs, and small molecule drugs (Bechara., 2013). Covalent conjugation is often used for the delivery of conjugates such as small drug molecules as well as peptides and proteins. However, due to the structural changes caused by covalent conjugation, it's been reported that this can change

the biological function of cargoes, reducing the overall therapeutic activity (Heitz et al., 2013 & Juliano et al., 2008). Conversely, non-covalent complexes are formed by electrostatic and/or hydrophobic interactions between bigger cargoes with negative charges and CPPs with positive charges, thereby not compromising the structure and function of the conjugate (Deshayes et al., 2010).

Macromolecules such as peptides, proteins, and nucleic acids are of great therapeutic interest for their ability to modulate biological mechanisms by targeting particular molecules and pathways, making them exceptionally useful as therapeutic agents involved in the diagnosing and treatment of many diseases. However, the cell membrane, a semipermeable barrier, restricts cell entry to compounds of small molecular size via channels and carrier proteins which macromolecules are unable to utilise (Ruseska & Zimmer 2020). To overcome the limitations of conventional therapeutics, an ideal delivery system would be able to efficiently transport macromolecules across cell membranes whilst providing stability and protection against enzymatic degradation, enhance target specificity, and minimise toxicity. CPPs are able to provide highly efficient delivery of macromolecule cargoes across cell membranes of many different cell types through non-invasive mechanisms without disrupting membrane integrity or eliciting an immunological response. The exact mechanisms by which cellular uptake occurs have not been fully elucidated but are typically divided into two main pathways: endocytosis (energy-dependent route) and membrane translocation (energy-independent route). The route used to gain cell entry is understood to be influenced by certain factors. These include the structure of the cell membrane, the physicochemical properties of the peptide and cargo (e.g., charge distribution and size), and the concentration used. Peptides with different physicochemical properties will interact with different cell surface molecules (e.g., proteoglycans) which can ultimately influence the cell entry mechanism (Wang et al., 2014).

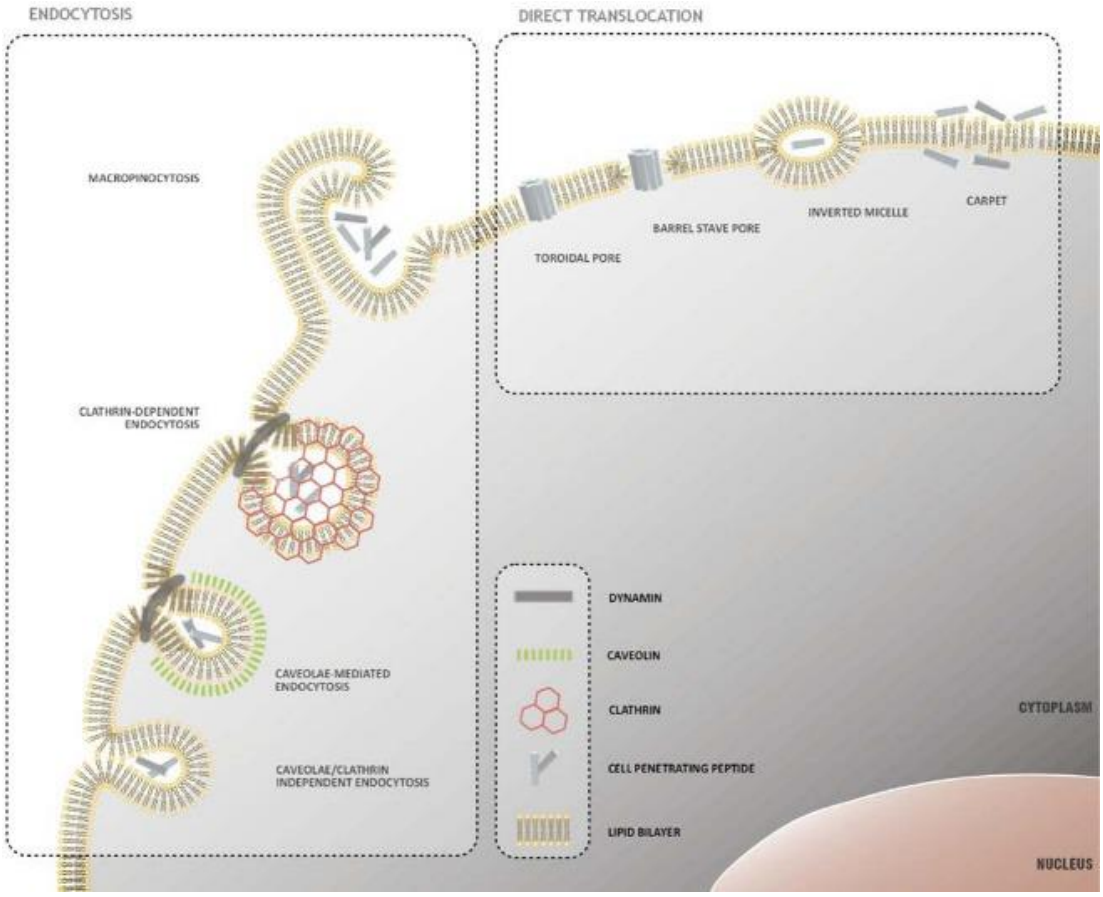

Figure 1. Cell-entry mechanisms of cell-penetrating peptides.
Several mechanisms have been proposed to explain cellular uptake of CPPs, which broadly fit

into two categories: endocytosis or direct translocation. Endocytosis occurs by energy-dependent pathways that involve vesicle formation, whilst direct translocation mechanisms are energy-independent and rely on the formation of hydrophillic pores or local destabilisation of the lipid bilayer. (Source: Trabulo et al., 2010).

Endocytosis is an energy-dependent cellular uptake mechanism that allows intracellular transport of macromolecules in vesicles or vacuoles that pinch off from the plasma membrane, involving two main steps: endocytic uptake and subsequent endosomal escape. Pinocytosis is the main process by which endocytosis occurs with relevance to CPP-mediated uptake, and is described by four distinct mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and clathrin- and caveolae-independent endocytosis (Trabulo et al., 2010). Routes of internalisation have previously been compared between several cationic and amphipathic CPPs conjugated with peptide nucleic acids (PNAs) in a study by Lundin et al., (2008). This study demonstrates that macropinocytosis is a preferred cell entry mechanism for cationic CPPs such as penetratin. However, amphipathic CPPs such as transportan were internalised primarily via CME. Overall, most studies support the idea that CPP-cargo uptake mainly occurs via the various endocytosis pathways (Nakase et al., 2007, Duchardt et al., 2007, El-Andaloussi et al., 2005).

Direct membrane translocation, however, is described by three distinct models: the inverted micelle model, the carpet model, and translocation via pore formation. The inverted micelle model proposes that CPP and cell membrane interaction causes structural changes in the membrane's phospholipid bilayer, causing inverted micelle structures to form which eventually destabilises and results in intracellular peptide release. This mechanism is incompatible with translocation of large molecule conjugates since they are unlikely to form these inverted micelle structures. The carpet model describes extensive association of CPP-cargo complexes and cell surface molecules, causing phospholipid reorganisation and transient membrane destabilisation, allowing cell entry (Hao et al., 2022, Lundin et al., 2008). Furthermore, toroidal pores are formed due to interaction between peptide hydrophilic groups and phosphate groups of the phospholipid bilayer, and barrel-stave pores are formed by interaction between peptide lipophilic groups and the lipid tails of the membrane's phospholipids, resulting in formation of transient pores which are highly destructive and cytotoxic (Langel & Zorko, 2021). It is now well established that generally, endocytosis is the primary method of cell-entry at low peptide concentrations, whilst direct translocation occurs at higher CPP concentrations (Ruseska & Zimmer, 2020 & Madani et al., 2011).

Although CPPs are generally reported as exhibiting low cytotoxicity, any substance can be toxic above a threshold concentration (Silva et al., 2019 & Guidotti et al., 2017). For example, high concentrations of two CPPs termed transportan 10 and model amphipathic peptide (MAP) have to been shown to cause significant membrane disruption in two human cancer cell lines, K562 (erythroleukemia) and MDA-MB-231 (breast cancer). A lactate dehydrogenase (LDH) assay showed that 40% of total LDH leaked from the K562 and MDA-MB-231 cells during a 10-minute exposure to 10μM of transportan 10 and MAP (Saar et al., 2005). This is an important metric to measure cytotoxicity by since extensive membrane disruption via direct translocation can cause cell death. Therefore, assessing cytotoxicity through the use of leakage and/or cell viability assays is crucial to the development of a CPP-mediated drug delivery system.

CPPs can be utilised to allow intracellular delivery of large molecules such as peptides with great therapeutic value, thereby bypassing the limitations of conventional therapeutics. Translocation of large molecules as described by any of the models that describe direct membrane translocation would cause significant membrane disruption, which greatly limits the therapeutic utility of CPP-cargo conjugates that exploit these pathways. In comparison, endocytosis is inherently less toxic and associated with greater epithelial permeation and successful delivery and absorption of peptides such as insulin (Xu et al., 2020, Yang et al.,

2018). However, endocytosis pathways are not without their own limits. Activity and localisation of cargo proteins delivered intracellularly via CPP-mediated endocytosis is limited via endosomal containment. Endosomal escape of CPP-cargo complexes is understood to be the primary limiting factor in cellular delivery of bioactive macromolecules, and the precise mechanism by which this occurs remains unknown. High extracellular concentrations of CPP cargo have been demonstrated *in vivo* to be an important factor in triggering endosomal escape and allowing the cargo to reach the cytosol and elicit its therapeutic effect (Guidotti et al., 2017, Kosuge et al., 2008).

1.3 Introduction to nanoparticle delivery

NPs are solid particles typically defined as being in the range of 1 – 100nm in size and categorised into three distinct groups: organic, inorganic, and carbon-based and can be further subdivided by morphology and dimension (Khan et al., 2019).

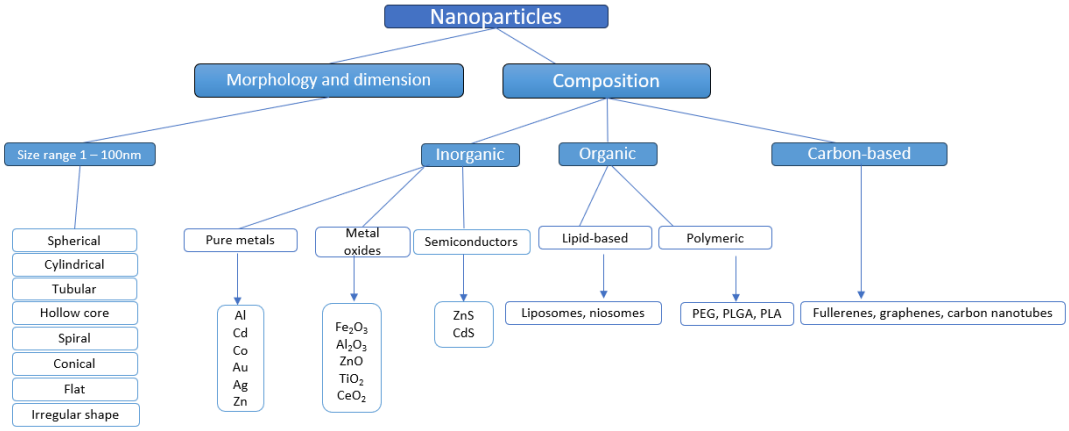

Figure 2. Nanoparticle classification.
Types of nanoparticles classified by composition, morphology, and dimension featuring a variety of examples. Poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid). Created with Microsoft PowerPoint (accessed on 12th September 2023).

The field of NP-based therapies (termed nanomedicine) is rapidly progressing and can provide a novel approach to the treatment and diagnosis of a wide range of disorders, including cancers, as well as neurological disorders (Khan et al., 2019). NPs possess a unique set of properties which are of great therapeutic interest, they can be engineered for controlled release of therapeutic molecules and targeted delivery to enhance bioavailability and biodistribution at the target site. Their ability to encapsulate therapeutic agents and release them in a controlled and specific manner may be utilised to enhance delivery to brain structures associated with neurodegenerative/psychiatric disorders, across the BBB or via alternative routes such as nose-to-brain pathways (Saraiva et al., 2016).

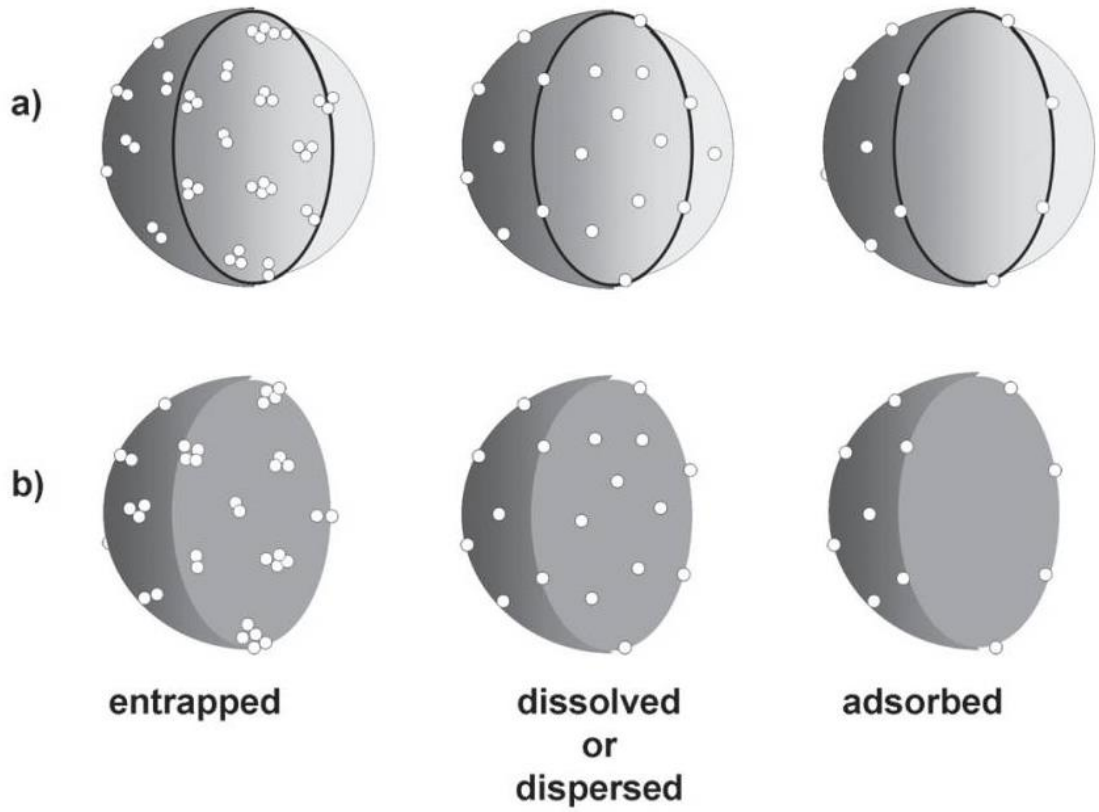

Figure 3. Encapsulation mechanism models.
Drug molecules (white spheres) can be entrapped in, dissolved or dispersed within, and adsorbed on: a) nanocapsules and b) nanospheres (Source: Guterres et al., 2007).

Polymeric NPs can be subdivided into nanocapsules or nanospheres depending on their composition. Nanocapsules feature a polymeric membrane whilst nanospheres feature a polymeric matrix. In either case, there are several mechanisms of encapsulation. The drug is either adsorbed on the NPs, or entrapped, dispersed, or dissolved within the NPs (Guterres et al., 2007). These NPs are able to efficiently conjugate CPPs to enhance cellular transduction of proteins such as insulin by facilitating membrane permeation (Oppen et al., 2019). This has wide-ranging implications for insulin-based therapies that aim to treat diabetes and associated metabolic disorders as well as neurological disorders associated with insulin resistance in the central nervous system (CNS).

1.3.1 Nanomedicine considerations & novel approaches for neurodegenerative disorders

Brain delivery of therapeutic molecules such as insulin poses a unique set of challenges which nanocarrier systems such as GET aim to overcome. The BBB limits drug influx and efflux through a comprehensive system of drug transporters (e.g., ATP-binding cassette transporters) and is composed of a continuous endothelial cell layer connected via tight junctions, adherent junctions, and gap junctions (Ahlawat et al., 2020, Dickens et al., 2016). The BBB is almost entirely bypassed by intranasal delivery via trigeminal and olfactory nerve pathways which constitute a direct connection between the nasal cavity and brain structures. However, the low permeability of nasal mucosa, rapid mucociliary clearance, and degrading enzymes contribute to poor bioavailability (typically < 1 %) (McMartin et al., 1987, Picone et al., 2018). These challenges emphasise the necessity for novel delivery methods to improve the brain uptake efficiency of insulin and other potential treatments of AD.

One of the main hallmarks of AD is a reduction in mitochondrial oxidative phosphorylation, leading to increased production of reactive oxygen species (ROS). The mitochondrial oxidative

phosphorylation cascade involves an important antioxidant, coenzyme Q10, the most common form of coenzyme Q in humans (Bhatt et al., 2021). Transgenic mouse models of AD (APP/PS1) treated with a caudal vein injection of PLGA NPs loaded with coenzyme Q-10 and modified with trimethylated chitosan demonstrated a significant improvement in cognitive and spatial memory, as well as reduced oxidative stress. Brain uptake was observed in the ventricles, choroid plexus, and cortex, demonstrating that polymer-based NP delivery systems are able to cross low-permeability epithelial barriers such as the BBB and efficiently deliver neurodegenerative therapies (Wang et al., 2010). Polymer-based NPs can also effectively deliver gene therapies via intranasal administration. In a previous study, BALB/c mice were treated with simultaneous intranasal administration of mouse-adapted influenza viruses (PR8) and chitosan/siRNA NCs designed for antiviral influenza treatment. Morbidity was significantly reduced compared to nonspecific siRNA-treated and control groups, and influenza virus replication was effectively inhibited (Jamali et al., 2017). This demonstrates that polymeric NPs can effectively deliver a variety of therapeutically valuable cargoes via several routes of administration.

ACIs such as donepezil are the main treatment options for AD patients and are all only available as an oral formulation to be taken once daily. Common side effects of these medications include gastrointestinal issues such as anorexia, nausea, diarrhoea, and muscle convulsions. Additionally, due to the deterioration of memory associated with AD, patients missing their scheduled dose is a common problem. To avoid these issues associated with oral delivery, reduce dose, and prolong its action, Bhavna et al., (2014) have previously intranasally administered a donepezil loaded chitosan nanosuspension, demonstrating higher Cmax and AUC vales in plasma and blood samples compared to ordinary donepezil solution via intranasal administration. Intranasal administration of insulin has also been enhanced by synthesised nanogels, significantly increasing delivery to brain regions such as the hippocampus, cerebellum, and cerebral cortex *in vivo* (Picone et al., 2018). No immunological response was observed, and no signs of toxicity were seen in tissue samples taken from kidneys and liver where drug accumulation is most likely to lead to organ damage. The encapsulation of insulin by nanocarriers such as the PLR (P21-LK15-8R) peptide offers protection throughout transport, increasing its stability, and allowing for controlled release of the drug (Rehmani et al.,2023).

Due to the size and light-weight of NPs, insulin benefits from improved muco-adhesion and increased contact time at site of absorption, leading to higher bioavailability. The distribution of NPs is very specific to a target area, allowing for more efficient uptake via olfactory and trigeminal nerve pathways (Borrajo et al., 2022, Shah et al., 2022). This leads to a comparatively higher drug uptake in target brain structures compared to conventional delivery, reduces the capacity for adverse effects since distribution to off-target sites are low, and reduces the required dose or frequency of dosing, making patient compliance easier (Ponchel & Irache., 1998).

1.3.2 Nanoparticle-based antidepressant treatment strategies

Various nano-formulations of antidepressants such as desvenlafaxine and tranylcypromine have been investigated in the hope of enhancing bioavailability in relevant brain structures and reducing adverse effects. For many of these NP-based antidepressant approaches, nose-to-brain pathways are of great interest. Currently available antidepressants such as selective serotonin reuptake inhibitors (SSRIs), the current main treatment option for depression, can only be taken as oral formulations. This route of administration limits bioavailability primarily due to the first pass effect and gastrointestinal (GI) enzymatic degradation, causing larger doses to be required which increases risk for adverse effects which in the case of psychoactive medications such as SSRIS, can be very severe (e.g., serotonin syndrome, mania). It's currently understood that reduced levels of monoamines (e.g., serotonin, dopamine) play a key role in the neuropathology of depression, SSRIs increase synaptic availability of serotonin by inhibiting reuptake of serotonin via the monoamine transporter SERT (serotonin transporter) (Patel et al., 2022).

PLGA-chitosan NPs have previously been loaded with the SSRI Desvenlafaxine for intranasal administration in rodent depression models (Wistar rats). In comparison to orally administered Desvenlafaxine, intranasally administered Desvenlafaxine-loaded PLGA-chitosan NPs significantly reduced symptoms of depression and enhanced monoamine brain concentrations (Tong et al., 2017). This showcases a promising strategy for the future of MDD therapies and how NPs as well as nose-to-brain routes can be utilised for the treatment of psychiatric disorders, which are poorly managed by current treatments. However, more detail is needed from future studies to assess toxicity and highlight the specific brain regions where intranasally administered SSRIs tend to accumulate and their relevance to MDD pathology, which could be achieved by single photon emission tomography (SPECT), positron emission tomography (PET), or immunohistochemistry analysis of brain slices for example (Kamila et al., 2009, Wang et al., 2010).

1.4 Introduction to Glycosaminoglycan-binding enhanced transduction (GET)

Glycosaminoglycan (GAG)-binding enhanced transduction (GET) is a novel CPP-based delivery system which uses membrane-docking peptides that bind membrane-bound heparan sulphates, allowing cell-targeting (Dixon et al., 2016). The GET peptide of interest combines the GAG - / heparan sulphate (HS) binding domain P21 (P), derived from heparin-binding epidermal growth factor (HB-EGF), with octa-arginine 8R (R), a cell-penetrating domain, an amphiphilic peptide LK15 (L), which facilitates endosomal escape, and is termed P21-LK15-8R (PLR) (Dixon et al., 2016). The application of the GET system has previously demonstrated effective enhanced intracellular transduction of a variety of cargos, including nucleic acids (e.g., plasmid DNA, siRNAs, modified nucleotide mRNA), recombinant proteins, and endogenous peptides (e.g., oxytocin, insulin) (Awwad et al 2017., Awwad et al 2020., Rehmani et al 2023., Wong et al., 2022). Additionally, GET has been utilised for improved delivery of lung gene therapy (Osman et al., 2018), programming cell fate (Eltaher et al., 2016), and regenerative medicine (Eltaher et al., 2022, Power et al., 2022, Raftery et al., 2019).

GET-mediated delivery of transcription factor conjugates has shown promising transfection efficiency (Dixon et al., 2016). For example, it has previously been shown that GET-mediated delivery of the transcription factor NANOG was able to efficiently enhance self-renewal of mouse embryonic stem cells (ESCs) CGR-8Z. This was demonstrated in the absence of leukaemia inhibitory factor, which is known to stimulate mouse ESC self-renewal (Kitzmann & Fernandez, 2001, Jorgensen & Puente, 2022). This effect was measured by changes in cell proliferation as well as pluripotency-associated alkaline phosphatase activity. Additionally, GET-mediated delivery of MYOD myogenic factor to human ESCs HUES7 effectively facilitated myogenic differentiation, which was measured by immumolabelling of multinucleated myogenin-positive myotubes as well as expression of MYOD and skeletal muscle-specific ACTA1 via quantitative polymerase chain reaction. Previously, NIH3t3 mouse embryonic cells treated with micropinocytosis inhibitors (e.g., amiloride, cytochalasin D) caused a dose-dependent decrease in cellular transduction, measured by GET-Cre recombinase activity in NIH3t3 cells expressing enhanced green fluorescent protein (eGFP) (Dixon et al., 2016). This indicates that this is the main CPP-mediated cellular delivery mechanism that GET relies on.

1.4.1 Insulin-GET complexation

Under normal metabolic conditions, elevated blood glucose signals beta cells of the pancreatic islets to produce the neuropeptide and hormone, insulin. Insulin receptors on insulin-sensitive tissues respond by absorbing glucose and thereby decreasing the blood glucose concentration, which the beta cells respond to by reducing insulin synthesis (Tokarz et al., 2018 & Rachdaoui 2020). Diabetes is a chronic condition characterised by excessive blood glucose and presents as two distinct types; type 1 diabetes is an autoimmune disorder in which cells of the immune

system target and destroy beta pancreatic cells (Chatterjee et al., 2017, Gillespie et al., 2006, Maahs et al., 2010). Type 2 diabetes is characterised by inadequate insulin production and insulin resistance (IR), an endocrinological condition that causes cells of tissues that are insulin-sensitive under normal conditions (muscle, fat etc) to lose their ability to uptake glucose from the bloodstream in response to insulin (Ndisang et al., 2017 & Taylor 2013). Type 1 and 2 are primarily treated by insulin therapies usually via subcutaneous injection, when other treatment options aren't effective. Therefore, there's great interest in an oral insulin formulation that can effectively regulate blood glucose due to non-invasiveness and ease of self-administration.

The P21-LK15-8R peptide has an overall negative electrostatic charge, which arises from the amino acid sequence of each domain: KRKKKGKGLGKKRDPCLRKYK (P21) – LLKLLLKLLLKLLK (LK15) – RRRRRRRR (8R) (Ferreras et al., 2021 & Dixon et al., 2016). Insulin's negative charge and GET's negative charge allows conjugation of the two peptides via electrostatic interaction, generating small and stable positively charged insulin-GET NCs, previously demonstrated by Rehmani et al., 2023.

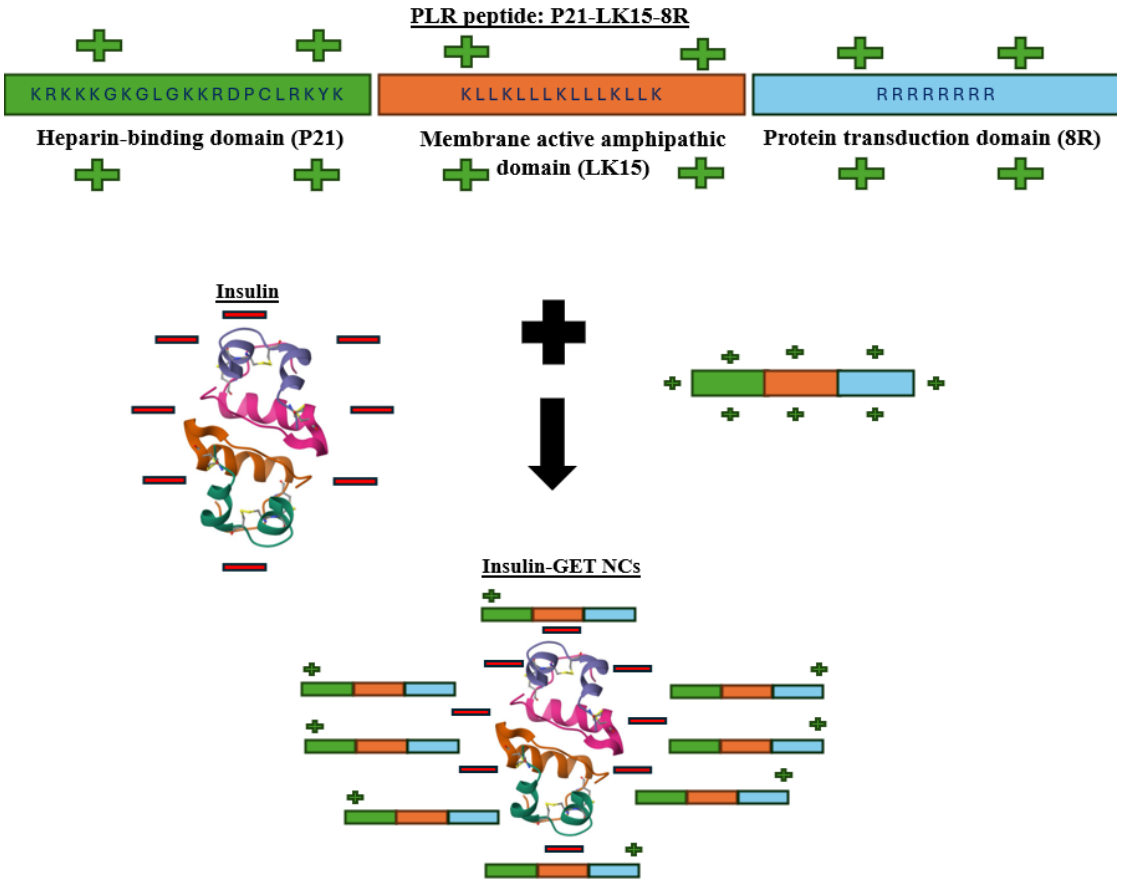

Figure 4. Insulin-GET nanocomplexes.
Insulin's negative charge and GET's positive charge allows for generation of Insulin-GET nanocomplexes via electrostatic interaction (source: Rehmani et al., 2023)**.**

A previous study by Rehmani et al., (2023) developed an oral formulation of insulin-GET NCs as a potential anti-diabetic therapy. This study involved *in vitro* assays to determine transepithelial permeability, cytotoxicity, and cellular uptake as well as *in vivo* studies of blood glucose control in diabetic mice, and extensive characterisation of the NCs in terms of size, charge and stability. Using differentiated Caco-2 monolayers cultured on transwells as a model of the intestinal epithelial barrier, insulin-GET NCs demonstrated significantly enhanced intracellular accumulation, apical and basal release of internalised insulin, and transcytosis compared to insulin alone without affecting viability or barrier integrity. Diabetes was induced

in overnight fasted male HsdOla:TO mice via intraperitoneal injection of streptozotocin (160 mg/kg body weight), hyperglycaemic conditions were determined by non-fasting blood glucose levels of >20 mmol/l. Twice-daily oral insulin (2iU/kg or 100 – 500iU/kg) was administered alone or in combination with several GET ratios (1:5, 1:0.5, and 1:0.05) by oral gavage. Blood samples were taken from the lateral tail vein before and after administration (9am and 3:30pm) to measure blood glucose levels and designated treatments administered 30 minutes later for 4 consecutive days. Orally administered insulin-GET at ratios of 1:0.5 and 1:0.05 were both able to consistently reduce non-fasting blood glucose at all timepoints. This demonstrates that low concentrations of GET can successfully stabilise diabetic glycemia for at least 4 days, when oral delivery is repeated for AM and PM dosages. These findings have global implications for the optimisation of insulin-based antidiabetic therapies, by improving bioavailability and ease of administration.

The current study utilises the same form of the GET peptide (P21-LK15-8R) as used by Rehmani et al., (2023) to generate insulin-GET NCs but for the purpose of assessing cellular uptake, cytotoxicity, and enhanced transcytosis of insulin across a differentiated RPMI 2650 monolayer as an *in vitro* model of nasal epithelium. Other forms of GET have been designed and studied for intracellular cargo delivery, such as FGF2B-LK15-8R (FLR). The only difference in structure being the replacement of the P21 domain with FGF2B, another heparan sulfate-GAG binding peptide, instead derived from fibroblast growth factor 2. FLR has not been used for delivery of endogenous peptides such as insulin but has demonstrated a higher transfection efficiency of pDNA in NIH3T3 cells compared to PLR (Osman et al., 2018). The difference in HS-GAG binding domains between these two peptides most likely causes targeting of different cell surface HS-epitopes. FLR-mediated *in vivo* DNA delivery requires the addition of PEG coatings (PEGylation) of NPs to enhance stability which ultimately reduced the efficiency of DNA transfection. PEGylated FLR has previously been used for *in vivo* intranasal delivery of 19.86nmol oxytocin (OT) (5:1, 1:1, 1:5, and 1:20 molar ratios of OT:PEGylated GET), another endogenous peptide, in male Lister hooded rats but failed to provide brain delivery of oxytocin. However, in another study, 100µg (99.29nmol) of OT was administered to male adult hooded Lister rats alone or in conjugation with the PLR peptide (1:1 OT-GET mass ratio) as NCs. Delivery to the olfactory bulb was significantly improved by PLR but failed to improve hippocampal delivery. Therefore, it's safe to say that PLR currently shows the most promise for improving delivery of insulin and other endogenous peptides across epithelial barriers (e.g., intestinal, nasal) and can even enhance brain delivery. Hence the use of the prototypical GET peptide, PLR, for conjugation to insulin in the current study.

1.5 In vitro techniques for assessing epithelial permeation

Limited bioavailability is the most important cause of new drugs failing to meet the standards required for approval and is a key problem for drugs targeting the CNS (Ahlawat et al., 2020). Preclinical *in vitro* models of intestinal epithelium, nasal epithelium, lung epithelium etc have been developed and optimised in recent years in order to replicate human physiology as closely as possible in a laboratory setting. These *in vitro* models provide an alternative to animal studies which aligns with the 3R principles: replacement, reduction, and refinement. However, successful *in vitro* permeation and absorption of drugs using cancer-derived epithelial cell lines often fails to provide an accurate prediction of *in vivo* outcomes. Whilst *in vivo* studies provide the most important test of a drug's absorption and permeability mechanisms, *in vitro* studies allow us to investigate crucial pharmacokinetic aspects of drug permeation in a controlled environment. Recently, oral, and nasal formulations of insulin are being researched for the treatment of diabetes mellitus, and neurodegenerative disorders (e.g., Alzheimer's disease) respectively. In each case, epithelial barriers limit bioavailability and delivery to the target site. Thus, *in vitro* assessment of epithelial permeation is a vital preclinical measure. Caco-2 and RPMI 2650 are both cancer cell lines, the former is derived from human intestinal epithelium whilst the latter is derived from human nasal epithelium. Both cell lines can be utilised in

transwell permeability assays, in which cells are cultured on semi-permeable filter inserts to form a well-differentiated monolayer. This transwell insert divides the culture wells into an apical (top) and basal (bottom) chamber, drugs are then administered to the top compartment and samples can be collected from the bottom to determine drug accumulation and permeability.

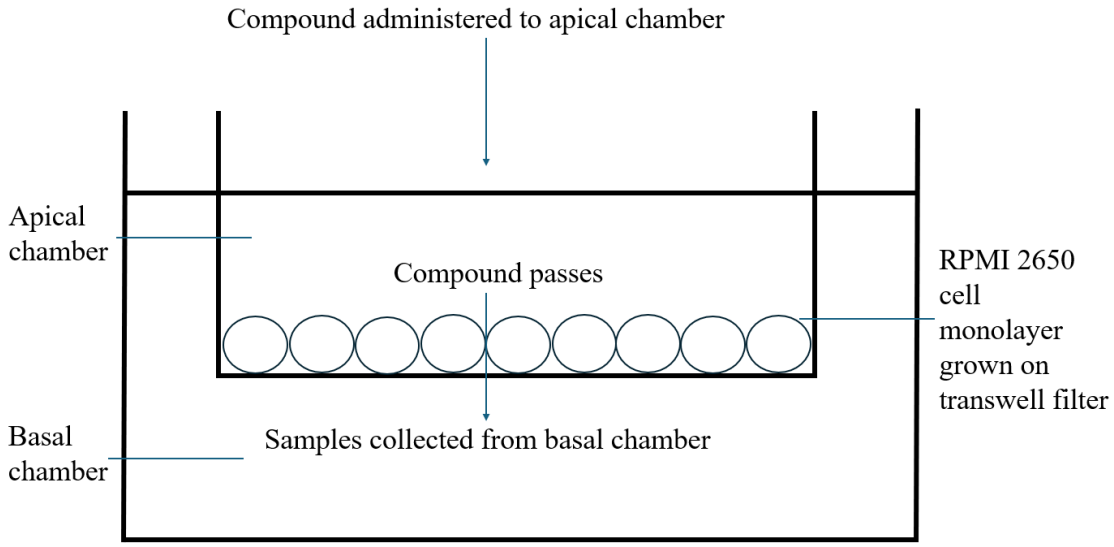

Figure 5. Transwell permeability assay.
Transwell permeability assays utilise cultured cell monolayers to assess drug permeability *in vitro*. Created with Microsoft PowerPoint (Accessed on 8th February 2024).

Air-liquid interface (ALI) culture is necessary for the development of a well differentiated monolayer with epithelial barrier function when using a cell line derived from nasal epithelium (e.g., RPMI 2650) or respiratory epithelium (e.g., 16HBE14o-) (Reichl & Becker., 2012). Typically, cells are grown under traditional liquid-culture conditions (LCC) until the 7th or 8th day when they are switched to ALI culture by removing media from the apical chamber, and on day 20/21 they are suitable for permeation studies (Sibinovska et al., 2019, Wong et al., 2022). This technique replicates the conditions found in the human nasal cavity and respiratory tract, providing optimal growth and differentiation conditions. Tight junction proteins form a continuous intercellular barrier between epithelia and modulate paracellular passage of drugs, a vital aspect of barrier function. Another important aspect of drug permeation is the expression of drug transporter proteins, which regulate the influx and efflux of several substrates including amino acids and drugs. Drug transporters are widely expressed by a variety of epithelial barriers including intestinal, nasal, and the BBB. Western blot analysis and immunofluorescence studies have shown that ALI stimulates a significantly higher expression of tight junction proteins such as ZO-1. Occludin, claudin-1, and E-cadherin as well as drug transporter proteins such as P-glycoprotein (P-gp) compared to growth under LCC, for several epithelial cell lines (Bai et al., 2007, Buckley et al., 2018, Ernhardt et al., 2003, Kreft et al., 2015, Wan et al., 2000). Monolayer integrity is also commonly assessed by measuring transepithelial electrical resistance values (TEER) which is a much more straightforward and widely applicable approach that measures ohmic resistance using electrodes connected to a voltohmeter (Srinivasan et al., 2015).

1.6 Nose-to-brain pathways

The intranasal route of administration allows insulin to non-invasively bypass the BBB and avoid first pass metabolism, with negligible risk of systemic side-effects. The trigeminal nerves originate in the brainstem and projects towards the nasal cavity, innervating the respiratory and olfactory regions (Crowe et al., 2018). Intranasal insulin first interacts with olfactory sensory neurons and is subsequently transported along olfactory and trigeminal nerves by intracellular

pathways, or by extracellular pathways via paracellular diffusion (Shaughness et al., 2020 & Hallschmid 2021).

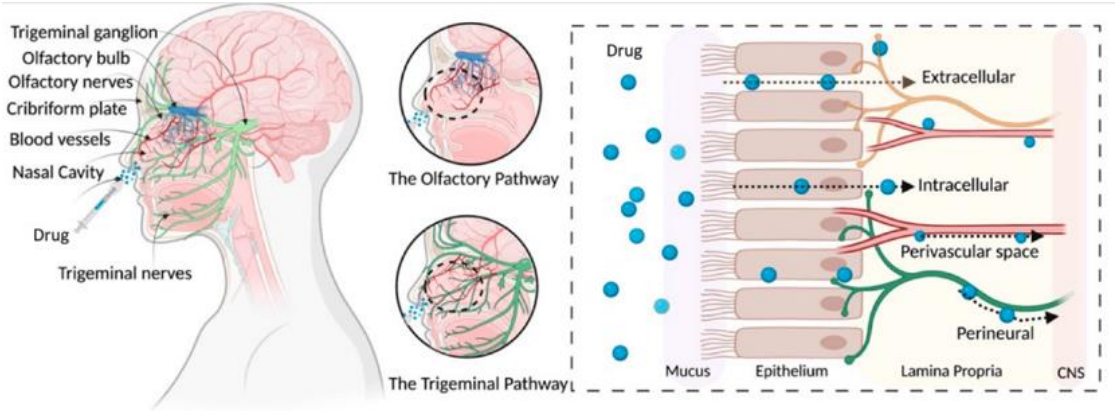

Figure 6. Nose-to-brain delivery routes.
Olfactory and trigeminal nerve pathways provide delivery routes to the central nervous system (CNS) following intranasal administration of therapeutics. Via intracellular or extracellular transport, compounds can reach the lamina propria and subsequently travel to the CNS primarily via bulk flow through the perineural and perivascular spaces (Source: Bharadwaj et al., 2021).

Intranasal insulin is rapidly detected within minutes in multiple brain regions of rodents such as the olfactory bulb, hippocampus, cortex, brainstem, and the cerebellum (Brabazon et al., 2017, Salameh et al., 2015, Thorne et al., 2004). Yet, intracellular transport of neuropeptides to the olfactory bulb via nasal passages takes many hours (Thorne et al., 1995). This suggests that extracellular transport pathways are the primary route for nose-to-brain insulin. Fluorescein isothiocyanate (FITC)-labelled insulin has been imaged in the trigeminal nerves of rats 30 minutes after nasal administration. The data shows a strong signal within the perineurium (area between axon bundles) and epineurium (area in outermost nerve layer), demonstrating that intranasal insulin reaches target brain regions along extracellular perineural spaces of the trigeminal nerve (Lockhead et al., 2019).

Nose-to-brain pathways are a compatible route for the delivery of many types of compounds, even antidepressants such as selegiline. Selegiline-loaded chitosan NPs have previously been delivered intranasally to male Wistar rats (5 or 10 mg/kg) to treat depressive behaviours. The forced swim and tail suspension tests were carried out to induce depression-like behaviours in the rats and immobility time was recorded. Immobility is referred to as behavioural despair in animals and is thought to reflect a condition similar to depression in humans. Whilst intranasal administration of selegiline alone had no effect on immobility time in depression-induced rats, selegiline-loaded chitosan NPs significantly reduced immobility time. Additionally, these NPs were measured at a significantly higher concentration in homogenised whole-brain samples following intranasal administration (Singh et al., 2015). In 2019, intranasal esketamine was approved for use in adults with treatment-resistant depression (TRD) and gained great interest as an NMDA (N-methyl-D-aspartic acid) receptor antagonist antidepressant. However, at present, it hasn't lived up to its initial reputation as what many believed to be an innovative antidepressant treatment that could rival the efficacy and demand of mainstay antidepressants such as selective serotonin reuptake inhibitors (SSRIs) (Bahr et al., 2019).

Intranasal esketamine is primarily absorbed by respiratory epithelium, suggesting brain delivery mainly occurs via the trigeminal nerve pathway (Doty et al., 2021). However, the preferred nose-to-brain pathway has not been investigated and the role of the olfactory pathway is unclear. The mean total absolute bioavailability of esketamine administered nasally is ~48%, much higher than that observed with oral administration (~8%) (Bahr et al., 2019, Doty et al., 2021).

However, the range of bioavailability following nasal administration has been estimated between 8 – 45%, a significantly large variation (Sanders et al., 2021). Evidence of efficacy is unclear but is regarded as mild and patient response seems to vary greatly (which could be associated with the large variation in bioavailability). Overall, intranasal esketamine raises more questions than it answers and with the lack of data on long-term safety, SSRIs and other more conventional antidepressants are a much more preferred treatment option (Schatzberg, 2019). Although efficient brain delivery can be achieved with nose-to-brain pathways, further investigation is greatly required.

1.7 Intranasal Insulin delivery

Due to the presence of biological barriers and metabolic routes, the route of drug administration can change the overall fate of a treatment. Subcutaneous delivery of insulin is commonly used for diabetes patients as this rapidly allows entry into systemic circulation and subsequent peripheral action, the desired effect (Shah et al., 2016). However, as evidenced, insulin's action in the brain is thought to be important for cognition and mood stability, and so administration for this purpose must optimise delivery into the CNS which the BBB makes very challenging (Hanson et al., 2008 & Thorne et al., 2001). To overcome these challenges associated with brain delivery, there is great interest in intranasal administration of insulin. Trigeminal and olfactory nerves provide neural connections to areas of the brain such as the brainstem and olfactory bulb that can be exploited for delivery to the brain (Lioutas et al., 2015 & Dhuria et al., 2010). Intravenous insulin has been shown to improve memory in AD patients. However, risk of systemic adverse effects e.g., hypoglycaemia and the invasive nature of this administration route restricts its safety and viability (Craft, S et al., 2000 & Craft, S et al., 1996). Intranasal insulin administration is non-invasive, bares little to no risk of systemic hypoglycaemia, and allows cerebral concentrations of insulin to reach levels 100-fold higher than intravenous delivery (Freiherr et al., 2013 & Hanson et al., 2008). Clinical research has investigated this treatment option for AD as well as mild cognitive impairment (MCI), which often develops into AD, both of which are poorly managed by current treatments (Petersen et al., 2001).

Intranasal insulin delivery avoids peripheral adverse effects and allows CSF insulin levels to rapidly rise within 10 minutes of administration and achieve their peak after 30 minutes (Born et al., 2002). After this 80-minute study had concluded, CSF insulin levels remained above baseline whilst blood glucose and insulin levels were unaltered, demonstrating that intranasal delivery allows insulin to access the CNS directly and the vast majority of the hormone to bypass the bloodstream without affecting circulating insulin and glucose levels (Born et al., 2002 & Benedict et al., 2003). This finding is significant in the context of using this approach to treat AD, which presents an associated decline in cognition and brain insulin signalling. As well as peripheral hyperinsulinemia, decreased CSF insulin levels are a typical characteristic of AD patients (Craft et al., 1998).

The beneficial effects on memory function as well as mood caused by long-term intranasal insulin administration have been demonstrated in a double-blind 8-week study by Benedict et al (2003). This study involved 19 healthy young participants (18 – 34 years old) with no personal or family history of diabetes. After one week of a treatment regime consisting of 4 doses of 40IU human insulin per day, self-confidence, well-being, and anger all improved. By the 8th week, delayed word recall significantly improved whilst depressive feelings significantly decreased and improvements in self-confidence and well-being were maintained. Blood glucose and plasma insulin levels were unaffected and no peripheral side effects were induced. This study suggests that intranasal insulin has therapeutic potential to improve memory function and emotional stability, which patients of MDD and AD could benefit from. However, it's important to note that this study involved 19 healthy participants, which is a small sample size and the benefits of intranasal insulin seen in healthy individuals are not consistently reported in those with neurological disorders.

Whilst such clinical evidence supports the promising effects of intranasal insulin on cognition in healthy patients, the findings are much more conflicting in studies that have used AD and MCI patients. One review from Avgerinos et al using clinical data up to 2017 examined a range of studies totalling 293 patients (172 with MCI, 121 with AD) (Avgerinos et al., 2018). Collectively, it's apparent that the only aspect of cognition significantly improved by intranasal insulin in patients with either condition, is verbal memory and story recall, when doses of 10IU, 20IU, and 40IU of regular insulin were administered. Long-acting insulin (Detemir) had neutral or negative effects on verbal memory when a similar range of doses (IU) were administered. Some of the data examined supports the idea that intranasal insulin follows a ∩-shaped function, and that by exceeding a threshold dose insulin can negatively impact several aspects of cognition (Reger et al., 2008). This study by Reger et al showed that 60IU doses of regular insulin impaired verbal memory and increased plasma Aβ42 levels. Other aspects of cognition (e.g., attention, executive function, visuospatial function) were neutrally impacted by intranasal insulin.

A more recent review using studies published up until December 2021 and totalling 899 patients, concluded that intranasal insulin shows no significant effects on cognitive improvement in MCI/AD patients (Long et al., 2022). Clearly the data is very conflicting and obviously limited by very small sample sizes. Many factors affect the observed effects of insulin, including treatment time-course, type of intranasal insulin (long-acting, regular etc), and device used. Potentially the single most significant factor impacting a patients response to treatment highlighted by both reviews, is the absence or presence of the APOE-ε4 gene. A clinical study by Reger et al demonstrated that intranasal insulin improved verbal memory in memory-impaired individuals without (ε4-) the APOE-ε4 gene (Reger et al., 2006). Compared to both healthy controls and memory-impaired individuals with (ε4+) the APOE-ε4 gene, this effect was more significant. In-fact, memory-impaired ε4+ subjects showed reduced cognitive performance for some measures of memory following treatment. Generally, ε4- AD and MCI patients respond better to various treatments, particularly at higher doses (Podewils et al., 2005, Reger et al., 2004, Risner et al., 2006) despite greater insulin resistance and poorer glucose utilisation compared to ε4+ AD patients (Craft et al., 2000). This selective response modulated by the APOE-ε4 genotype may be partially explained by ε4- AD patients' greater memory improvement in hyperinsulinemic states (Craft et al., 1999).

A randomised, double-blind, placebo-controlled clinical trial by Cha et al., (2017) investigated the therapeutic use of intranasal insulin to treat cognitive and emotional dysfunction in adults with MDD. This included 35 participants who received 4 x 40IU intranasal insulin (n = 19) or placebo (n = 16) for 4 weeks. Intranasal insulin failed to demonstrate significantly significant improvements of neurocognitive function, overall mood, emotional processing, or self-reported quality of life. As it stands, intranasal insulin for the treatment of cognitive and emotional deficits clearly demonstrates some therapeutic value. However, the results supporting the efficacy of this treatment are very inconsistent and in cases where this treatment is efficacious, the effect is often mild. These issues could be improved with a more efficient delivery system that effectively enhances bioavailability and therapeutic effect. This can be achieved through the use of NP-based delivery approaches such as GET, which promotes controllable insulin release to facilitate specific drug delivery and allows for effective bioavailability by enhancing insulin absorption *in vivo* (Rehmani et al., 2023). As well as insulin, there are other peptides that are of great interest for intranasal administration to treat neurological disorders. At present, the pro-cognitive effects of intranasal insulin in patients suffering from neurodegenerative conditions, particularly AD and MCI, is inconclusive and requires further research.

1.7.1 Novel nose-to-brain delivery approaches for CNS disorders

Oxytocin has been suggested as a novel therapy to reduce anxious symptoms and improve social deficits in a variety of psychiatric disorders. A study by Neumann et al., (2013) administered

oxytocin intranasally to rats and mice. Compared to basal levels, significant increases in oxytocin concentration were measured via simultaneous microdialysis in both the amygdala and dorsal hippocampus of rats and mice, paralleled by changes in plasma oxytocin concentration. Peak oxytocin levels were measured in the hippocampus and amygdala 30 – 60 post-administration. These two brain regions are of particular relevance to psychiatric and neurobehavioural disorders since they are involved in the regulation of memory and emotional stability (Neumann et al., 2013).

The optimisation of nose-to-brain delivery of peptides such as insulin is an ongoing process requiring constant innovation. Ijeoma Uchegbu and her group have consistently worked with nanotechnology approaches for the delivery of CNS agents. Recently, they have investigated a novel nano-in-microparticle formulation of L-DOPA (GCPQ-L-DOPA). L-DOPA (Levodopa) is a Parkinson's disease drug and the precursor to the neurotransmitter dopamine. 2-hours after nasal administration of GCPQ-L-DOPA, rat brain tissues analysed by liquid chromatography and mass spectrometry showed significantly higher dopamine levels in the brain compared to L-DOPA alone, which resulted in undetectable dopamine brain levels (Dimiou et al.., 2022). Additionally, nasal GCPQ-L-DOPA resulted in elevated L-DOPA plasma concentration compared to L-DOPA alone (17-fold higher Cmax). There are many ways to approach the problems associated with the delivery of CNS drugs. The consistent efforts of her group and others have contributed significantly to the collective optimisation and understanding of current approaches and utilisation of nanomedicine, particularly concerning nose-to-brain pathways (Mellor & Uchegbu et al., 2022).

1.8 Mechanisms linking CNS insulin and neurological disorders

Until a few decades ago, the brain was thought to be an insulin-insensitive organ. Since then, insulin's role in the brain has been a point of extensive research and has been linked to the pathology of several psychiatric and neurodegenerative disorders, including major depressive disorder and AD. Havrankova et al (1978) initially discovered that insulin receptors are widely distributed throughout the CNS of rats and shortly thereafter, expression of insulin receptors in the human brain was observed (Sara, V et al., 1982). Insulin receptors are expressed at high concentrations in the olfactory bulb, hypothalamus, and hippocampus and insulin signalling in the CNS is associated with many brain functions involved in the regulation of cognition, memory, mood, and emotional stability.

Insulin resistance is the term commonly used to describe a deficient biological response to endogenous or exogenous insulin, requiring an increased amount of insulin to trigger IR-mediated signalling than is expected under healthy physiological conditions, whether this is due to an inhibition of IR activation or insufficient insulin availability (American diabetes association, 1997). Considering the role of insulin signalling in the CNS, central insulin resistance has emerged as a characteristic of psychiatric and neurodegenerative conditions. Insulin resistance is considered a major risk factor for AD and has been shown to increase risk of AD onset by at least two-fold. Furthermore, samples of brain tissue from AD patients have been shown to display impaired insulin signalling and decreased mRNA has been measured in post-mortem brain tissue samples of AD patients, indicating a significant association between insulin resistance and neurodegeneration. Several mechanisms have been proposed to explain this association, such as the accumulation of amyloid-beta (Aβ) peptides which are suggested to contribute to defective insulin signalling. Aβ is a 4kDA peptide that commonly self-associates in aqueous medium, forming soluble aggregates or Aβ-Oligomers (AβOs) typically within the synapse, contributing to synaptic loss and the neurodegenerative events that characterise AD (Querfurth & LaFerla., 2010, Nisbet et al., 2015, Gabuzda et al., 1994,).

Aβs have been shown to cause the removal of IRs from the plasma membrane of hippocampal neurons *in vitro* and impair IR activation (Zhao et al., 2008). Additionally, *In vivo* evidence

demonstrates that insulin resistance induced in transgenic AD mice via a high fat diet results in increased levels of brain Aβ as well as enzymes such as γ-secretase that cause Aβ production. Furthermore, memory impairment as well as Aβ brain levels in mice with genetically induced AD-like neuropathology were subsequently lowered in response to a single injection of insulin (Vandal, M et al., 2014). It has been shown that increased brain insulin signalling improves memory in cognitively healthy humans, therefore loss of insulin sensitivity and receptor expression may be an underlying mechanism for the loss of memory function in AD pathology.

Insulin receptors are expressed in areas associated with mood regulation such as the nucleus accumbens, ventral tegmental area, amygdala, and raphe nuclei, where insulin signalling is associated with neurogenesis, synaptic plasticity, and neurotransmission (Figlewicz et al., 2003, Woods et al., 2016). However, the link between insulin resistance and depression is much less clear than it is for AD and even other psychiatric disorders such as schizophrenia (Leonard et al., 2020, Silva et al., 2019). A recent study showed that three surrogate measures of insulin resistance can be used to positively predict incidence of major depressive disorder. These factors include high triglyceride-HDL (high density lipoprotein) ratio, fasting plasma glucose level, and waist circumference. This study involved 601 participants (18 – 65 years old) without a lifetime history of anxiety disorders or depression and observed that moderate increases in these parameters used to measure insulin resistance correlate to an 89% increase in the incidence of major depressive disorder in a 9-year follow up period (Watson et al., 2021). Genetic evidence also suggests a significant overlap and positive genetic correlation between depression and insulin-related conditions (type 2 diabetes, obesity, and metabolic syndrome) of which insulin-resistance is a primary characteristic (Fanelli et al., 2022). Disruptions to brain insulin signalling have been shown to affect the dopaminergic mesolimbic reward system, which could thereby increase the risk of developing depression or exaggerate already present depressive symptoms (Cai et al., 2018). *In vivo* studies have shown that knockout of insulin receptors in the astrocyte of mice causes anxious and depressive behavioural symptoms, caused by altered dopaminergic and purinergic signalling. Neuronal-specific knockout of insulin receptors in mice as well as hypothalamic knockout in rats also induces these behavioural changes (Grillo et al., 2011, Kleinnridders et al., 2015).

There are currently no available treatments for conditions such as AD and major depressive disorder that aim to alleviate symptoms or address underlying neuropathology by either increasing available brain insulin or restore sensitivity. As previously discussed, insulin therapy is suggested to improve aspects of cognition such as mood and memory function and by exploiting nose-to-brain pathways, bioavailability and therapeutic activity can be enhanced. However, as it stands currently, data from clinical trials and *in vivo* studies is lacking and can be very conflicting in regard to what aspects of cognition are improved by insulin therapy and how significant the effects are. We aim to demonstrate that with the use of a highly efficient CPP-based NP delivery system, we can enhance the delivery of insulin across nasal epithelium *in vitro*, a biological barrier that significantly limits bioavailability through conventional means of delivery.

1.8.1 Psychedelic therapy for MDD

Insulin resistance is thought to be associated with neuroinflammation as an aspect of MDD pathology (Leonard et al., 2019). However, clinical trials using intranasal insulin have failed to demonstrate a long-lasting improvement of the severe symptoms that MDD patients struggle to manage, with the psychoactive therapies currently available (Cha et al., 2017). Whilst intranasal insulin shows some promise as a potential therapy, there is much greater interest in and more impressive results reported from the use of novel psychedelic therapies for the treatment of depression, particularly, 'treatment-resistant' depression (TRD). COMP360 is a novel psilocybin formulation developed by Compass Pathways for the treatment of TRD and post-traumatic stress disorder (PTSD). In phase 2 clinical trials using COMP360 to treat TRD, 233

participants received a single dose of psilocybin, either 25mg, 10mg, or 1mg. Three weeks post-dosing, 25mg, and to a lesser extent, 10mg of psilocybin improved measures of patient-reported depression severity, anxiety, affect, functioning, and quality of life as well as symptoms of depression assessed by clinicians (Goodwin et al., 2023). Following on from these very promising results, COMP360 has become the first of its kind as a psilocybin therapy to reach phase 3 clinical trials. This randomised, double-blind trial began in January of 2023, is estimated to conclude in October 2024, and will involve almost 1000 participants (ClinicalTrials.gov., 2023).

1.9 Project aims & hypotheses

Current investigations into intranasal insulin as a treatment for neurodegenerative disorders such as Alzheimer's struggle to achieve therapeutic concentrations in target brain regions, due to the presence of biological barriers such as nasal epithelium. Given the prevalence, and global burden associated with such disorders, this project aims to explore and demonstrate a nanocomplex between insulin and the CPP delivery system, GET (specifically, the PLR peptide) which can efficiently permeate nasal epithelium *in vitro* using a transwell barrier model. In summary, a combination of permeation assays, cell viability assays, and confocal imaging allowed us to demonstrate significantly enhanced permeation of insulin across RPMI 2650 human nasal epithelium over time using the GET system, without compromising cell viability or barrier integrity.

Hypothesis:

- Transport of insulin across a differentiated nasal epithelial barrier *in vitro* will be significantly enhanced by GET-mediated delivery and without loss of barrier function or toxicity.

2 Methods

2.1 Cell culture

In order to evaluate the permeation efficiency of insulin-GET compared to insulin alone, the RPMI 2650 human nasal epithelium cell line was used for all assays included in this study. RPMI 2650 cells (ATCC, UK) were cultured in complete Eagle's Minimum Essential Media (EMEM; ATCC, UK) media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine in T75 flaks and incubated at 37^{o}C and 5% CO_2. Cultures were passaged every 3 to 4 days when 70 – 90% confluency was achieved. The RPMI 2650 cell line is derived from anaplastic squamous cell carcinoma of the human nasal septum and is a widely used model of human nasal mucosa to assess the permeation efficiency and tolerability of nasally administered peptides. With the use of ELISA and immunofluorescent staining, pharmacological characterisation studies have assessed expression of four adenosine triphosphate (ATP)-binding cassette (ABC) transporters (P-glycoprotein (P-gp), multidrug resistance associated protein (MRP) 1, MRP2, as well as breast cancer resistance protein (BCRP) (Mercier et al., 2018a). Functional bioactivity of all transporters has been detected at a cellular level. The RPMI 2650 cell line is the only immortalised human nasal cell line currently available for assessing drug permeability (Bai et al., 2008). RPMI 2650 cultures proliferate and reach confluence much faster than human primary nasal cell cultures (Merkle et al., 1998). Therefore, the RPMI 2650 cell line is considered to be far superior (Kreft et al., 2015).

2.2 Insulin-GET NC formulation

FITC-labelled insulin (termed FITC-ins) (Sigma, Cat: I3661) was mixed with P21-LK15-84 (PLR) peptide (Biodiscovery Institute, University of Nottingham) (1 mM) in serum-free EMEM in polypropylene tubes. Following combination, the mixture was incubated at room-temperature for 15 minutes to generate FITC-tagged insulin-GET NCs. The final concentration of FITC-ins administered both alone and in insulin-GET NC formulations for *in vitro* testing (0.5ml total volumes throughout) was always 3.4µM (10µg, 20µg/ml). Final concentrations of PLR peptide varied from 0.017µM - 68µM (molar ratios of 200:1, 20:1, 2:1, 1:5, and 1:20) in preliminary uptake and corresponding metabolic activity assays. For studying transcytosis, metabolic activity of monolayers, and confocal imaging, final concentrations of either 1.7µM (4.72µg, 9.44 µg/ml) or 0.17µM (0.472µg, 0.944 µg/ml) PLR were used in combination with 3.4µM FITC-insulin to generate FITC-tagged insulin-GET NCs. The other PLR concentrations initially tested were excluded from later experiments due to cytotoxic effects or insignificant effects on cellular uptake. 3.4µM FITC-ins and the range of molar ratios tested were chosen based on a previous study which had successfully enhanced transcytosis of 3.4µM insulin across epithelial barriers *in vitro* and provided effective bioavailability *in vivo*, using similar PLR concentrations (Rehmani et al., 2023). Additionally, similar PLR concentrations have previously improved *in vitro* permeation across nasal cell barriers and brain penetration of oxytocin (Wong et al., 2022).

Previous work by Rehmani et al., (2023) characterised the size, morphology, and surface charge of insulin-GET NCs by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The hydrodynamic diameter and zeta potential of insulin-GET NCs were measured at pH 7.0. The average particle size of insulin-GET NCs measured by DLS was 140nm, and surface potential was measured at 28.16mV by laser doppler anemometry. TEM analysis showed that the diameter of NCs ranged between 100-350nm and 500-700nm for larger aggregates. Final concentrations of insulin and PLR in these NC formulations (0.5ml volumes) were 20µg/ml (10µg, 0.275iU) and 10µM (27.77µg, 55.53 µg/ml).

2.3 Uptake assay

Previous studies using GET to facilitate delivery of peptides such as oxytocin and insulin in epithelial cell *in vitro* indicate that significant uptake and permeation can be seen around 4-6 hours post-administration and continues to rise up to the 24-hour mark, so we based our time-course examinations around this (Rehmani et al., 2023, Wong et al., 2022). The same timepoints were included for the permeation assays.

RPMI 2650 cells (ATCC, UK) were seeded in 1ml of fully supplemented EMEM media containing 200,000 cells, per well, in a 12-well plate and left to incubate overnight to allow adhesion to the well surface. The following day, each well was washed with 500µl of phosphate buffer saline (PBS) and media was replaced with 450µl of fresh EMEM. Treatment groups for these assays included control (serum-free EMEM), FITC-ins alone, and the full range of FITC-tagged insulin-GET NCs at different molar ratios as mentioned above. Each treatment was assigned triplicate wells, and they were administered in total volumes of 50µl (diluted in serum-free EMEM) to the designated wells.

After 24 hours, samples were harvested for flow cytometry. Cells were trypsinised in 0.5ml (trypsin-EDTA, Sigma, Cat: T4049) 0.5ml complete EMEM was added before pipetting each sample into an Eppendorf tube. Samples were then centrifuged at 300G for 5 minutes before removing 850µl of media and fixing the cells in 0.5ml of 4% Paraformaldehyde (PFA) (Sigma, Cat: 158127) solution. Samples were analysed on a CytoFlex S Flow Cytometer with Kaluza software and mean fluorescent intensity (MFI) after gating was used for statistical analysis. FITC-labelled insulin was excited using the blue laser channel (488nm) and emission was

detected at 525nm. Cells harvested after 24-hour incubation with FITC-tagged insulin-GET NCs of a 200:1 molar ratio (3 4µM insulin, 0.017µM PLR) showed no significant improvement in uptake compared to insulin alone and were subsequently excluded from future studies. Parallel cell viability assays (see **section 2.5**) highlighted that high concentrations of PLR (17µM and 68µM specifically) significantly compromised cell viability whereas low concentrations did not, and so were also excluded from future permeation studies. FITC-tagged Insulin-GET NCs of a 20:1 molar ratio (3 4µM insulin, 0.17µM PLR) significantly improved uptake compared to insulin alone and had an insignificant effect on cell viability. Subsequently, uptake assays were repeated for insulin-GET 20:1 and samples were harvested at 1, 4, and 6 hours.

Although fixing cells in PFA can weaken fluorescent signal intensity, it's very helpful for time-course assays because cells can be fixed at specific time points to preserve their structure (Schmid et al., 1999). Samples can typically be stored for up to 1 week at $4^{O}C$, allowing them to be analysed all together when flow cytometry equipment becomes available. Contrastingly, live cells must be analysed in a narrow time frame to avoid cell death (Tang et al., 2021).

2.3.1 Confocal imaging

Cellular uptake and localisation of FITC-tagged insulin-GET NCs was examined using confocal laser scanning microscopy (CLSM). RPMI 2650 cells were seeded on glass coverslips that fit a 12-well plate, at 200,000 cells/per well before and treated with labelled insulin or Ins-GET NCs for 1, 4, and 6 hours. Cells were washed with PBS, fixed with PFA (4% in PBS) at RT for 10 minutes, washed with PBS, permeabilised with 0.1ml of 0.1% Triton x-100 at RT for 12 minutes, and washed again before adding one drop of Fluoroshield mounting medium with DAPI (4′,6-diamidino-2-phenylindole) fluorescent stain (Sigma-Aldrich, item no. F6057) to each sample. DAPI is a blue-fluorescent stain used as a nuclear counterstain for the FITC-labelled treatment (FITC-insulin alone and FITC-tagged insulin-GET), since there is very little overlap between DAPI and green-fluorescent fluorophores (e.g., FITC). Stained samples were visually examined by CLSM (Zeiss LSM 880) with a 10x water immersion lens. DAPI and FITC were imaged using 405nm and 488nm laser lines, respectively. Emission was recorded between 505 – 585nm nm and 426 – 491nm. Frame size was set at 1024 x 1024, and images were acquired sequentially. Laser power and gain were kept constant across all samples imaged. Potential drawbacks of the method applied and issues regarding the quality of images collected is discussed later (see **section 4.5.3**).

2.4 Transwell assays

Air-liquid conditions allow RPMI 2650 cells to form confluent monolayers which express tight junctions and transporter proteins as well as transepithelial electrical resistance (TEER) values comparable to human nasal mucosa (Mercier et al., 2018a). TEER is a technique that allows quantification of tight junction integrity, an important aspect of any cellular barrier model (Srinivasan et al., 2015).

2.4.1 Permeation assay

RPMI 2650 cells were seeded at $2x10^5$ cells/well (in complete EMEM; 500µl apical/top chamber, 1ml basal/bottom) in transwells that fit a 12-well plate (Greiner Bio-one polystyrene cell culture inserts; Item no. 665641, 13.85 mm diameter transwell inserts with 0.4µm pore size) and grown under liquid covered culture (LCC) for 8 days, before shifting to air-liquid interface (ALI) by removing media in the apical chamber, until day 21 when permeability or cytotoxicity was assessed. Each well was aspirated, washed with 500µl PBS twice, and 450µl of EMEM replaced. FITC-tagged Insulin-GET NCs were generated at molar ratios of 2:1 and 20:1 (3.4µM insulin + 1.7 and 0.17µM PLR respectively) as detailed in **section 2.2**. The 21-day growth and

maturation period is necessary to allow confluent and well-differentiated monolayers of RPMI 2650 cells to form (Mercier et al., 2018b).

The prepared insulin-GET treatments (including FITC-insulin alone) were then administered (50µl) to the apical chamber (500µl total volume) and incubated under standard conditions. Media was collected from the basal chamber (50µl) in triplicate at 1, 2, 4, 6, and 24 hours and the volume removed (150µl) replaced with fresh EMEM. After the samples at 24 hours were collected, the apical chamber was aspirated, washed twice with PBS, and replaced with 500µl fresh EMEM. Samples were then taken from the basal and apical compartments at a further 1, 2, 4, 6, and 24 hours and volume removed replaced with fresh EMEM, to assess bidirectional cumulative transport of FITC-ins alone and FITC-tagged insulin-GET NCs. Samples were taken to a plate reader (Tecan infinite 200PRO multimode microplate reader) and fluorescence measured in a black, 96 well plate (Costar flat-bottom microplate; Item no. 10695951) using excitation (Ex)/emission (Em) of 490nm/520nm for detection of FITC-labelling to determine percentage drug transport/transcytosis and permeability co-efficient (P_{app}).

In order to determine % transport from fluorescence values, a standard curve was established. A series of standard solutions with known concentrations of FITC-ins were prepared, ranging from 3.4 (100%) – 0.017µM (0.5%). All solutions were prepared in total volumes of 0.5ml to keep experimental conditions identical to the conditions of the permeation assay. Samples were taken from the range of standard solutions to a plate reader and FITC fluorescence was measured in a black, 96 well plate using an Ex/Em of 490nm/520nm. Fluorescence values were then plotted against the known FITC-ins concentrations (µM). After the permeation assays were performed, the standard curve was used to interpolate the FITC-ins concentration in the unknown samples based on their fluorescence values. Cumulative values were derived from adding each time point's concentration to the total concentration derived from previous time points. Cumulative concentration values were then used to determine what % of the 3.4µM FITC-ins initially administered to the apical chamber (either alone or in the presence of PLR) had successfully transported into the basal and apical chambers. Cumulative concentration values were also used to determine the permeability rate (mmol/sec) of FITC-insulin alone and FITC-tagged insulin-GET NCs across RPMI 2650 monolayers.

The apparent permeability co-efficient which quantifies the rate at which a molecule crosses a membrane was calculated using the following equation (Gao et al., 2001):

$$P_{app} = dQ/dt \times C_0 \times A$$

Where dQ/dt = permeability rate of compound across the barrier (mmol/sec), C_0 = initial concentration of compound in apical compartment (mmol/ml), and A = surface area of the cell monolayer (cm^2).

2.4.2 Assessment of TEER

Using electrodes, the voltohmmeter applies a brief AC electrical signal to either side of the transwell insert, and resistance values (ohm, Ω) are measured based on Ohm's law and displayed. Measurements were taken every 2 – 4 days, apical and basal chambers were replaced with 0.5ml and 1ml of fresh EMEM, and values were taken 5 minutes later using the EVOM2TM epithelial voltohmeter (World Precision Instruments, Sarasota, Florida, USA). Transwell inserts with no cells seeded were used as blanks and their values deducted from Ω measurements. TEER values of human nasal mucosa typically range between 60 and 180 Ω/cm2 (Mercier et al., 2018a) and so, permeability was only assessed using monolayers with TEER values above 60 Ω/cm2.

2.5 Prestoblue cell viability assay

To assess potential cytotoxic effects of FITC-tagged insulin-GET on RPMI 2650 cultures, cell viability assays were run in parallel to both the uptake and transwell permeation assays respectively, using the PrestoBlue Cell Viability Reagent (Thermo Fisher, UK). This reagent contains resazurin, which is converted by metabolically active, viable cells, into fluorescent resorufin (Lall et al., 2013). Therefore, the intensity of the fluorescent signal emitted is directly proportional to the quantity of viable and metabolically active cells. The inclusion of an untreated control group representing 100% metabolic activity allows cytotoxic effects of a treatment to be determined by percentage decreases relative to the control. PrestoBlue assays are very easy to prepare, compatible with a large variety of cell lines (including epithelial) and can be used for short or long-term incubation periods (Wong et al., 2022). The information these assays provide is very useful in the early stages of *in vitro* drug development.

The first cell viability assays were conducted to assess potential cytotoxic effects of FITC-tagged insulin-GET NCs on RPMI 2650 cells, made up at the full range of molar ratios as described in **Section 2.2.** Cells were seeded at 25,000 cells/well in a 96 well plate before treatments were added in quantities of 12.5µl per well. After incubating at 37^{o}C in a 5% humidified CO2 incubator for 24 hours, wells were aspirated and washed with 0.2ml PBS, and replaced with PrestoBlue Cell Viability Reagent (Thermo Fisher, UK) diluted 10-fold in HBSS. This process was repeated for 1, 4-, and 6-hour incubation with FITC-tagged insulin-GET NCs at a 20:1 molar ratio. Therefore, we were able to replicate all the timepoints and treatments used to assess cellular uptake, to produce parallel cell viability data.

Cell viability assays were repeated, this time to replicate the conditions of the transwell permeation assay. The same process was followed as described above (see **sections 2.4.1** and **2.4.2**) to cultivate RPMI 2650 monolayers, assess TEER, generate FITC-tagged insulin-GET NCs at 2:1 and 20:1 molar ratios, and administer them to monolayers. On day 21, wells were aspirated and washed with 0.5ml PBS twice and replaced with 450µl EMEM before adding treatments to the apical chamber of their assigned wells in triplicate. After incubating for 1, 2, 4, 6, and 24 hours respectively, FITC-tagged insulin-GET NCs were removed from the apical chamber and replaced with PrestoBlue Cell Viability Reagent diluted 10-fold in HBSS. In all cases, cells were incubated again for 60 minutes and 50µl samples were pipetted into a 96 well black walled plate in triplicate. Fluorescence was then measured on a Tecan fluorescence plate reader using Ex/Em of 560nm and 590nm and results expressed as percentage of control.

2.6 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 9. Normal distribution of data was tested using D'Agostino-Pearson, Anderson-Darling, Shaprio-Wilk, and Kolmogorov-Smirnov tests. Data from the uptake assay measured after 24-hours failed normality tests and so a log transform was performed, normalising the distribution. A one-way analysis of variance (ANOVA) with the Tukey's multiple comparisons post-hoc test determined statistical significance by comparing every group to each other. One-way ANOVA analysed data from the initial cell viability assays at all timepoints, Tukey's was used to determine significant effect of treatments by comparing between treatments and control at each timepoint. Data from repeated uptake assays (1, 4, and 6-hours) was log transformed and analysed with two-way ANOVA without repeated measures and Tukey's post-hoc was used to determine significant effect of treatments by comparing all treatment groups at each timepoint. Two-way ANOVA was also used to analyse transwell permeability and viability assay data. Overall effect of time, treatment and their interaction were measured. Repeated measures were used to analyse permeability data and Tukey's determined significant effects of treatments by comparing every group at each timepoint. Transwell viability data was not analysed with repeated measures and Tukey's test

determined no significant effect of treatments compared to control. All data are represented as mean ± SEM. P<0.05 was considered statistically significant.

3 Results

3.1 Cellular uptake and cytotoxicity studies

We began by using flow cytometry to assess whether uptake of FITC-labelled insulin into RPMI 2650 cultures could be enhanced by conjugation with GET, at a range of concentrations based on previous *in vitro* and *in vivo* demonstrations of GET-mediated enhanced delivery of insulin using similar concentrations. After a 24-hour incubation period, there was a main effect of treatment on mean fluorescence intensity (MFI) ($F_{(6, 14)} = 37.85$, $P < 0.0001$). Although uptake of FITC-labelled insulin did occur in the absence of GET (P<0.01 versus control), the extent of uptake was significantly enhanced by GET at concentrations of 0.17µM and above ($P < 0.05$ – 0.0001 versus insulin alone) and so 0.017µM PLR was excluded from future work. Effective molar ratios of insulin-GET in this assay therefore range from 20:1 to 1:20 (**Figure 7A)**. Prestoblue assays using the same treatment groups and incubation period (24-hour) revealed that high PLR concentrations (17µM and 68µM) severely compromised the metabolic activity of RPMI 2650 cultures and were therefore excluded from future experiments. Additionally, 1.7µM PLR was also excluded due to reducing metabolic activity of cultures, although this effect was not statistically significant. However, 0.17µM PLR significantly enhanced uptake of FITC-insulin after 24-hours by **10-fold** compared to FITC-insulin alone and had no effect on metabolic activity (**Figure 7A – B**). Uptake assays were then repeated using this concentration of PLR and cells were harvested for analysis by flow cytometry after 1, 4, and 6-hour incubation periods, to gain more insight into the temporal effects of GET. This revealed that by 6 hours, uptake is significantly improved by 0.17µM PLR compared to insulin alone. PrestoBlue assays were repeated to include 1,4- and 6-hour timepoints, metabolic activity was not significantly affected.

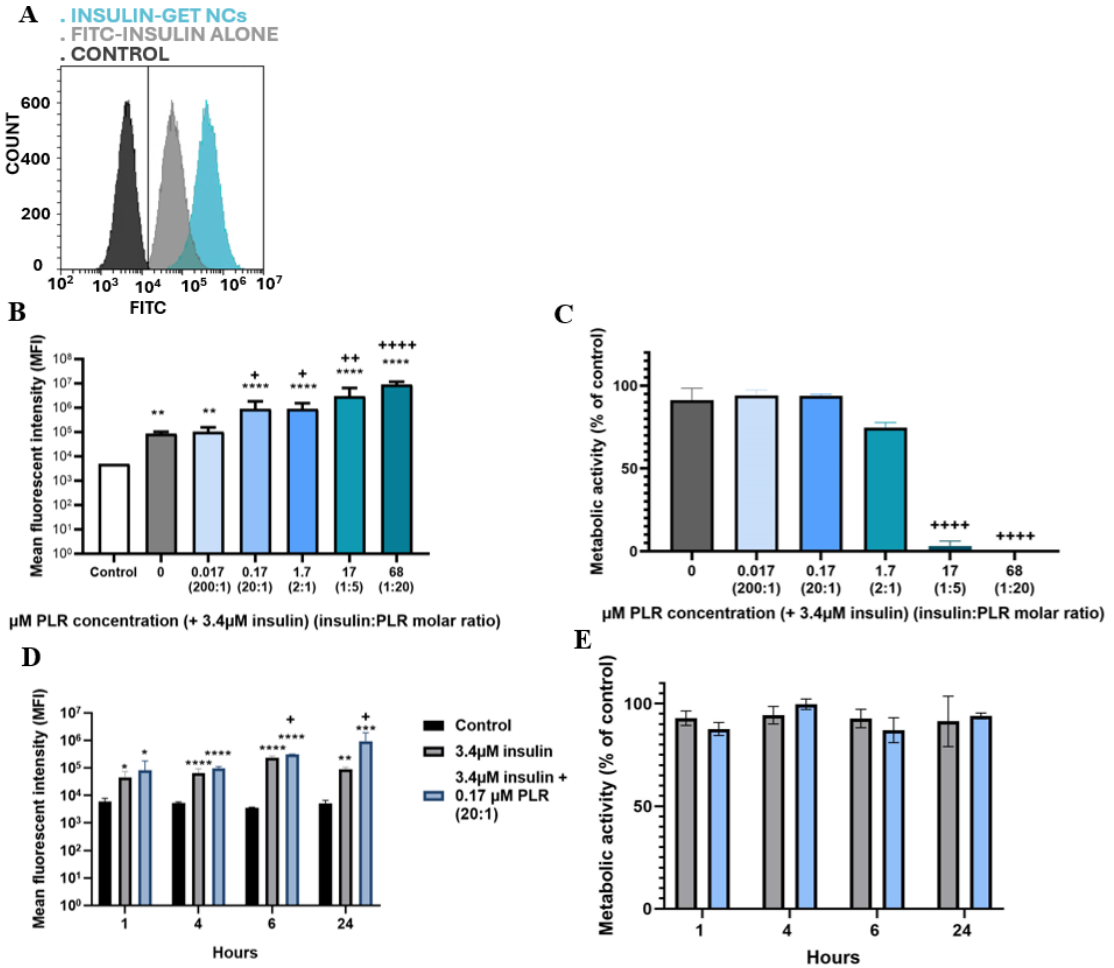

Figure 7. GET increases uptake of FITC-labelled insulin into RPMI 2650 nasal epithelial cells, at concentrations that do not reduce cell viability.
(A) Flow cytometric histogram analysis demonstrating GET enhances cellular internalization of FITC-insulin (B) cellular uptake measured by flow cytometry detection of FITC-insulin and (C) cell viability after 24-hour incubation with FITC-insulin alone (3.4µM) or in the presence of PLR (0.017 - 68µM, insulin-GET molar ratios of 200:1 – 1:20), as well as more detailed temporal examination of (D) uptake and (E) viability comparing insulin alone and in the presence of a single effective PLR concentration (0.17µM PLR, insulin-GET molar ratio of 20:1). Concentrations displayed (µM) are the final concentrations of reagents. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus control and +P<0.05, ++P<0.01, +++P<0.001, ++++P<0.0001 versus FITC-insulin alone; one-way (B-C) or two-way (D-E) ANOVA followed by Tukey's multiple comparisons post-hoc test. Data are mean ± S.D. from n = 3 independent replicates and 3 technical replicates per treatment group.

0.17 - 68µM PLR significantly improved cellular uptake of FITC-insulin over a 24-hour period (**Figure 7B**), however concentrations above 0.17µM compromised cell viability (**Figure 7C**) (in the case of 1.7µM this effect was just shy of statistical significance, P = 0.0594). Based on these results and the ability of 0.17µM PLR to significantly improve cellular uptake of insulin compared to insulin alone at 6-hours (**Figure 7D**), without affecting viability (**Figure 7E**), we chose this concentration for progression to transwell permeation assays. We also included 1.7µM GET to see if this concentration would have a greater or lesser effect on insulin permeation compared to 0.17µM.

3.1.1 Confocal imaging

Confocal microscopy using FITC-ins and nuclear staining (DAPI) displays a distinct difference in relative insulin delivery with and without GET. The same concentrations of PLR as used in the permeation study (0.17µM and 1.7µM) improved cellular delivery and internalisation of insulin over time. Prior to staining with DAPI, RPMI 2650 cultures were fixed with 4% PFA and permeabilized with 0.1% Triton x-100, which allows DAPI to enter the cells and bind DNA. PFA forms covalent cross links between molecules, thereby preventing the decay of cells due to processes such as autolysis and preserving their biochemical and physical properties (Kim et al., 2017). DAPI inefficiently permeates through intact cell membranes and is therefore preferred for staining fixed/dead cells. However, poor staining with DAPI and the use of 10x zoom makes some images unclear. Potential explanations for poor DAPI staining and the quality of images collected is discussed in detail later (see **section 4.5.3).** The images collected provide qualitative evidence of GET-mediated enhanced cellular delivery of insulin which was previously quantified using flow cytometry.

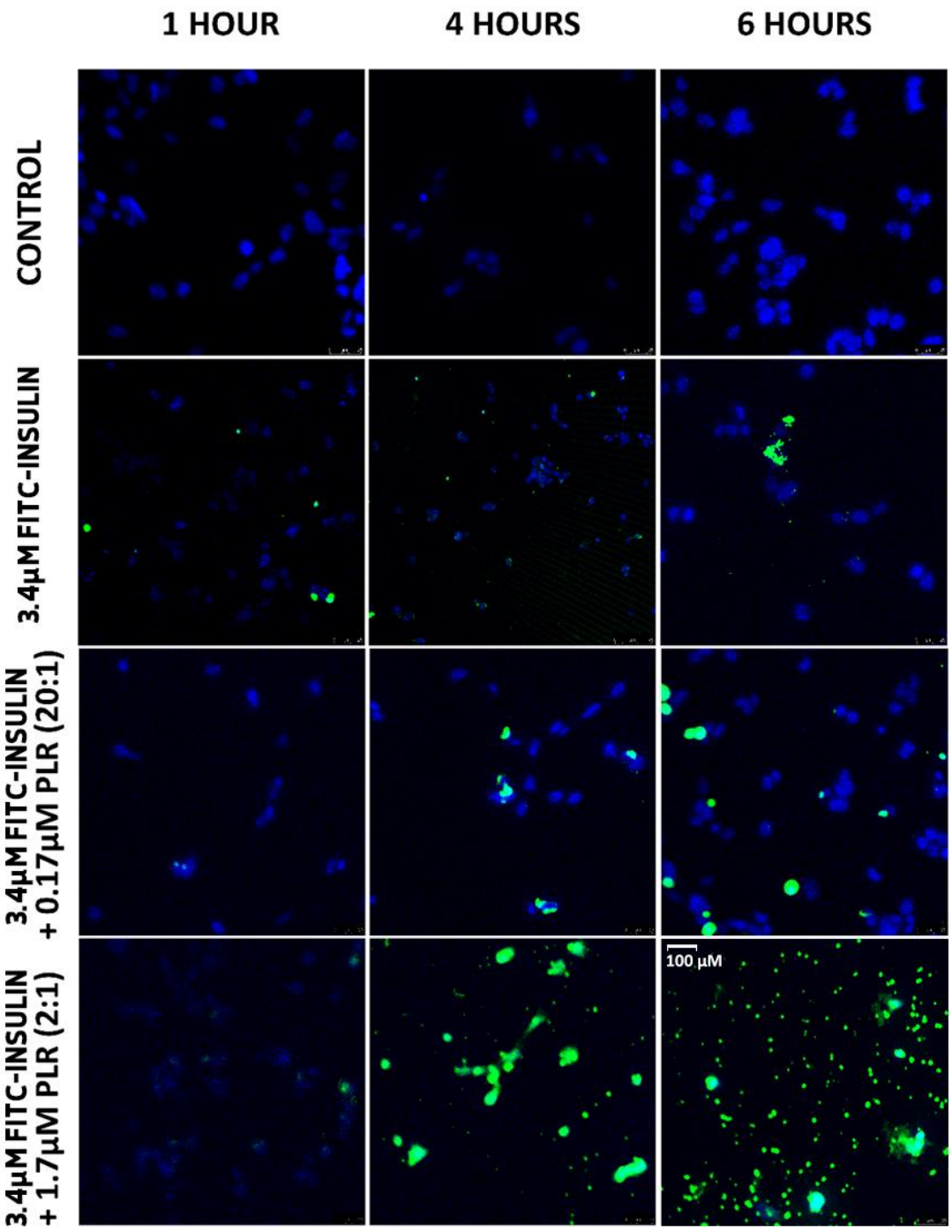

Figure 8. GET-mediated enhanced cellular insulin delivery using RPMI 2650 cultures. Confocal microscopy of FITC-insulin (green-fluorescent peptide) administered as 3.4µM FITC-insulin alone as well as 3.4µM FITC-insulin + 0.17 and 1.7µM PLR (final reagent concentrations) respectively, counter-stained with 4′,6-diamidino-2-phenylindole (DAPI, blue-fluorescent DNA dye) to indicate presence of cell nuclei. Controls were treated with serum-free EMEM. Images were taken using a Zeiss LSM 880 confocal laser scanning microscope (Zeiss LSM 880 META, Germany) and Zen Black 2012 Imaging Software. Laser emission of 405nm and 488nm were used to visualise DAPI and FITC respectively with magnificent of 10X water immersion lens, n = 1.

3.2 Permeation studies

3.2.1 TEER measurements

RPMI 2650 monolayers were cultivated on transwell inserts at ALI to form *in vitro* nasal barrier models for assessing the permeation of (FITC-tagged) insulin alone and insulin:GET NCs. ALI allows RPMI 2650 cells to differentiate and express transporter proteins as well as tight junctions, the integrity of which can be quantified by transepithelial electrical resistance (TEER) values. Measurements were taken every 2 – 4 days using a voltohmeter and cultures were switched from liquid-culture conditions to ALI on day 8.

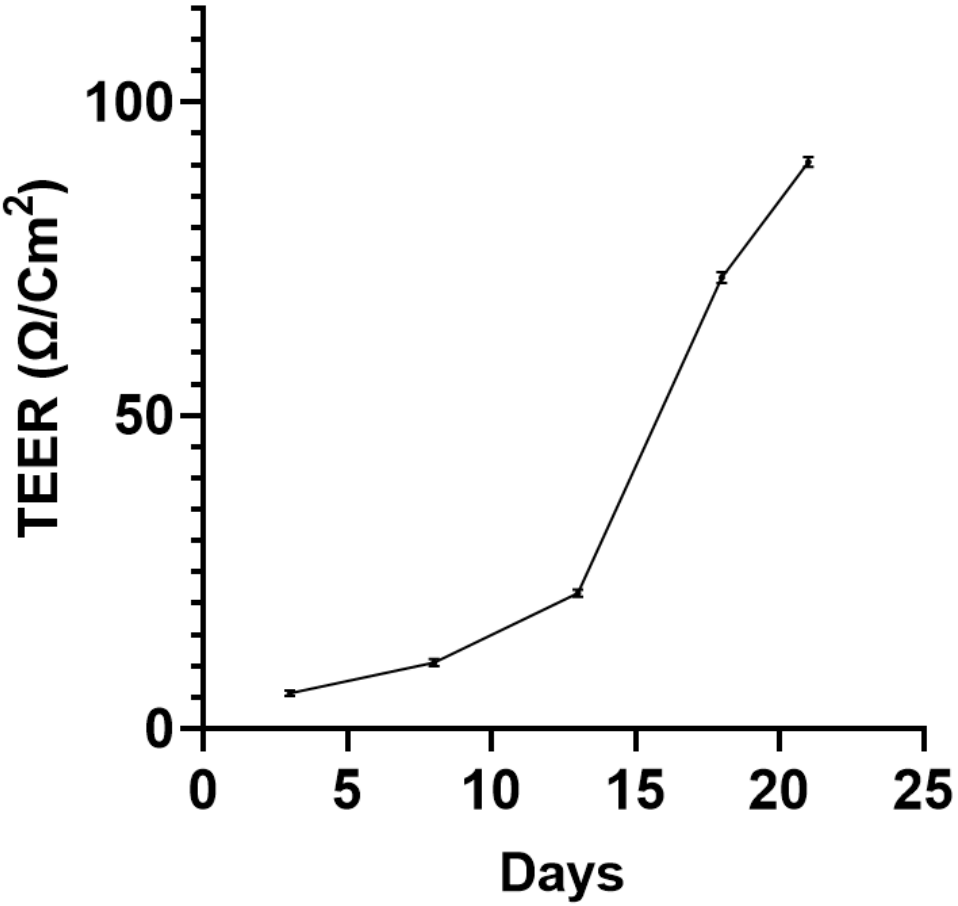

Figure 9. TEER values of RPMI 2650 monolayers.
TEER values of RPMI 2650 cells cultured under ALI conditions from day 8 onwards (EMEM + 10% FCS) was measured with a voltohmmeter every 2 – 4 days over the course of 21 days. From day 17 onwards TEER values rose above 60 Ω/cm2 making them viable for assessing permeation and cytotoxicity. Results are mean ± S.D. values from n = 3 independent replicates.

3.2.2 Insulin-GET NCs enhance permeability across nasal cells models in vitro without affecting barrier integrity

We've demonstrated that particular final concentrations of the PLR peptide (e.g., 0.17μM) were able to significantly improve cellular internalization of FITC-ins by RPMI 2650 cultures without compromising metabolic activity. By growing RPMI 2650 cultures under ALI conditions on transwells, *in vitro* nasal barrier models were cultivated. These barrier models

better resemble the physiology of human nasal epithelium (e.g., transporter protein expression) and integrity could be validated using TEER measurements (Bai et al., 2007, Wan et al., 2000, Srinivasan et al., 2015). Therefore, providing a suitable model to assess GET-mediated permeation of FITC-ins across nasal epithelium *in vitro*. FITC-ins and FITC-tagged insulin-GET NCs were administered to the apical chamber of transwells and samples were then collected from the basal chamber after 1- , 2-, 4-, 6-, and 24-hours for fluorescence analysis by a plate reader. Fluorescence data was analysed to measure cumulative apical to basal transport (%) and determine apparent permeability coefficients (P_{app}) (see section 2.4.1). 100% Cumulative apical to basal transport is defined by complete transport of the 3.4µM (10µg, 20µg/ml) FITC-ins initially administered to the apical chamber, to the basal chamber. Volumes in the apical and basal chambers were kept the same (0.5ml). We found that at each timepoint considered (1 – 24 hours), FITC-ins apical to basal transport across RPMI 2650 monolayers was significantly improved by both 0.17 and 1.7µM PLR (final concentrations) compared to FITC-ins alone. 24 hours post-administration, the cumulative transport of FITC-ins mediated by 0.17 and 1.7µM PLR were 8-fold and 11-fold greater than that of FITC-ins alone.

Furthermore, although CPPs that utilise endocytotic pathways are generally regarded as safe, PrestoBlue viability assays and permeability assays were carried out on differentiated RPMI 2650 monolayers in parallel under identical experimental conditions (e.g., same exact treatments and timepoints) (Trabulo et al., 2010). PrestoBlue assays confirmed that neither of these PLR concentrations (+ FITC-ins) or FITC-ins alone at any timepoint compromised the metabolic integrity of our *in vitro* barrier models. If our GET-mediated delivery of FITC-tagged insulin was to compromise barrier integrity and damage the monolayers, this would allow the FITC-tagged insulin-GET NCs to collect in the basal chamber, thereby leading to false positive data. Barrier integrity is a very important aspect of permeation assays for this reason and information on cytotoxicity is crucial to every step of drug development (Saar et al., 2005).

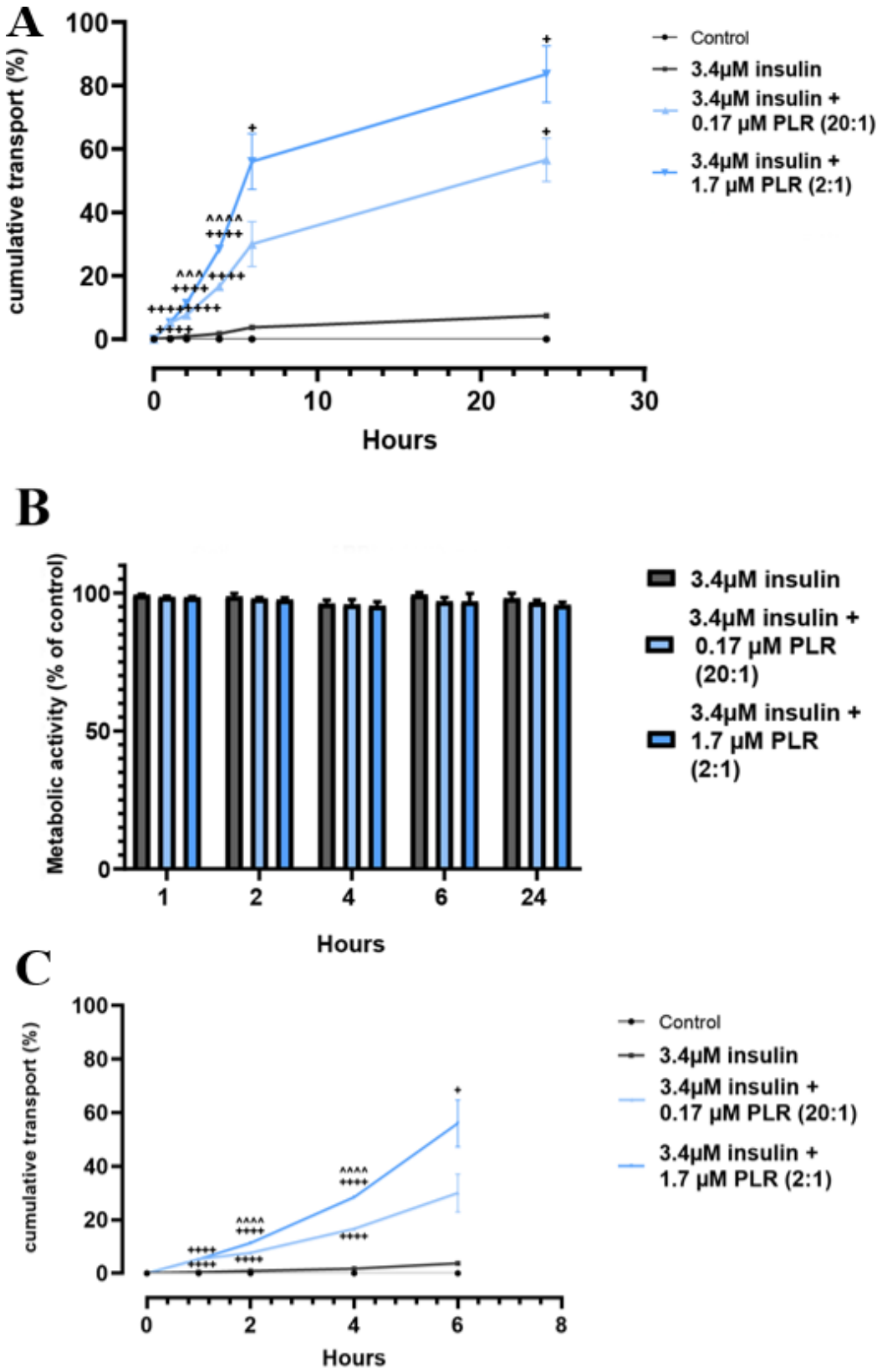

Figure 10. GET enhances apical to basal permeation of insulin across RPMI 2650 human nasal epithelium monolayers without compromising barrier viability.
1.7 and 0.17µM PLR enhanced FITC-insulin (3.4µM) transcytosis across differentiated RPMI 2650 monolayers (apical to basal) by as much as 11x and 8x compared to FITC-insulin alone respectively (A) after 24-hours exposure, without affecting cell viability (B). No significant

difference was found between treatment groups at any timepoint. (C) Earlier timepoints from permeation assay displayed separately to appear more visible. Concentrations displayed (µM) are the final concentrations of reagents in apical chambers. + P<0.05, ++++ P<0.0005 versus FITC-insulin alone and ^^^P<0.001, ^^^^P<0.0001 versus FITC-insulin + 0.17µM PLR. Based on Two-way ANOVA with Tukey's multiple comparisons test. Data are mean ± S.D. from n = 3 independent replicates and 3 technical replicates per treatment group.

Apical to basal transport of FITC-ins alone and FITC-tagged insulin-GET NCs across RPMI 2650 monolayers was assessed over 24 hours. From this permeation assay, P_{app} values (10^{-6} cm/s) were calculated at 24-hours post-administration (see **section 2.4.1**) to determine the rate at which FITC-ins and FITC-tagged insulin-GET NCs crossed RPMI 2650 monolayers. There was a main effect of GET on the P_{app} values calculated ((F(2, 6) = 426.8 , P<0.0001; one-way ANOVA; **figure 11**). Both 0.17 and 1.7µM PLR significantly improve the permeation rate of FITC-ins across RPMI 2650 monolayers (P<0.001 and P<0.0001 respectively). Furthermore, FITC-tagged insulin-GET NCs with a molar ratio of 2:1 had a significantly greater permeability rate compared to NCs with a molar ratio of 20:1 (*P*<0.0001). Both the cumulative transport data and P_{app} values demonstrate that GET-mediated delivery of FITC-ins is significantly more effective than FITC-ins alone and is even a more efficient delivery system compared to previous demonstrations of NP-based insulin delivery (Akel et al., 2021).

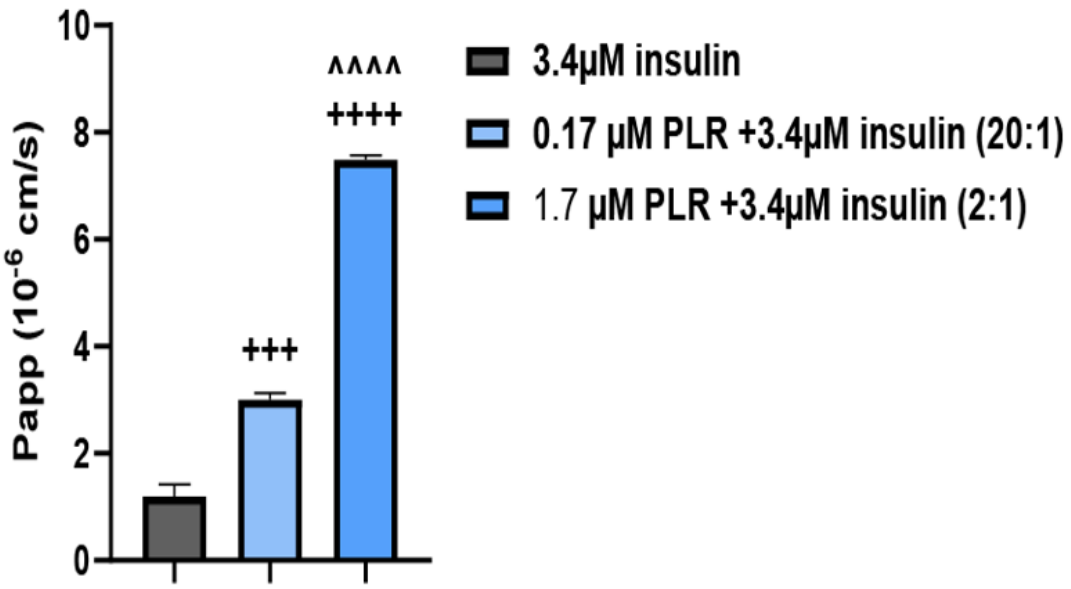

Figure 11. Permeability rate of insulin and insulin-GET nanocomplexes.
P_{app} of FITC-insulin delivered alone (3.4µM) or in the presence of PLR (0.17µM or 1.7µM) across differentiated RPMI 2650 monolayers calculated after 24-hours. Concentrations displayed (µM) are the final concentrations of reagents used. One-way ANOVA revealed a main effect of insulin-GET treatment (F(2, 6) = 426.8 , P<0.0001), +++P<0.001, ++++P<0.0001 versus FITC-insulin alone ^^^^P<0.0001 versus FITC-insulin + 0.17µM PLR; Tukey's post-hoc. Data are mean ± S.D from n = 3 independent replicates and 3 technical replicates per treatment group.

3.2.3 GET enhances bidirectional permeation of insulin post-delivery

Apical to basal permeation of FITC-ins and FITC-tagged insulin-GET NCs was initially assessed for 24 hours (see **figure 10A**). This figure shows that after 24 hours, the initial concentration of FITC-ins administered to the apical chamber (3.4µM) has not completely transported to the basal chamber. We can assume this remaining FITC-ins and FITC-tagged insulin-GET NCs either hasn't permeated into the monolayer, has permeated but is retained by the 24-hour mark or transported back into the apical chamber by drug efflux transporter proteins. Therefore, apical to basal transport was assessed for a further 24 hours (up to 48-hours post-delivery). During this time period, samples from apical chambers were also assessed (1-, 2-, 4-, 6-, and 24-hour timepoints) to determine the extent of basal to apical transport following on from the initial apical to basal permeation assay. We've demonstrated below that apical to basal transport of FITC-tagged insulin-GET NCs persists up to 48-hours post-delivery. To a lesser extent, these NCs also accumulate in the apical chamber, indicating bidirectionally.

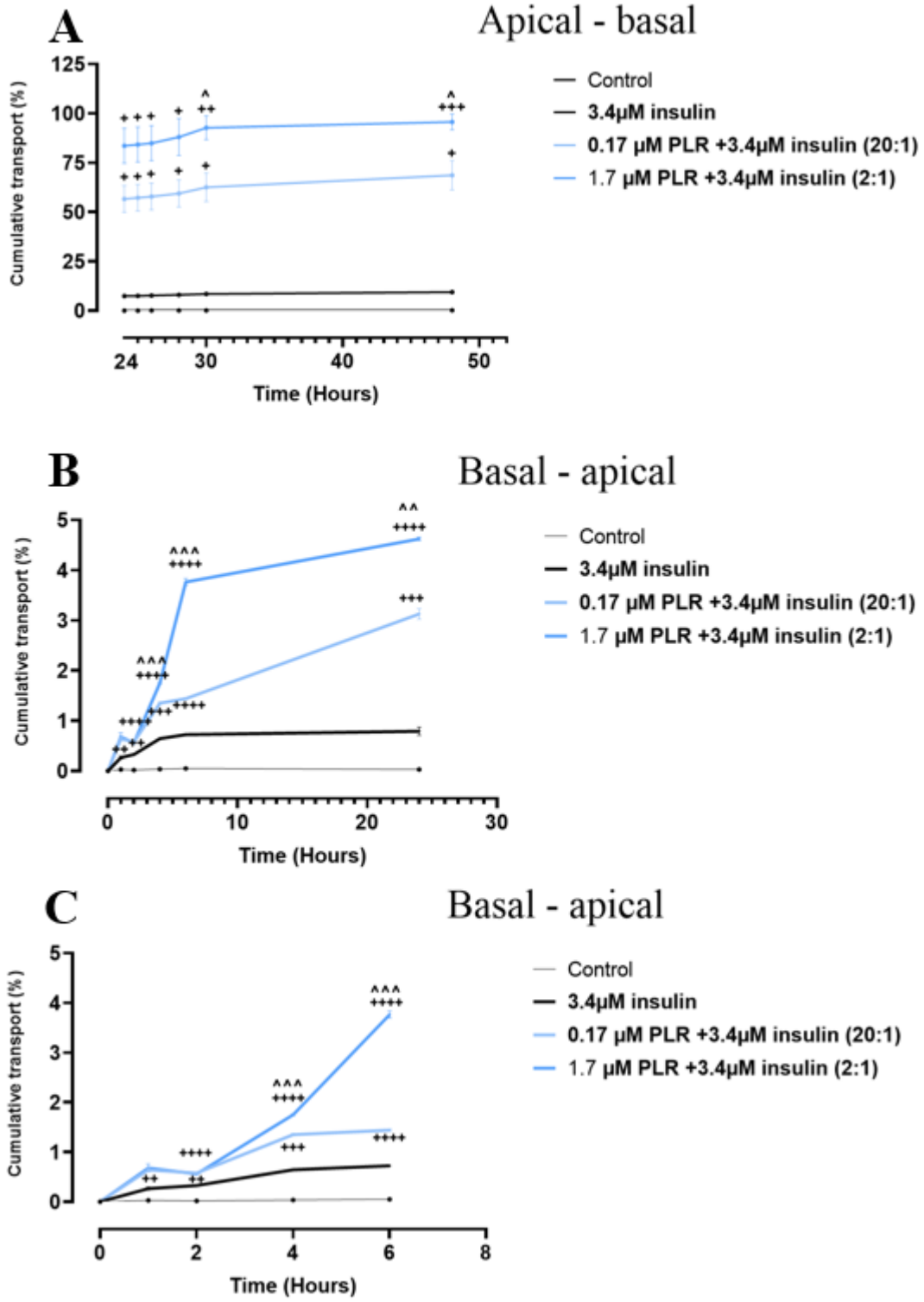

Figure 12. GET facilitates bi-directional release of insulin into apical and basal chambers.

Following the 24-hour period of the initial apical – basal permeation study (See **Figure 9A**), transport of FITC-insulin alone (3.4μM) or in the presence of PLR (0.17 or 1.7μM) into basal chambers was assessed for a further 24 hours (A) (up to 48-hours post-administration). Basal to apical transport was also assessed for 24 hours (B). (C) Earlier timepoints of basal – apical transport displayed separately to enhance visibility. Concentrations displayed (μM) are the final concentrations of reagents. ++ P<0.01, +++ P<0.001, ++++ P<0.0001 compared to FITC-insulin alone and ^P<0.05, ^^P<0.01 ^^^P<0.001 compared to FITC-insulin + 0.17μM

PLR. Based on Two-way ANOVA with Tukey's post-hoc. Data are mean ± S.D. from n = 3 independent replicates and 3 technical replicates per treatment group.

4 Discussion

4.1 Key Findings

Through a combination of assays, we have been able to observe and quantify a clear GET-mediated enhancement of cellular insulin internalisation and permeation across a differentiated RPMI 2650 transwell barrier. We have determined that low concentrations of PLR (0.17 μM, 1.7μM) most effectively facilitate the transcytosis of FITC-ins (3.4 μM) across an *in vitro* nasal barrier with no significant effects on barrier integrity or monolayer viability, in contrast to the higher concentrations tested. These observations support my hypothesis (see **Section 1.9)**.

4.2 GET improves insulin uptake in RPMI 2650 cultures

GET has previously been demonstrated to efficiently improve transcytosis of peptide cargoes such as insulin and oxytocin, across differentiated intestinal and nasal epithelial monolayers respectively *in vitro* (Rehmani et al., 2023, Wong et al., 2022). We have presented both quantitative and qualitative data that support GET-mediated enhanced cellular internalisation of insulin by RPMI 2650 cells. Firstly, data measured by flow cytometry and presented in **figure 7B** clearly shows that GET significantly increases the accumulation of insulin compared to insulin alone over time, up to 24-hours after administration. This was further investigated using confocal imaging to assess cellular uptake of insulin by RPMI 2650 cultures treated with FITC-tagged insulin-GET NCs and FITC-ins alone, presented in **Figure 8**. The data from both confocal imaging and flow cytometry demonstrate that GET-mediated uptake of insulin is most significantly improved between **6** and **24 hours** after administration. We recognise that the DAPI staining, and resolution used to image cultures treated with insulin-PLR NCs inhibit our ability to fully assess the interaction between cells and the NCs, and the extent of NC internalisation. Although not very clear, these images do demonstrate aggregation of NCs interacting with cell membranes and some level of internalisation. We believe that the combination of these findings sufficiently demonstrates GET-mediated enhanced delivery of insulin.

4.3 GET enhances insulin transduction without compromising viability or integrity of nasal barriers

For effective nose-to-brain delivery, cellular uptake by nasal epithelium is not sufficient, effective delivery into brain structures requires permeation across the nasal barrier for subsequent delivery via trigeminal and olfactory nerve pathways. Using a transwell barrier model with differentiated RPMI 2650 monolayers, insulin-GET 20:1 and 2:1 enhanced insulin delivery across the barrier with significantly higher insulin P_{app} values and cumulative insulin concentration in the basal chamber after 24 and 48 hours compared to insulin alone. Additionally, we have shown that GET-mediated enhanced permeation occurs bidirectionally. Cumulative insulin concentration in the apical chamber 48-hours post administration was significantly enhanced by insulin-PLR 20:1 and 2:1 compared to insulin alone.

It has been demonstrated that the uptake of insulin-GET NCs by epithelial barrier cells most likely occurs via macropinocytosis (Rehmani et al., 2023). GET has been shown to facilitate cell entry via heparan sulphate-membrane binding, promoting macropinocytosis and uptake of NCs into vesicles, allowing transcytosis to occur. The GAG-binding motif P21 which improves cell surface binding and the octa-arginine 8R CPP domain which promotes endocytosis are very

important for this uptake/export transcytosis mechanism. Additionally, the LK15 domain facilitates endosomal escape, thereby improving transcytosis compared to insulin alone.

We have shown that high concentrations of GET have significant adverse effects on cell viability (see **Figure 7C**) and were therefore excluded from the permeation study. If exposure to FITC-tagged insulin-GET NCs damaged barrier integrity causing them to become leaky, this would falsely indicate transcytosis occurring. For this reason, it is crucial for us to confirm that loss of barrier integrity is not responsible for enhanced insulin permeation and that this was in fact a GET-mediated effect. TEER values were measured frequently prior to permeation studies (~90 ± 0.85 Ω/cm2), confirming appropriate membrane tight-junction integrity for a valid nasal barrier model (Mercier et al., 2018b). Resazurin-based PrestoBlue assays (see **Figure 10B**) were used to assess any effect on monolayer viability upon exposure to FITC-tagged insulin-GET NCs. Insulin alone as well as insulin-GET 20:1 and 2:1 had no significant metabolic effect, confirming that the GET system does not mediate insulin transport through cytotoxic effects and effectively enhances insulin transport without compromising barrier viability.

The P_{app} value measured for insulin is similar to the value reported previously by Akel et al., (2021), who also investigated the use of a NP insulin formulation to improve delivery across a RPMI 2650 nasal barrier model. This study utilised uncoated and chitosan-coated insulin-loaded solid lipid nanoparticles (SLNs) and poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs). The P_{app} value reported for our insulin-GET 2:1 NCs exceeds those reported for all four of their formulations, indicating more efficient transcytosis.

Insulin-GET NCs have previously been shown to permeate across epithelial barriers through transcytosis by analysis of insulin-GET treated epithelial cultures with flow cytometry and microscopy. These findings show that GET-mediated insulin uptake and exocytosis occurs mainly through intracellular trafficking, ruling out a significant role of paracellular routes in the transport of insulin (Rehmani et al., 2023). We've demonstrated that compared to conventional CPP-based delivery systems that rely on high extracellular concentrations to facilitate transduction, low GET concentrations significantly enhance transcytosis *in vitro*, which may reduce the risk of adverse effects (Dixon et al., 2016). Evidence suggests that GET progressively saturates cells with endosomal insulin depots, thereby inhibiting further accumulation of subsequent insulin which is instead exported via transcytosis. By allowing cells to function as depots, GET-mediated delivery appears to be an effective method of sustained release and chronic delivery of insulin. Post-transcytosis, insulin-GET NCs have been shown to stay intact and when administered again on to fresh monolayers, they even retain biological activity. Since the electrostatic interaction between GET and insulin remains stable post-transcytosis, NCs may be able to successfully permeate nasal tissue via transcytosis and enter brain structures via trigeminal and olfactory nerve pathways without compromising biological activity. It remains unclear how complexation with GET affects insulin's ability to bind and interact with its receptors, and whether or not binding may promote decomplexation, allowing it to exert its pharmacological effects. Previous findings suggest that insulin may not require full decomplexation and that some insulin remains accessible (Rehmani et al., 2023).

4.4 In vivo findings

Despite these advantages and the apparent *in vitro* success as presented in the current study and others, translating these findings into *in vivo* studies can still be very challenging. Our group has previously used a modified version of GET with the attachment of polytheylene glycol polymer chains (PEG-GET) to form NCs with oxytocin for *in vivo* intranasal delivery (King et al., 2023). In this case, the GET peptide FGF2B-LK15-8R (FLR) was used, not PLR. Using ELISA, deeper nasal tissues samples from rats showed elevated oxytocin concentration 30-minutes after administration, indicative of improved tissue penetration. Plasma oxytocin concentration was also elevated after 30 minutes. However, we were unable to detect enhancement of brain regional levels by PEG-GET (olfactory bulb, pons, amygdala; 30 or 60

minutes later). Rhinarium oxytocin levels were also not enhanced by PEG-GET. Although results were underwhelming, these are very preliminary findings which require repeats and similar analysis following insulin-GET administration. Nasal administration and absorption of insulin-GET NCs has not yet been assessed *in vivo* and differences should be expected compared to oxytocin-GET. Insulin and oxytocin have very different molecular weights and charges, which significantly affects complexation with GET and the characteristics of NCs formed. Not only are these two very different peptides themselves, but the GET peptide used (e.g., FLR or PLR) and presence of PEG also have a significant impact on cargo bioavailability and distribution (Dixon et al., 2016, Osman et al., 2018).

4.4.1 Methods of nasal administration in vivo

Conventional formulations of drugs designed for intranasal delivery such as gels, solutions, suspensions, powders, and emulsions suffer from many issues that affect bioavailability and overall efficacy. One of the main issues is that the dose administered is not precisely targeted at the intended treatment site. Drug instability, high viscosity, and large particle size also limit the therapeutic potential of drugs administered in these formulations. Novel drug formulations such as nanogels, liposomes, and polymer-based NP delivery systems are being assessed for their potential to overcome these issues associated with conventional intranasal drug administration. Nanogels are able to encapsulate NPs in a polymer network. They provide many advantages such as reduced mucociliary clearance due to their high viscosity which also reduces postnatal drip, reduced anterior leakage, and target-specific delivery to nasal mucosa allowing for more efficient absorption. The mucoadhesive properties of nanogels facilitates drug transport by increasing residence time of the drug at the absorption site. This nano-formulation has demonstrated compatibility with a range of small molecule and large macromolecular drugs such as insulin. Poly(N-vinyl pyrrolidone)-based nanogels covalently attached to insulin have previously been injected into the nasal cavities of male C57BL/6J mice. Individual brain regions were then homogenised 30 and 60 minutes after administration, showing significantly enhanced bioactivity of insulin and biodistribution into the olfactory bulb, cerebral cortex, hippocampus, cerebellum, and whole-brain samples (Picone et al., 2018). Nanogels require specially-designed delivery devices and gelling agents to allow for effective distribution in the nasal cavity and without this, distribution only occurs in a narrow area, inhibiting therapeutic potential (Kumar et al., 2016).

Liposomes are another form of nano-based delivery formulations, composed of phospholipid bilayers that act as protective shells and enclose an aqueous inner core in which therapeutic agents ranging from small to large molecules can be encapsulated. Liposomes have been utilised for the delivery of influenza vaccine in chickens. Intranasal delivery of liposomes encapsulating inactivated H5N3 avian influenza virus with bioadhesive polysaccharides effectively enhanced the immunogenic response elicited by chickens, measured by increased mucosal secretory immunoglobulin A (s-IgA) and serum immunoglobulin G after two vaccinations (Chiou et al., 2009). Encapsulation by liposomes protects drugs and genetic material from enzymatic degradation and increases retention time in the nasal mucosa, enhancing permeation. Furthermore, liposomes have previously been used to enhance nasal absorption of peptides such as insulin in rabbits following administration by inserting a syringe into the nasal cavity. The ability of liposomes to provide a rigid membrane that protects the drug from enzymatic degradation and prevents leakage is essential to increasing nasal mucosa permeation (Muramatsu et al., 1999).

In addition to the drug formulation, the device used for intranasal drug delivery is an important aspect that effects the efficiency of nose-to-brain delivery. The correct delivery device is highly dependent upon the formulation of the drug being administered (e.g., liquid, powder, semisolid). It's very common for *in vivo* studies of nasal administration to involve direct application into the nasal cavity via a micropipette or syringe (Picone et al., 2018). However, our own *in vivo* studies with oxytocin-GET involved administering the treatment bilaterally directly on the

rhinarium of rats with a pipette whilst they are held in an up-right position for about 1 minute to ensure the drug has time to absorb and doesn't run off from the target area (King et al., 2023). The rhinarium is a hairless region of skin surrounding the nostrils of rats, mice etc and is highly innervated by free nerve endings including a branch of the trigeminal nerve which facilitates nose-to-brain delivery (Tuminaite & Kroger, 2020). This technique is non-invasive which makes administering the treatment easier but requires careful and precise application to the desired area (Lukas & Neumann, 2012).

4.5 Limitations and improvements

4.5.1 RPMI 2650 as a valid nasal barrier model

RPMI 2650 cells cultured on 0.4μm pore size polyester filter inserts for 21 days, at ALI from day 8 onwards, were able to form confluent monolayers expressing TEER values similar to those previously reported in literature. Studies from Mercier et al., (2018a), and Wengst and Reichl (2010) report a range of 80 - 130 Ω/cm^2 for ALI RPMI 2650 cultures. Our TEER values increased rapidly once culture conditions were switched to ALI, reaching a maximal average of 90 ± 2.22 Ω/cm^2 which falls into this range, as well as the 60 - 180 Ω/cm^2 range measured in human excised nasal mucosa (Mercier et al., 2018a). ALI cultivation is crucial for valid and reproducible RPMI 2650 nasal barrier models, which is emphasised by studies from Wengst and Reichl (Wengst & Reichl., 2010) and Reichl and Becker (Reichl & Becker., 2012). These studies share the same time-course of cultivation and day of media switch, the only difference being that filters used by Reich and Becker (2012) were polyethylene terephthalate whilst those used by Wengst and Reichl (2010) were polycarbonate. Such studies have standardized cultivation by identifying optimal conditions for producing a valid *in vitro* cell culture model. Prior to this, the RPMI 2650 cell line was assessed by De Fraissinette et al., (De Fraissinette et al., 1995) and others to be a very limited *in vitro* nasal barrier model with poor suitability to drug permeation studies. Cultivated under liquid culture conditions, De Fraissinette et al., (1995) observed the formation of cell clusters with no expression of tight junctions, microvilli, or cell polarisation. Whilst successful cultivation of RPMI 2650 monolayers was claimed by some, however, no TEER values or microscopy validating this were reported in this case (Byrd et al., 1993), Werner and Kissel (Werner & Kissel., 1996) and Schmidt et al., (Schmidt et al., 1998) criticised this cell line for poor differentiation and lack of polarisation, both failing to produce monolayers. RPMI 2650 was largely disregarded and excised human nasal tissue persisted as a preferred model.

Since these initial criticisms, optimisation studies have emphasised the importance of ALI cultivation (Mercier et al., 2018a), seeding density, pore size, and type of transwell coating to provide optimal growth conditions, addressing these challenges (Mercier et al., 2018b, Bai et al., 2008). As demonstrated in the current study, the correct combination of these factors allows for well differentiated cells that form a tight monolayer and express electrical resistance comparable to human nasal tissue. These factors have been shown to promote expression of tight junction proteins such as ZO-1, Claudin-1, Occludin, and E-cadherin (Bai et al., 2008, Kreft et al., 2015, Kurti et al., 2013). Although techniques to validate tight junction presence such as western blot analysis are not included in this study, the TEER values of cultures used are indicative of a confluent epithelial membrane with organotypic features. Despite now being well-characterised, several issues with this cell line still persist. RPMI 2650 cells are strictly composed of squamous cells whereas human nasal epithelium is heterogeneously composed of four distinct cell types: ciliated, goblet, columnar, and basal (Goncalves et al., 2016, Pozzoli et al., 2016). This distinct morphological difference could limit the pharmacokinetic relevance of this cell line, lack of cilia means mucociliary clearance cannot be assessed, which is an important factor for intranasal drug administration (Ladel et al., 2019, Mercier et al., 2018b). Additionally, the composition of mucus secreted by this cell line is not well understood, with

several conflicting reports. Mucoadhesion is an important property of novel nasally administered therapeutics, many of which aim to increase retention time in the mucosa.

Nevertheless, the RPMI 2650 cell line is held in higher regard than alternatives such as primary nasal cell lines, which may only be passaged up to three times in order to maintain desirable morphological features (Wu et al., 1985). Optimal cell culture conditions for RPMI 2650 have been standardised and well recorded, making the cancer-derived cell line the best option available.

4.5.2 Octa-arginine

The PLR peptide (P21-LK15-8R) allows for a novel nanotherapeutic approach by combining enhanced membrane-docking peptides that bind heparan sulphate glycosaminoglycans (GAGs) with octa-arginine, a CPP that comprises the 8R domain. Octa-arginine has been shown to facilitate cell adhesion and effectively enhance cellular uptake and internalisation of cargoes, including peptide NPs, siRNA, and cancer treatments, to therapeutic levels (Li et al., 2015, Oppen et al., 2019, Rompicharla et al., 2018). Additionally, Khafagy et al., 2009 have previously demonstrated the ability of octa-arginine to significantly enhance nasal absorption of insulin when delivered as a simple complex. The inclusion of octa-arginine/insulin complexes as another treatment group in the present study would allow us to assess how the other domains of the PLR peptide (P21, LK15) which facilitate cell-targeting and endosomal escape respectively, encourage cell entry in combination with CPP activity. Based on findings from Rehmani et al., we could expect to see that when compared to octa-arginine/insulin complexes, the GET system significantly enhances cellular uptake and internalisation of insulin based on the activity of the P21 and LK15 domains facilitating CPP activity (Rehmani et al., 2023).

4.5.3 Confocal Imaging

In **Figure 8** we aimed to show GET-mediated enhanced delivery of insulin into RPMI 2650 cultures using DAPI as a counter-stain for the FITC-tagged treatment. The DAPI staining for the control group, particularly at 6 hours shows a very clear image. However, for the Insulin-GET 2:1 treatment group, the DAPI staining of samples collected at all timepoints is very poor and creates an unclear image of the cells and their interaction with FITC-tagged insulin-GET NCs. At the 4 and 6-hour timepoints, there is a high level of aggregation of these NCs clearly interacting with cells but due to the staining and resolution used, the extent of internalisation is unclear and seems poor. Particularly at 6 hours, there appears to be a scattering effect of FITC-labelled particles. It's unclear whether this is cellular debris, an unwanted effect caused by 1.7µM GET or if this represents unbound FITC-tagged insulin-GET NCs with no cellular interaction. Furthermore, fixing cells in PFA is known to cause a reduction in the intensity of fluorescent signals emitted by fluorophores (e.g., DAPI, FITC) (Tang et al., 2021). The aldehyde group in the paraformaldehyde molecule is able to bind with amino groups of cellular proteins, which creates carboxymethyl groups and thereby forms intermolecular crosslinks which fix proteins and alter their configurations. Ultimately, these molecular structural changes may interfere with protein interactions of fluorophores and fluorescent intensity (DiDonato & Brasaemle., 2003). Therefore, fixation in PFA (4%) may have caused DAPI nuclear staining as well as FITC-labelled particles to become less visible, obscuring the extent of their internalisation.

The poor DAPI staining seen in some of the images displayed in **figure 8** could indicate that the cells were dying under these conditions. By fixing cells in PFA, metabolism is halted which allows them to be stored for analysis much longer than live cells. Therefore, in this instance, cells would not be dying unless one of the reagents used is inducing cytotoxicity. However, our results from cell viability assays suggest that neither FITC-ins alone or either of the two PLR

concentrations used, are cytotoxic to RPMI 2650 cultures. Therefore, if cell death is the reason behind poor DAPI staining, it is more likely that either DAPI, PFA, or Triton x-100 at the concentrations used were the sources of cytotoxicity. DAPI can be toxic but usually only to live cells at very high concentrations, fixed cells should tolerate dilute DAPI (Zink et al., 2003). PFA can be severely cytotoxic, but this is unlikely when it is very diluted (1 – 8% is typical for fixing cells) and exposure time is limited (10 minutes) (Ke et al., 2014).

It has previously been shown that triton x-100 can be significantly cytotoxic at concentrations much lower than used in this experiment. 0.01% (w/v) Triton x-100 can cause a cell death rate of approximately 90% in cultures of various human hepatoma cell lines after 150 minutes of incubation (Ahn et al., 1997). Although this suggests very low concentrations of triton x-100 can be severely cytotoxic, 150 minutes exposure is 10-fold greater than the exposure time involved in the current study.

However, mean fluorescent intensity (MFI) measured by flow cytometry, which is a much more sensitive technique, shows very high levels of insulin uptake at 4 and 6 hours, when the same concentrations of GET are used (0.17µM, 1.7µM). If repeated, a clearer image could be achieved by including more washes when preparing cultures for confocal imaging, to remove cellular debris, and a higher resolution would allow us to assess the extent of insulin internalisation more accurately. Additionally, flow cytometry results show that insulin uptake continues to rise after 24-hour exposure compared to 6 hours. Confocal imaging of RPMI 2650 cultures after 24-hour exposure should show a similar increase in internalisation of insulin-PLR NCs.

4.6 Future Work

4.6.1 Insulin-GET NC characterization

Insulin-GET NCs have previously been analysed via to determine characteristics such as zeta potential, morphology, and size distribution (see **section 2.2**) by employing techniques such as DLS, TEM, and laser doppler anemometry. Images can be taken using TEM at a much higher resolution than other techniques. Future studies could see TEM used in combination with CLSM to assess the subcellular location and accumulation of insulin-GET NCs post-endocytosis in greater detail (Mourdikoudis et al., 2018). This could provide an insight into shape and size requirements for effective cellular uptake and vesicle escape (Yue et al., 2017).

4.6.2 In vitro inhibition studies

Previous work by Rehmani et al., 2023 describes *in vitro* chemical inhibition of endocytosis pathways, leading to a significant decrease in cellular internalisation and maximum cumulative transport of insulin-GET NCs across differentiated caco-2 monolayers after 24 hours. Similar inhibition studies using the insulin-GET formulations and *in vitro* nasal barrier model described in the present study would provide novel insight into how these mechanisms influence insulin-GET delivery across nasal barriers. Repeats of our cellular uptake and permeation assays utilising RPMI 2650 monolayers incubated with FITC-tagged insulin-GET NCs in the presence of endocytosis inhibitors would highlight the role of endocytosis-mediated uptake. For example, 5-N-ethyl-isopropyl amiloride (EIPA) is an inhibitor of NA+/H+ exchanged required for macropinocytosis, one of the main mechanisms by which endocytosis occurs (Wadia et al., 2004). Bolinaquinone (BLQ) is a natural marine product which when modified with the addition of the a,o-diaminopolyethylene glycol chain inhibits CME (Szewczyk-Roszczenko et al., 2023). Dose-dependent inhibition of CME by modified BLQ has been demonstrated via flow cytometry and microscopic analysis (Margarucci et al., 2010). Furthermore, it has been demonstrated that incubation of Hep-2 cell cultures in 10mM Methyl-b-Cyclodextrin (MBCD) significantly reduces CvME (Rodal et al., 1999). Cholesterol is essential for the formation of

typically invaginated caveolae. MBCD is able to inhibit CvME by specifically removing cholesterol from the plasma membrane.

Functional expression of ABC drug transporters (e.g., P-gp, BCRP, and several MRPs) has previously been demonstrated in RPMI 2650 cells cultured under ALI conditions (Mercier et al., 2018b). ABC transporter proteins regulate cellular drug efflux, one of many pharmacological factors that determines cellular internalisation of locally administered drugs (Choi et al., 2014). Future studies involving the inhibition of these transporter proteins would shed light on the extent to which efflux mechanisms mediate the internalisation and permeation of insulin-GET. Probenecid, verapamil, and Ko143 are all ABC transporter inhibitors (MRPs, P-gp, and BCRP respectively) and have demonstrated compatibility with RPMI 2650 cultures in previous drug efflux studies (Gollapudi et al., 1997 & Mercier et al., 2018b). Repeats of our permeation and uptake assays that include incubation of RPMI 2650 monolayers with ABC transporter inhibitors would provide further insight into the mechanisms that mediate intracellular accumulation and transcytosis of insulin-GET NCs. We could expect that by inhibiting drug efflux mechanisms, this would lead to an increase in cellular internalisation of FITC-tagged insulin-GET NCs (measured by flow cytometry) and less efficient transcytosis, demonstrated by lower P_{app} values.

4.6.3 Extensions to the transwell model

Some studies have demonstrated that ALI-cultured RPMI 2560 monolayers can be cultivated on transwells coated with extracellular matrix proteins such as collagen (Mercier et al., 2018a). The extracellular matrix provides a physiological microenvironment that allows cellular proliferation and differentiation to occur as well as the development of specialized epithelial functions such as barrier formation. Several studies demonstrate that the inclusion of collagen-coated transwells provides a useful extension to the RPMI 2650 nasal barrier model by providing an intact surface for nasal epithelial cell layers to grow upon. Fibronectin is another extracellular matrix protein, however fibronectin-coated transwells have been shown to have an insignificant or negative effect on barrier integrity development. Collagen-coated transwells have been reported to increased cell differentiation and TEER in ALI-cultured RPMI 2650 monolayers compared to non-coated controls (Bai et al., 2008, Kurti et al., 2013). This is likely related to the fact that the respiratory basement membrane is primarily made up of collagen fibres.

This idea has been expanded upon even further by Wengst & Reichl (2009). This study demonstrates that human nasal fibroblasts (HNF) and RPMI 2650 can be seeded together on collagen-coated transwells under ALI conditions to develop a three-dimensional human nasal mucosa construct composed of a collagen matrix with embedded HNF, covered by a layer of RPMI 2650. However, the TEER values of these human nasal mucosa constructs were insignificantly greater than those reported for RPMI 2650 cultivated on transwell inserts (both ALI-cultivated). Furthermore, TEER values of ALI-cultivated RPMI 2650 monolayers reported more recently are consistently greater compared to those measured in these constructs (Wong et al., 2022 & Kurti et al., 2013). This is likely due to the fact that ALI cultivation has since been optimised.

4.6.4 In vivo studies

We've demonstrated that low concentrations of the PLR peptide (0.17μM and 1.7μM) can form NCs with insulin to significantly enhance transport across differentiated nasal epithelial barriers *in vitro* without compromising barrier integrity or causing toxicity. GET-mediated delivery of insulin is cytocompatible, insulin-GET NCs can be generated quickly via simple electrostatic interaction and only require low concentrations to be effective. These findings provide early proof-of-concept data to support future *in vivo* work to assess whether GET-mediated intranasal

delivery of insulin can provide effective delivery to brain regions such as the olfactory bulb, pons, amygdala, hypothalamus, and hippocampus where insulin receptors are widely distributed. The amygdala and hippocampus are of particular interest due to their association with aspects of cognition (e.g., memory, emotional stability) that decline with neurological disease. An important aspect to consider is whether or not insulin delivered to the brain by GET retains its biological activity. Nose-to-brain delivery *in vivo* is much more complex than *in vitro* epithelial permeation since this requires perineuronal and/or perivascular transport following epithelial penetration. Insulin's biological activity be measured *in vivo* by levels of phosphorylated protein kinase B (Akt), either in whole brain or regional brain samples (Picone et al., 2018). Upon binding to its receptor, insulin induces a conformation change which in turn activates Akt by phosphorylation.

4.7 Concluding remarks

We have successfully managed to culture differentiated RPMI 2650 monolayers on transwells at ALI to form an effective *in vitro* nasal barrier model for studying insulin transcytosis enhanced by GET. Using both quantitative and qualitative assays, we were able to demonstrate that the insulin-GET NCs generated are cytocompatible and compared to insulin alone display significantly higher P_{app} values as well as greater cumulative concentration in both apical and basal chambers up to 48-hours post administration. We have developed a GET-mediated insulin formulation for nasal delivery which we can demonstrate significantly enhances insulin permeation across nasal barriers *in vitro* without adverse effects on barrier integrity. It is our hope to see this system translated to *in vivo* studies where GET-mediated intranasal insulin delivery can effectively enhance insulin bioavailability in behaviourally relevant brain regions (e.g., hippocampus, amygdala) without loss of biological activity.

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