Investigation of purinergic receptors in human BE(2)-M17 neuroblastoma cells and their role in neurotransmitter release

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Abstract

Adenosine 5'-triphosphate (ATP) is a neurotransmitter of postganglionic sympathetic nerves and non-adrenergic, non-cholinergic nerves of the enteric nervous system. Norepinephrine (NE) is a key monoamine neurotransmitter in the central nervous systems and peripheral organs of vertebrate organisms. ATP and NE are co-stored in synaptic vesicles in sympathetic nerves and when co-released act post-junctionally to evoke contraction of visceral and vascular smooth muscle. However, little is known about the effect of ATP on pre-junctional NE release. Here we show that undifferentiated human BE(2)-M17 neuroblastoma cells demonstrate a calcium response to nucleotides with UDP evoking a statistically greater potency and maximal response than ATP. We also successfully transient-transfected undifferentiated human BE(2)-M17 neuroblastoma cells with the $GRAB_{NE}$ biosensor, which we used to detect changes in extracellular NE concentrations when exposed to the nucleotides. We isolated the significant difference in the maximal response of NE compared to Epinephrine and Dopamine demonstrating that NE had a greater efficacy on the $GRAB_{NE}$ biosensor. However, the EC_{50} between the three catecholamines was not significantly different suggesting that the catecholamines are equipotent to the $GRAB_{NE}$ biosensor. Finally, we found no significant difference in the change of fluorescence in $GRAB_{NE}$ transfected cells following exposure to ATP and UDP. Thus, suggesting nucleotides do not excite NE release but may inhibit it.

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Acknowledgements

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Chapter 1: Introduction

1.1 Calcium signalling

1.1.1 Basic Principles and Mechanism

The universality of calcium is key to its effectiveness as an intracellular messenger and its flexible use across physiological systems. Cells possess a calcium toolkit with many components that can be combined to create a wide range of spatial and temporal signals. The flexibility is exploited in a diverse range of biological processes including fertilisation, proliferation, development, learning and memory, contraction and secretion, and must be accomplished within a range of calcium being highly toxic. Exceeding its homeostatic boundaries can result in cell death through both necrosis and apoptosis (Berridge, 2000).

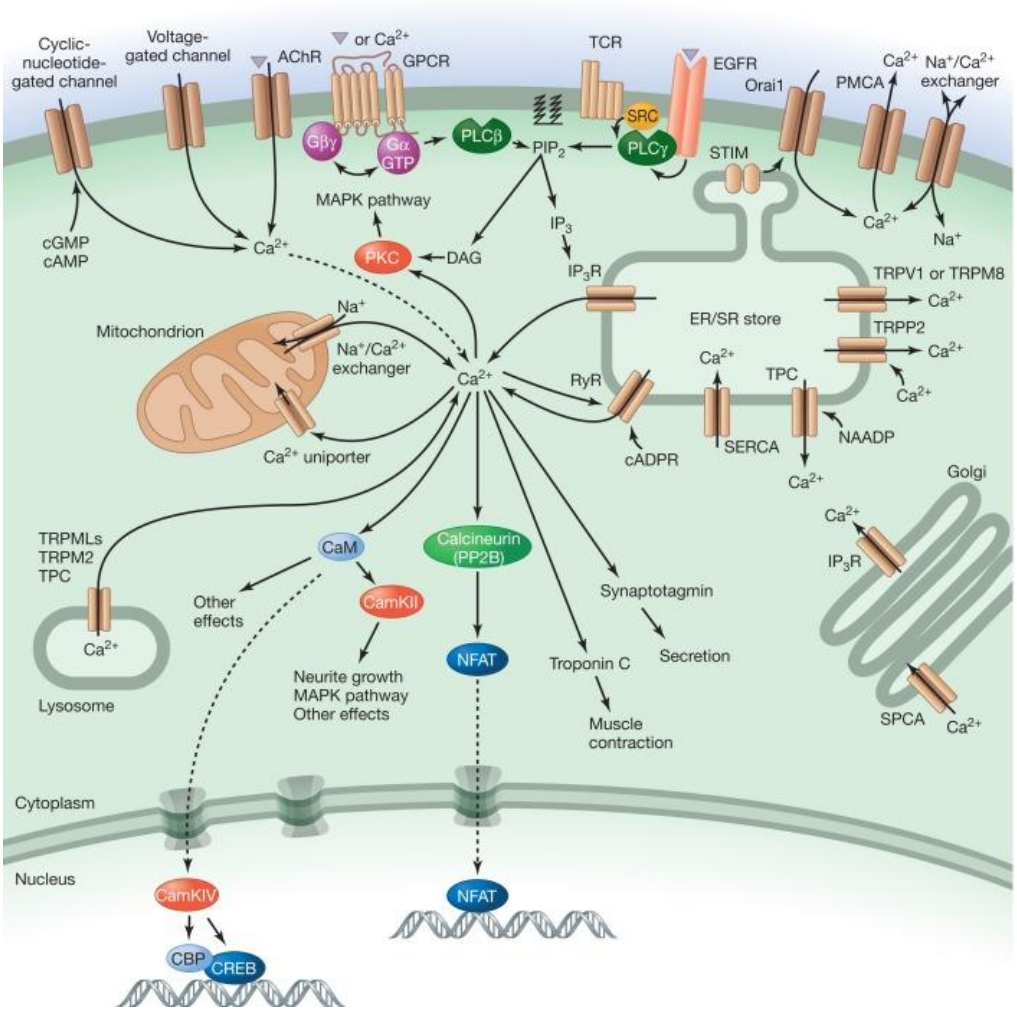

Figure 2: The versatility of intracellular calcium signalling.

Ca^{2+} is integral to a variety of processes within the cell. Numerous proteins are modulated directly or indirectly by calcium. These include kinases and phosphatases, transcription factors such as NF-AT and CREB, and the ubiquitous calcium-binding protein calmodulin. Figure taken from Bootman, 2012.

Cells invest large sums of energy to effect changes in Ca^{2+} concentration due to the underlying rate and effectiveness of a 20,000-fold gradient maintained by cells between their intracellular (~100 nM free) and extracellular (mM) concentrations. Unlike complex molecules, Ca^{2+} cannot be chemically altered. Therefore, cells chelate, compartmentalise, or extrude Ca^{2+} to exert control and maintain conditions. Cells across the body hold a large array of cellular proteins which have adapted to Ca^{2+}-binding over a diverse range of affinities, which can result in the increasing, buffering or lowering of Ca^{2+} levels or result in the activation of intracellular processes, including vesicle release (Clapham, 2007).

1.1.2 Storage and Influx

In addition to intracellular influx, Ca^{2+} signals comprise their release from intracellular stores as either a result of Gq-coupled receptor activation, or secondary to the initial intracellular Ca^{2+} rise. Calcium is predominantly stored in the endoplasmic reticulum which can accumulate Ca^{2+} to concentrations of 10–100 mM. Endoplasmic reticulum Ca^{2+} release is mediated by RyRs, and by inositol phosphate 3 receptors (IP3Rs). The function of the endoplasmic reticulum Ca^{2+} channels is to amplify or trigger Ca^{2+} rises initiated by Ca^{2+} influx resulting from transmembrane receptor activation. In addition, mitochondria have also been shown to act as intracellular Ca^{2+} stores and play a prominent role in determining the shape, amplitude, and duration of Ca^{2+} release (Kawamoto *et al.,* 2012).

1.1.2 Calcium Signalling in Neurotransmission

Ca^{2+} entering neurons during the initiation of synaptic transmission at fast conventional synapses is mediated through CaV2.1 and CaV2.2 channels. CAV2.1 channels conduct P/Q-type Ca^{2+} currents and play a major role in synaptic transmission in the Central Nervous system (CNS). CaV2.2 channels, which conduct N-type Ca^{2+} current, are most important at synapses of the peripheral nervous system. However, in some central synapses, including a subset of inhibitory interneurons of the hippocampus, CaV2.2 channels are predominant in neurotransmitter release (Catterall & Few, 2008).

Upon entering a presynaptic terminal, an action potential opens Ca^{2+} channels, triggering Ca^{2+} exocytosis, increasing the local Ca^{2+} concentration at the presynaptic active zone characterised by an assembly of electron-dense material (Weingarten *et al*., 2017; Catterall & Few, 2008). Ca^{2+} then triggers neurotransmitter release by activating synaptotagmins Ca^{2+} within a few hundred microseconds. Synaptotagmins bind Ca^{2+} via two C2-domains, and transduce the Ca^{2+} signal into a nanomechanical activation of membrane fusion machinery; mediated by the Ca^{2+}-dependent interaction of the synaptotagmin C2-domains with surrounding phospholipids and SNARE proteins (Südhof, 2012). Formation of the SNARE complex causes a conformational change from a trans to a

cis state, resulting in the fusion of opposing membranes and the release of neurotransmitters into the synaptic cleft (Catterall & Few, 2008).

1.2 Purinergic signalling

Purines and pyrimidines were first recognised as a fundamental element of bioenergetics in 1929 through studies by Szent-Gyorgyi and Alan Drury using intravenous injection of adenine into guinea pigs to disturb cardiac rhythm (Huang *et al.,* 2021). Following this, Feldberg and Hebb described Adenosine Triphosphate (ATP) as having a "powerful stimulating action on the perfused superior ganglion of the cat" (Feldberg & Hebb, 1948). Holton's research in 1959 demonstrated that antidromic stimulation of sensory nerves "liberated" ATP (Holton. 1959) resulting in the generation of the theory that purines play a role in the nervous system and transmitter release.

ATP, originally assumed to be exclusively the universal energy metabolite of cells, was proposed in 1972 by Geoffrey Burnstock to be an extracellular, non-adrenergic, non-cholinergic neurotransmitter in smooth muscle organs of the guinea pig gastrointestinal tract (Burnstock, 1972; Illes *et al.* 2021).

However, this concept was not well accepted at first until the 1990s when further research into the molecular cloning of receptors that mediate transmitter release in response to extracellular ATP was published (Burnstock, 2017).

The concept of purinergic neurotransmission and the potent actions of extracellular ATP on many different cell types implied the presence of purinergic membrane receptors. Purinergic receptors were described first in 1976 and two years later, a basis for distinguishing two families of purinoceptors was proposed, identifying P1 and P2 receptors (for adenosine and ATP/adenosine 5'-diphosphate (ADP), respectively (Burnstock, 2018). These receptors are further sub-classified into several subtypes, which are diffusely expressed in tissues and are activated by different purine derivatives (Huang *et al*, 2021).

1.2.1 P1 receptors

P1 receptors are G-protein coupled receptors activated by the nucleoside agonist adenosine, earning the nomenclature Adenosine receptors, and are divided into four subtypes: A_1, A_{2A}, A_{2B}, and A_3. The intracellular segment of the membrane-bound receptor interacts with the appropriate G-protein, with subsequent activation of intracellular calcium (Burnstock, 2007; Ribeiro *et al,* 2002). Adenosine receptors are present in the peripheral nervous system including autonomic nerves and results obtained from motor nerve endings have shown A_1-mediated presynaptic inhibitory actions as well as A_{2A}-mediated presynaptic excitatory actions (Riberio *et al*, 2002).

1.2.2 P2 Receptors

P2 receptors exist as two distinct families: the P2X ligand-gated ionotropic channel family and the P2Y metabotropic, heptahelical G-protein coupled receptor family (Burnstock & Williams, 2000).

Table 1: Comprehensive list of key P2-receptor agonists

P2 Receptor	Agonist	Source
P2X1	ATP	(Bianchi *et al.,* 1998; Roberts & Evans. 2004)
	ADP	(North & Surprenant. 2000)
	α,β-meATP	(Bianchi *et al.,* 1998: Sim *et al.,* 2007)
	β,γ-MeATP	(Orriss *et al.,* 2012)
	2-meSATP	(Wildman *et al.,* 2002)
	BzATP	(Bianchi *et al.,* 1998; Wildman *et al.,* 2002)
	AP_6A	(Wildman *et al*., 1999)
P2X2	ATP	(North & Surprenant. 2000)
	ADP	(North & Surprenant. 2000)
	ATP-γ-S	(King *et al.,* 2009)
	BzATP	(Lynch *et al.,* 1999; Bhargava *et al.,* 2012)
	2-meSATP	(Lynch *et al.,* 1999)
P2X3	ATP	(Jarvis & Khakh. 2009)
	ADP	(Lewis et al., 1995; North and Surprenant, 2000)
	α,β-meATP	(Orriss *et al.,* 2012)
	2-meSATP	(Jarvis & Khakh. 2009)
	β,γ-MeATP	(Orriss *et al.,* 2012)
	BzATP	(Bianchi *et al.,* 1999; Jarvis & Khakh. 2009)
P2X4	ATP	(Bo *et al.,* 1995; Soto *et al.,* 1996; Stokes *et al.,* 2011)
	α,β-meATP	(Soto *et al.,* 1996)
	2-meSATP	(Soto *et al.,* 1996)
	CTP	(Soto *et al.,* 1996; Maynard *et al.,* 2022)
	BzATP	(Stokes *et al.,* 2011)
P2X5	ATP	(Bo *et al.,* 2003)
	2-meSATP	(Bo *et al.,* 2003)
	α,β-meATP	(Wildman *et al.,* 2002; Ruppelt *et al.,* 2001)
P2X6	ATP	(Jones *et al.,* 2004)
	α,β-meATP	(Jones *et al.,* 2004)

P2X7	ATP	(Donnelly-Roberts *et al.,* 2009; Müller *et al.,* 2022)
	BzATP	(Donnelly-Roberts *et al.,* 2009)
P2Y1	ADP	(Palmer *et al.,* 1998)
	2-meSADP	(Palmer *et al.,* 1998)
	ADPβS	(Palmer *et al.,* 1998)
	ATP	(Léon *et al.,* 1997 *Palmer et al.,* 1998)
	ATP-γ-S	(Palmer *et al.,* 1998)
	ATP-α-B	(Nahum *et al.,* 2002)
	2-meSATP-α-B	(Nahum *et al.,* 2002)
	2ClATP-α-B	(Nahum *et al.,* 2002)
P2Y2	UTP	(Lazarowski *et al.,* 1995; Sromek & Harden., 1998)
	UTPγS	(Lazarowski *et al.,* 1995)
	ATP	(Lazarowski *et al.,* 1995; Sromek & Harden., 1998)
	5BrUTP	(Lazarowski *et al.,* 1995; Knoblauch *et al.,* 1999)
	2ClATP	(Lazarowski *et al.,* 1995)
	dCp4U	(Kellerman *et al.,* 2002)
	Up4U	(Diego-Garcia *et al.,* 2017)
P2Y4	UTP	(Communi *et al.,* 1996; Herold *et al.,* 2004; Rafehi *et al.,* 2017)
	UTPγS	(Brunschweiger *et al*., 2006)
	5BrUTP	(Communi *et al.,* 1996)
	UDP	(Communi *et al.,* 1996)
	ITP	(Communi *et al.,* 1996)
	ATP	(Nicholas *et al.,* 1996*;* Herold *et al.,* 2004)
P2Y6	UDP	(Communi *et al.,* 1996; Brinson & Harden. 2001)
	UDPβS	(Jacobson *et al.,* 2002)
	5BrUTP	(Communi *et al.,* 1996)
	UTP	(Communi *et al.,* 1996; Nicholas *et al.,* 1996)
	ADP	(Communi *et al.,* 1996; Nicholas *et al.,* 1996)
	ATP	(Nicholas *et al.,* 1996)
	2-meSATP	(Communi *et al.,* 1996)
P2Y11	ATP	(Communi *et al.,* 2009)
	ADP	(Communi *et al.,* 2009)
	ATPγS	(van der Weyden *et al.,* 2000; Communi *et al.,* 2009)
	BzATP	(Communi *et al.,* 2009)

	dATP	(van der Weyden *et al.,* 2000; Communi *et al.,* 2009)
	ADPβS	(van der Weyden *et al.,* 2000; Communi *et al.,* 2009)
	2-meSATP	(Communi *et al.,* 2009)
	AR-C67085	(Communi *et al.,* 1999)
P2Y12	ATP	(Waldo & Harden. 2004; Ohsawa *et al.,* 2007)
	2-meSADP	(Zhang *et al.,* 2014)
	2-meSATP	(Zhang *et al.,* 2014)
	ADP	(Zhang *et al.,* 2014; Jairaman *et al.,* 2022)
	ADPβS	(Zhang *et al.,* 2014)
	ATPγS	(Zhang *et al.,* 2014)
	2ClATP	(Zhang *et al.,* 2014)
P2Y13	2-meSADP	(Zhang *et al.,* 2002; Dsouza & Komarova. 2021)
	ADPβS	(Zhang *et al.,* 2002; Dsouza & Komarova. 2021)
	ADP	(Dsouza & Komarova. 2021; Wang *et al.,* 2005)
	2-meSATP	(Zhang *et al.,* 2002; Marteau *et al.,* 2003)
	AP_3A	(Zhang *et al.,* 2002; Marteau *et al.,* 2003)
	ATP	(Zhang *et al.,* 2002; Marteau *et al.,* 2003)
	IDP	(Zhang *et al.,* 2002; Marteau *et al.,* 2003)
P2Y14	UDP-glucose	(Chambers *et al.,* 2000)
	UDP-galactose	(Chambers *et al.,* 2000)
	UDP-glucuronic acid	(Chambers *et al.,* 2000)
	UDP-*N*-acetylglucosamine	(Chambers *et al.,* 2000)

1.2.2.1 P2X receptors

P2X receptors (P2XRs) are non-selective cation channels that open in response to ATP binding, allowing the rapid flow of ions (K^+, Na^+, Ca^{2+}) across the membrane (Stokes *et al,* 2017). There are seven different P2X subunits (P2X1-7) which share a common topology assembling as trimers (Habermacher *et al*, 2016; Stokes *et al*, 2017), with evidence of both homomeric and heteromeric assembly such as the P2X2/3 and P2X1/5 receptors (North, 2016). The members of the P2X family share 45% identity in amino acid sequence and a common subunit structure (Martinez, 2019) but differ vastly in physiological function (North, 2002).

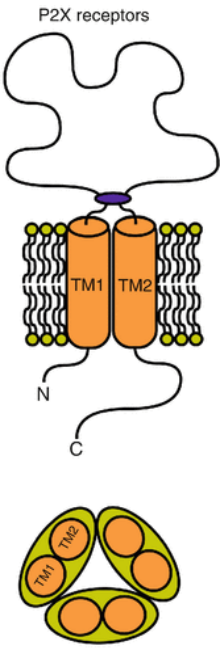

Figure 2: Schematic representation of the common P2XR structure.

Figure adapted from Shen *et al*. 2018

Each subunit has several common underlying features. The C- and N-termini are located intracellularly, linked by two membrane-spanning segments (TM1 and TM2) and a large extracellular domain (ectodomain), where the ATP-binding sites are nestled (Habermacher *et al.*, 2016)

P2XRs exhibit little discrimination between Na^+ and K^+ but, at resting membrane potentials, currents are carried by the movement of Na^+ down its electrochemical gradient and into the cell. The result is membrane depolarisation. All P2XRs also conduct Ca^{2+}, with the permeability of Ca^{2+} relative to Na^+ varying depending on the subunit makeup of the functional channel. Nucleotide activation of P2XRs results in Na^+-mediated depolarisation of the plasma membrane, and an increase in the concentration of free cytosolic Ca^{2+} from internal stores (Samways *et al.*, 2014).

1.2.2.2 P2X Receptors in the Nervous System

The relatively high Ca^{2+} permeability of P2X receptors makes them excellent mechanisms for initiating neurotransmitter release by Ca^{2+} influx. Previous research using *In situ* hybridisation using specific riboprobes and immunocytochemical studies using antibodies against individual P2X receptor subunits revealed vast expression of all seven P2X receptors across the nervous system. However, sub-units demonstrated distinct distribution and function (Sperlágh *et al.* 2007).

P2X1 receptors are widely expressed in the smooth muscle and homotrimeric P2X1 receptors have been linked to mediating smooth muscle contraction in response to nerve stimulation in the vas deferens (Mulryan et al., 2000), bladder (Fong et al., 2021), and arteries (Vial & Evans., 2002). In P2X1 knockout mice, vasoconstriction (Vial & Evans., 2002) and bladder contraction (Vial & Evans., 2000) induced by nerve stimulation were reduced compared to wild-type mice. Furthermore, c-terminal

peptide sequence-specific antibodies to P2X1 have demonstrated the receptors to be expressed in the superficial dorsal horn of the spinal cord (Vulchanova et al., 1996) and electron-immunocytochemical localisation has demonstrated the expression of P2X1 in synapses between varicosities of parallel fibres of granule cells and dendritic spines of Purkinje cells (Loesch & Burnstock., 1998). In neurotransmission, P2X1 has been shown to be involved in the facilitation of glutamate release (Rodrigues et al., 2005) and the inhibition of GABA release (Watano et al., 2004). There has also been research suggesting that P2X1 may be responsible for the facilitation of norepinephrine in the rat hippocampus (Papp et al., 2004) and vas deferens (Queiroz et al., 2003).

The P2X2 receptor is widely expressed in the CNS including the cerebral cortex (Kanjhan et al., 1999), hippocampus (Lommen et al., 2021), and spinal cord (Vulchanova et al., 1997). Immunocytochemistry has demonstrated P2X2 and P2X3 to be highly colocalised in the rat dorsal root and nodose ganglia (Vulchanova et al., 1997). Furthermore, P2X2 has been demonstrated to be endogenously expressed in the presynaptic and extrasynaptic regions of Supraoptic nucleus neurons (Vavra et al., 2011). In Neurotransmission, P2X2 has been linked to facilitating excitatory transmission onto stratum radiatum interneurons (Khakh & North. 2012) and has been linked to the stimulation of glutamate and GABA release (Vavra et al., 2011).

P2X3 receptors are primarily associated with sensory pathways, and play a key role in the development of neuropathic pain (Dong et al. 2022). P2X3 receptors have been localised to peripheral nerves that innervate tissues important in chemosensation and the sensing the stretching of hollow organs (Krajewski. 2020) as well as nociceptive primary sensory neurons (Bradbury et al., 1998) in the dorsal root, trigeminal and nodose ganglia (Burnstock. 2000). In neurotransmission, P2X3 receptors have been linked the outflow of norepinephrine in the rat hippocampus (Papp et al., 2004) and as Deferens (Queiroz et al., 2003). P2X3 has also been linked to the sensation of taste, P2X3 knockout mice demonstrated no taste-evoked activity in the chorda tympani and glossopharyngeal nerves when stimulated (Eddy et al., 2009).

The P2X4 receptors has been shown to be expressed throughout the CNS and PNS including ganglion neurons (Wheeler-Schilling et al., 2001), supraoptic neurons (Vavra *et al.,* 2011), rat spinal dorsal horn neurons (Ducza et al., 2023; Bardoni et al., 1997). In the CNS, P2X4 has been linked to controlling co-transmission of GABA and glutamate. In mouse models, P2X4 knockout mice demonstrated significant brain-regional alterations including the composition of glutamate inotropic receptors (Wyatt et al., 2013). Furthermore, P2X4 knockout mice have been observed to have differential changes in the GABA AR α1 subunit expression in several regions of the brain including the cerebellum, prefrontal cortex, and midbrain (Wyatt et al., 2014). Immunohistochemistry of rat striatum and substantia nigra

has also demonstrated P2X4 expression in GABAergic interneurons and GABAergic spiny neurons (Amadio et al., 2007)

P2X5 subunits are strictly located in the brain with strong localisation in certain areas such as the nucleus tractus solitarii (Yao et al., 2001), cerebellum (Xiang et al., 2005), hypothalamus (Xiang et al., 2006), and the paraventricular nucleus (Cham et al., 2006). Furthermore, P2X1 and P2X5 subunits have been demonstrated to form a functional P2X receptor in Mouse Cortical Astrocytes (Lalo et al., 2008). P2X6 has been shown to be localised to the CNS with predominantly localised to the peri- and extrasynaptic space of post-synaptic neurons (Boué-Grabot & Pankratov, 2017).

P2X7 receptors are widely distributed throughout the central and peripheral nervous system. P2X7 has been localised to areas such as the frontal cortex (Calovi *et al.,* 2020), hippocampus (Papp *et al.,* 2004), amygdala (Csölle *et al.,* 2013), and brainstem (Papp *et al.,* 2004). Immunocytochemistry has linked P2X7 expression to different areas of the nervous including sensory nerve terminals, sympathetic neurons, the hippocampus, the spinal cord, and neuromuscular junctions (Kanellopoulos & Delarasse. 2019; Sperlágh et al. 2006)

In knockout mice models, an absence of P2X7 has been linked to a decrease in dopamine release in the prefrontal cortex (Calovi *et al.,* 2020). Furthermore, P2X7 absence has been linked to a decrease in the release of both glutamate and GABA in the hippocampus (Papp *et al.,* 2004). In contrast, the absence of P2X7, basal norepinephrine levels were shown to be elevated in the amygdala (Csölle *et al.,* 2013).

1.1.2.4 P2Y receptors

P2Y receptors (P2YRs) are seven transmembrane G-protein coupled receptors (GPCRs) for extracellular nucleotides. Eight human subtypes of the P2YR family have been defined (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) (Kügelgen & Hoffmann, 2016).

P2YRs are further sub-divided into the G_q protein-coupled “P2Y1-like” receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) and the G_i protein-coupled “P2Y12-like” receptors (P2Y12, P2Y13, and P2Y14).

The P2Y11 receptor additionally couples to G_S proteins besides its ability to activate G_Q proteins (Figure 2; Neumann *et al.*, 2020).

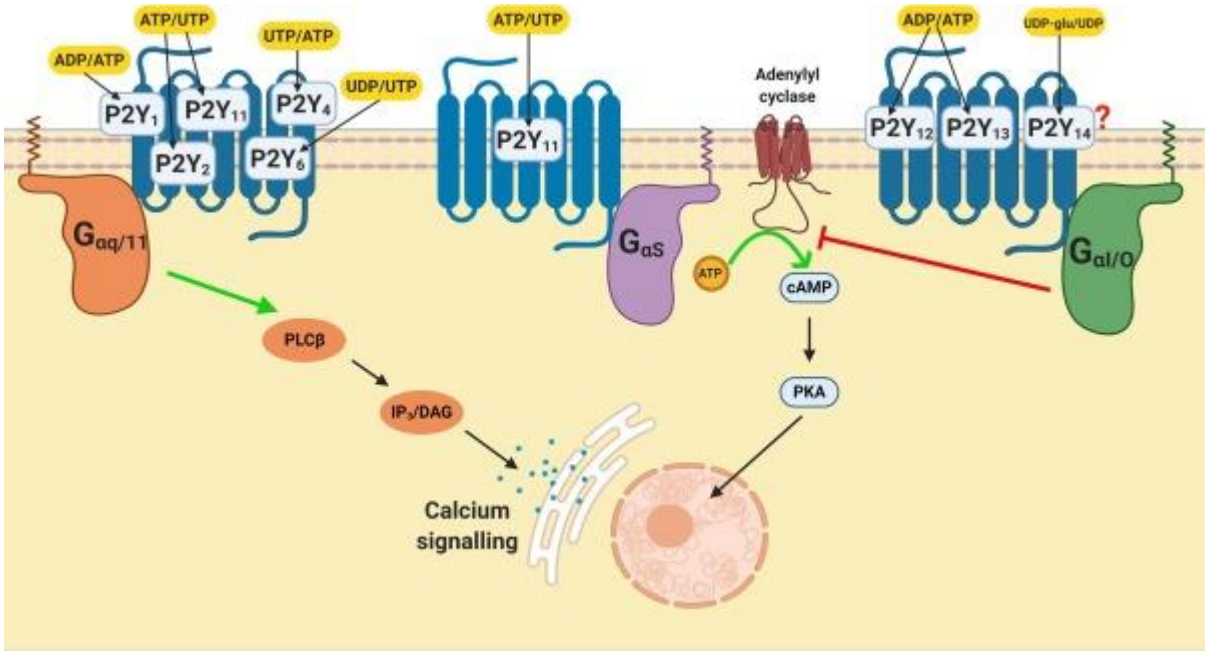

Figure 3: A schematic representation of P2YRs, their preferred endogenous agonists, and their downstream signalling effects.

The endogenous agonists activating the respective receptor are at physiological relevant conditions and the specific G-protein coupling is shown (Neumann *et al.*, 2020). P2YRs signal through either G_q/G11 proteins resulting in phospholipase C activation or Gs proteins leading to activation of the adenyl cyclase pathway. Signalling via Gi/Go proteins inhibits the adenyl cyclase pathway. Figure taken from Lovászi *et al*. 2021.

Unlike the P2XR, P2YRs can be activated by a variety of adenosine- and uridine-based nucleotides, however, the ability of the nucleotides varies between subtypes (Figure A; Abbracchio *et al.* 2006; Neumann *et al*., 2020). ATP can bind to all P2YRs with the exception of P2Y6 (Jacobson *et al.* 2012).

P2Y receptors are linked to PLC activation that catalyses the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate into the intracellular messenger IP3 and diacylglycerol (DAG) (Van Kolen & Slegers. 2006; Kügelgen & Wetter, 2000). Activation of PLC occurs via Giα- and/or Gqα-dependent mechanisms (Communi *et al.,* 2000). IP3 binds and activates the IP3R causing calcium release from the endoplasmic reticulum resulting in increased intracellular calcium concentrations. Both IP3Rs and RyRs are sensitive to Ca^{2+} concentrations in the lumen of the endoplasmic reticulum and sarcoplasmic reticulum and high cytosolic Ca^{2+} can lead to negative feedback resulting in inhibition (Woll & Van Petegem. 2021).

1.1.2.5 P2Y receptors in the Nervous system

Endogenous P2Y receptor agonists are released from cells in the CNS under various conditions, including exocytosis at nerve terminals, the opening of pannexin 1 hemichannels, hypoxia and apoptosis. All 8 subtypes of P2Y receptors are expressed in cells that comprise the CNS (e.g., neurons, astrocytes, microglia, and endothelial cells) (Weisman *et al*., 2012).

P2Y1 receptors are widely expressed in the brain, including the cerebral cortex, hippocampus, caudate nucleus, putamen, globus pallidus, habenula, subthalamic nucleus, midbrain, and cerebellum (Weisman *et al.* 2012; Morán-Jiménez & Matute., 2000). In the prefrontal cortex, previous research has found P2Y1 receptors to be colocalised with synaptophysin and vGLUT3 suggesting P2Y1 expression in presynaptic terminals involved in the release of neurotransmitters including glutamate, acetylcholine, serotonin and GABA (Koch *et al*., 2015).

The P2Y2 has been found to be upregulated in cell and animal models under a variety of conditions associated with inflammation or injury, including spinal cord injury and brain trauma, suggesting P2Y2 may exert a protective role in the brain (Weisman *et al.,* 2012). Various P2Y receptors have been linked to the inhibition of neurotransmitter release. P2Y1, P2Y2, P2Y4, P2Y12, and P2Y13 receptors have been shown to inhibit glutamate release from the sensory terminals in the spinal cord, from Schaffer collateral synapses of the hippocampus, and in the cerebral cortex. P2Y4 receptors have been linked to the inhibition of GABA release in the cerebellum. Furthermore, serotonin release was shown to decrease in response to P2Y activation in the cortex. P2Y1 has been shown to have inhibitory effects on NE and DA release, with P2Y12 and P2Y13 also affecting the NE release cortex (Sperlágh *et al.,* 2007; Guzman *et al.,* 2016). It is worth noting that dopaminergic terminals in the prefrontal cortex do not contain P2Y1 receptors, however, inhibition of DA occurred, suggesting the introduction of a multi-synaptic mechanism.(Guzman et al., 2016).

In P2Y1 knockout mice and after antagonism of P2Y1 receptors, decline in cognitive function and glial neuroinflammatory response were observed after middle cerebral artery occlusion was observed in both rats and mice (Chin et al., 2013). P2Y12 knockout mice showed more anxiety-like behaviour, impaired memory, and abnormal locomotor activity (Zheng et al., 2018). Furthermore, P2Y12 deficient mice have been shown to exhibit reduced seizure-induced increases in microglial process numbers and worsened KA-induced seizure behaviours (Eyo *et al.,* 2014).

1.3 Adrenergic signalling

1.3.1 Catecholamines

The sympathetic nervous system plays a pivotal role in maintaining the body's homeostasis by secreting different neurotransmitters including catecholamines, acetylcholine, and glutamine. The synthesis of catecholamines begins with the rate-limiting step managed by the enzyme tyrosine hydroxylase, which converts tyrosine into L-DOPA (L-dihydroxyphenylalanine). Following this, L-dopa is processed to DA by L-aromatic amino acid decarboxylase. From there, NE is formed by dopamine-β-hydroxylase. Finally, epinephrine (Epi) is synthesised by the binding of a methyl group to NE facilitated by phenylethanolamine-N-methyltransferase. NE and Epi both act as neurotransmitters for sympathetic innervation (Chhatar & Lal, 2021).

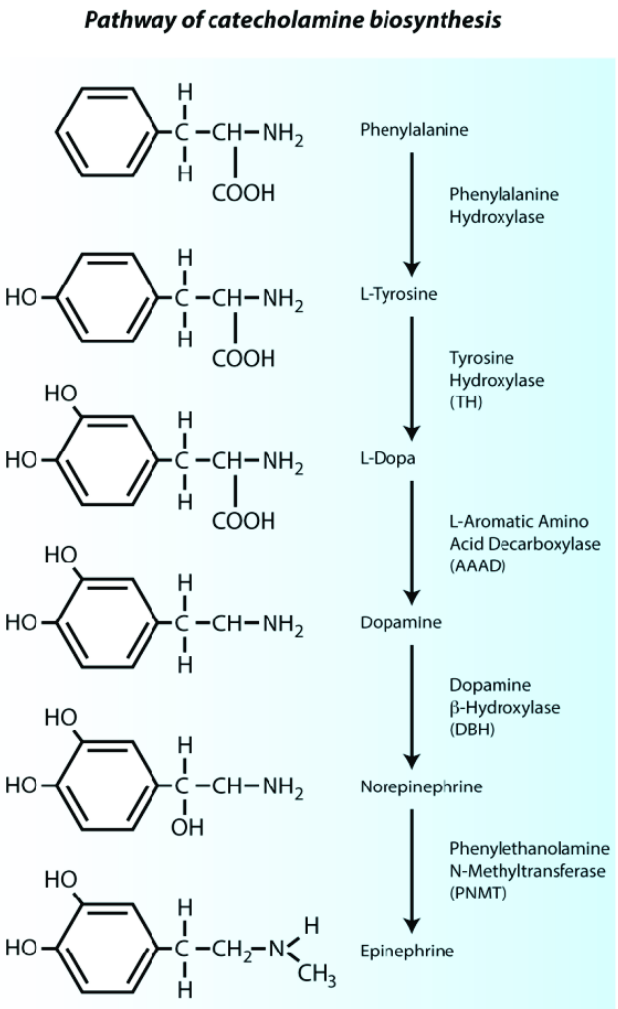

Figure 4: Synthesis of catecholamines.
Figure taken from Végh, A.M.D *et al.,* 2016

1.3.2 Adrenergic receptors

Adrenergic receptors (ARs) are membrane proteins mediating the actions of NE and Epi in the human body. (Wu *et al.,* 2021). These receptors possess seven hydrophobic transmembrane regions, an intracellular C-terminal domain, and an extracellular N-terminal domain, along with 3 intracellular and extracellular loops. The N-terminal domain contains sites for N-linked glycosylation (Chhatar & Lal. 2021).

Similarly, to P2Y receptors, ARs belong to the class of GPCRs that can be classified, based on specificity to Epi (Chhatar & Lal. 2021), into three major subtypes: α_1, α_2, and β, each having three subtypes: α_{1A}, α_{1B}, α_{1D}; α_{2A}, α_{2B}, α_{2C}; and β_1, β_2, β_3, respectively. (Qiao *et al.*, 2018; Wu *et al.,* 2021). Members within the major types share highly similar sequences and functions, while these aspects differ among the three major types. (Wu *et al.,* 2021).

α_1-ARs play an important signalling role within vasculatures and mediate vasoconstriction. Exogenous and endogenous Epi and NE activate these receptors through the Gq family of G proteins stimulating

the hydrolysis of membrane phospholipids resulting in the subsequent generation of IP3 and diacylglycerol (DAG). IP3 binds to its receptor (IP3R) leading to the mobilisation of calcium from intracellular storage sites in the endoplasmic reticulum resulting in subsequent smooth muscle contraction (Archer *et al*., 2021; Hawrylyshyn *et al.,* 2004).

The α_2-ARs are located on both pre-and postsynaptic neurons, where they exert an inhibitory role in the CNS and periphery (Drouin *et al.,* 2017). Contrary to α_1-ARs, α_2-ARs respond to both Epi and NE through the G_i family of G proteins, which inhibit adenyl cyclase resulting in the inhibition of NE release from presynaptic neurons (Archer *et al.,* 2021). In the CNS, these receptors can regulate neurotransmitter release as an autoreceptor when located on NE nerve terminals or as a heteroreceptor when bound on non-NE nerve terminals. In addition, α_2-ARs have been linked to the stimulation of Ca^{2+} influx, as well as the activation of K^+ channels, phospholipase A2 and Na^+-H^+ exchange (Drouin *et al.,* 2017).

In addition to α1-ARs, β-ARs are responsible for mediating many of the effects of NE released from the sympathetic nervous system and Epi released from the endocrine system. Through the Gs family of G proteins, they activate the adenylyl cyclase signalling pathway resulting in Ca^{2+} release (Archer *et al.,* 2021).

1.4 Neuroblastoma Model

BE(2)-M17 is a clone of the SK-N-BE(2) neuroblastoma cell line that was established in November of 1972 from a bone marrow biopsy taken from a 2-year-old male with disseminated neuroblastoma after repeated courses of chemotherapy and radiotherapy. BE(2)-M17 cells are multipotential concerning neuronal enzyme expression e.g., choline acetyltransferase, acetylcholinesterase and dopamine-β-hydroxylase implying cholinergic, dopaminergic and adrenergic properties (Andres *et al*, 2013). We used the BE(2)-M17 human neuroblastoma cell line due to its limited exploration and its prominent expression of proteins associated with dopamine and norepinephrine release to model neurotransmitter release. BE(2)-M17 neuroblastoma cells have been shown to differentiate after prolonged exposure to Retinoic Acid, Staurosporine, phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Due to the aims of this study, we decided to use Staurosporine as this had been shown to have to most significant contrast of DA and NE release compared to undifferentiated cells (Filograna *et al.,* 2015)

1.5 Wider Scientific Applications

The identification of P2 receptors BE(2)-M17 neuroblastoma cell line has yet to be established. Here we explore the expression of P2 receptors on these cells. Identification may allow future use of this cell line as an alternative to the SH-SY5Y cell line and allow the exploration of the relationship between purinergic signalling and noradrenergic and dopaminergic signalling. On a wider scale, many key diseases have been linked to norepinephrine and dopamine release including hypertension (FitzGerald et al., 1981), Parkinson's disease (Franco et al., 2021), Alzheimer's disease (Pan et al., 2019), and ADHD (Blum et al., 2008)

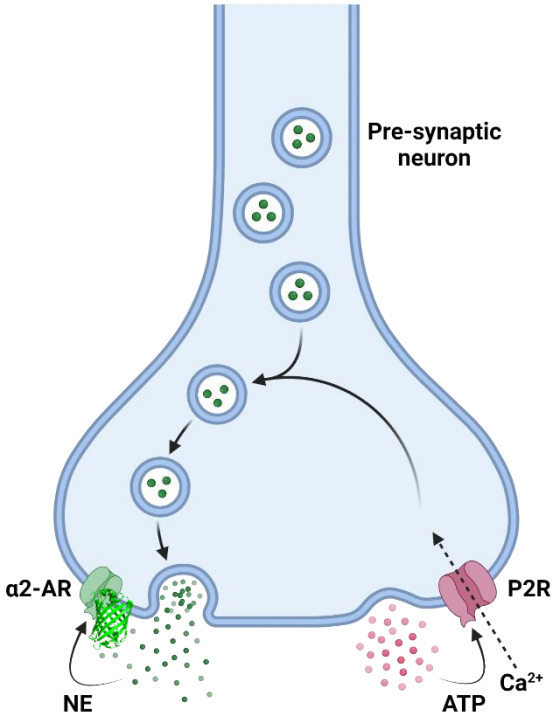

Figure 5: Proposed mechanism for ATP modulation of NE release.

We hypothesise that ATP released from pre-synaptic neurons may induce or inhibit the release of NE. NE release activates pre-synaptic α2-ARs resulting in an increase in fluorescence from the $GRAB_{NE}$ biosensor attached.

Figure created using Biorender.

1.6 Aims and Hypotheses

In this study, we hypothesise that similarly to SH-SY5Y and SK-N-BE neuroblastoma cells, BE(2)-M17 neuroblastoma cells express P2 receptors. Furthermore, we suggest that ATP- and/or UDP- activation of these P2 receptors may enact a modulatory role on the α2-adrenegric receptor. The aims of this study were to confirm the expression of P2 receptors through ratiometric calcium mobilisation assays in both undifferentiated and Staurosporine-mediated differentiated BE(2)-M17 neuroblastoma cells. Furthermore, we aimed to successfully transiently transfect undifferentiated BE(2)-M17 neuroblastoma cells with the $GRAB_{NE}$ biosensor to measure norepinephrine release in response to endogenous nucleotides.

Chapter 2: Materials & Methods

2.1 BE(2)-M17 human neuroblastoma cell line

2.1.1 General maintenance and cell passage

BE(2)-M17 cells were grown in Dulbecco's Modified Eagle Media (DMEM) with L-glutamine, 15 mM HEPES, and sodium bicarbonate supplemented with 15% (v/v) FBS and 1% (v/v) penicillin and streptomycin solution containing 50 U/ml of Penicillin and 50 µg/mL of Streptomycin.

All cells were cultured in adhesion in vented T75 flasks maintained in a humidified environment at 37°C and 5% CO_2/95% air. Cells that reached 70-80% confluency were sub-cultured. Cells were washed twice with 10 mL of sterile Phosphate Buffered Saline and treated with 1.5 mL of trypsin/EDTA for 5 minutes in the incubator. The detached cells were diluted with 1.5 mL culture media to stop the trypsin/EDTA-mediated enzymatic reaction and centrifuged at 1200 rpm for 5 minutes. The cell pellet was resuspended in 1 mL of culture media and a 10 µL aliquot was pipetted into a Neubauer haemocytometer. Labelled flasks were seeded with a 250 µL aliquot of the culture media containing 5×10^5 cells/mL. Experimental repeats were conducted on BE(2)-M17 cells between 3 and 12 passages. BE(2)-M17 cells beyond 12 passages demonstrated issues with cell growth and yield for ratiometric calcium assays..

2.1.2 Cryopreservation and Thawing

Early passage BE(2)-m17 cells that were not required, were stored in liquid nitrogen for future use. Cells were washed twice with 10 mL of sterile Phosphate Buffered Saline, treated with 1.5 mL of trypsin/EDTA for 5 minutes in the incubator, and centrifuged at 1200 rpm for 5 minutes. The resulting pellet was resuspended in media containing 75% (v/v) DMEM and 25% (v/v) FBS. The suspension was transferred into cryovials containing 5% (v/v) DMSO and immediately placed in a Mr Frosty™ freezing container to achieve a cooling rate of -1°C/min and stored at -80°C. After at least 24 hours, the cryovials were transferred to a liquid nitrogen tank for long-term storage.

Cells were thawed in a water bath at 37°C for 60 seconds and added to a labelled T75 flask containing 20 mL of fresh media. Cells were grown in a humidified environment at 37˚C with 5% CO_2/95% air. Once 80% confluence was reached the cells were passaged and seeded for experimental use.

2.1.3 Staurosporine-mediated human BE(2)-M17 neuroblastoma cell differentiation

Cells were passaged and placed in a T75 flask containing 10 nM of Staurosporine in 20 mL of fresh culture media supplemented with 15% (v/v) FBS and 1% (v/v) penicillin/streptomycin. Every 48 hours, the media was removed, and cells were supplemented with fresh media containing 10 nM of Staurosporine. After 7 days, the cells were observed under a confocal microscope to determine if

differentiation had occurred through observable changes to neurite length and cessation of proliferation. If differentiation confluency was adequate, cells were passaged and counted. Differentiated cells BE(2)-m17 cells were seeded at 2.5 x 10^4 cells per well in 200 µL of culture media containing 10 nM of Staurosporine in a 96-well culture plate and maintained in cell culture conditions overnight for experimental use.

2.2 Ratiometric calcium indicator

Fura-2-acetoxymethyl ester (Fura-2-AM), is a membrane-permeable, non-invasive derivative of the ratiometric calcium indicator fura-2. Fura-2-AM crosses cell membranes and once inside the cell, the cellular esterases remove the acetoxymethyl group (Martinez *et al.,* 2017). Fura-2 possesses an excitation spectrum at 380 nm (emission at 500 nm) as a Ca^{2+}-free form. When the dye binds to Ca^{2+}, the excitation is shifted to 340 nm (with the same emission wavelength at 500 nm). Thus, the increase in $[Ca^{2+}]_i$ excites the dye in the Ca^{2+}-binding form at 340 nm, along with a decrease in fluorescence intensity at 380 nm from the free form of the dye (Grynkiewicz *et al.,* 1985) allowing the calculation of an F_{ratio} to quantify a nucleotide-mediated Ca^{2+} response.

2.2.1 Salt-buffered saline and loading buffer

Salt buffered saline (SBS) was prepared in deionised water and contained (mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, D-Glucose 8, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10. SBS was titrated with NaOH to a pH of 7.4 at a 1 mL final volume. A loading buffer (LB) solution was prepared by adding 0.01% (w/v) Pluronic F-127 to 200 mL of SBS.

2.2.2 Calcium mobilisation assays

BE(2)-m17 cells were seeded at 2.5 x 10^4 cells per well in 200 µL of culture media in a 96-well culture plate and maintained in cell culture conditions overnight to ensure an adherent monolayer forms. After 24 hours, the culture media was carefully removed. Cells were washed twice with 200 µL of SBS and then incubated in the dark in 200 µL of LB supplemented with 2 µg/mL of Fura-2-AM for 1 hour at 37°C. After 1 hour, cells were carefully washed three times with 200 µL of SBS and then left to rest in 200 µL SBS. A concentration range of 1 µM to 1 mM (ATP) and 1 nM to 3 µM (UDP) was prepared and 80 µL of each concentration was added to the corresponding wells of a 96-well U-bottomed plate. SBS and 100 µM ionomycin was also added to the plates as negative and positive controls, respectively.

Cells were exposed to 50 µL of nucleotides administered by the FlexStation III microplate reader. Fluorescence readings of 3 reads per well were recorded at two excitation wavelengths (340 nm and 380 nM) and a 510 nm emission wavelength every 3.42 seconds over 250 seconds, calculating F_{ratio} values. All experiments were performed at 37°C.

2.3 GRAB$_{NE}$ transient transfection

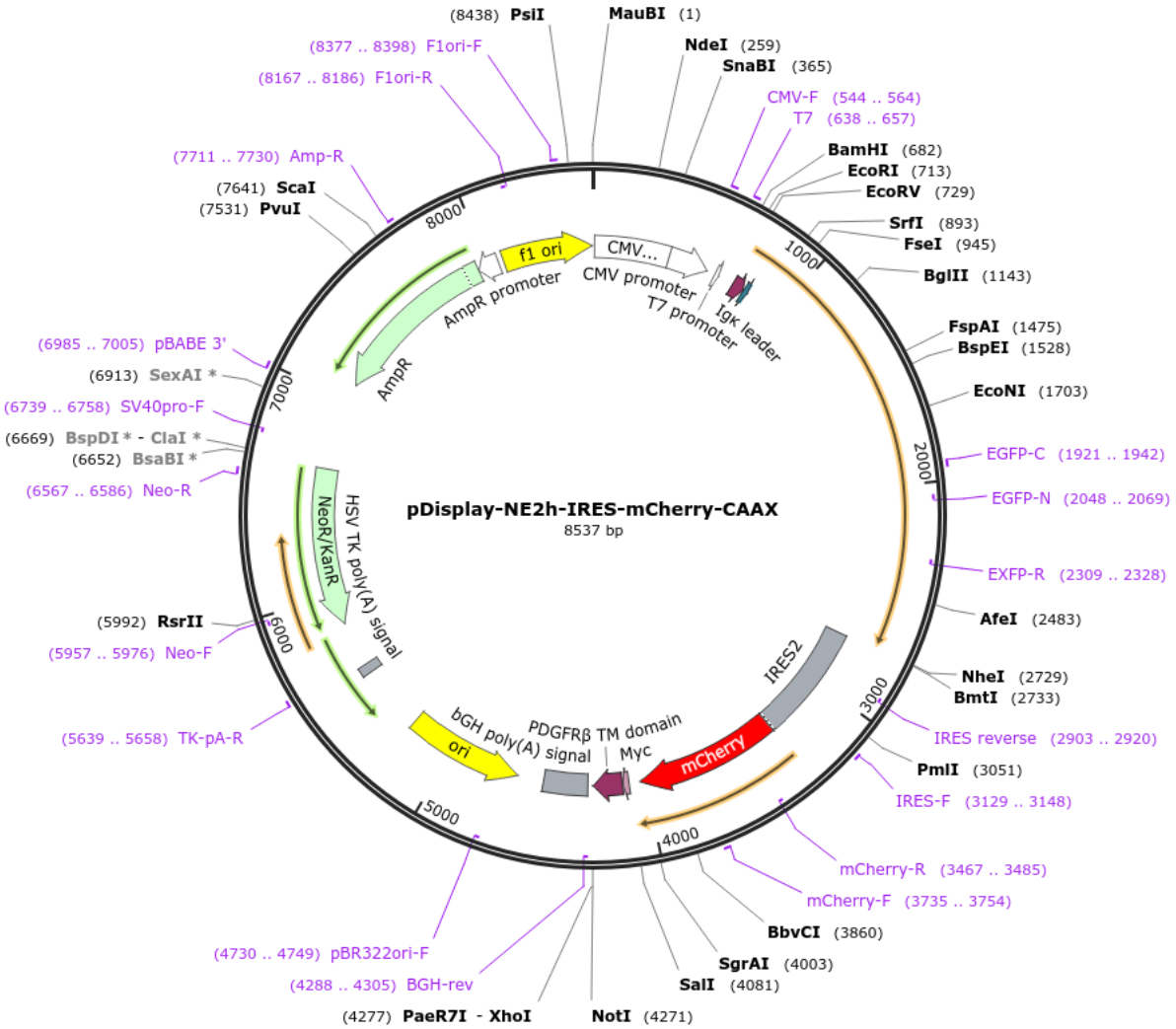

Figure 6: Comprehensive diagram of the GRAB$_{NE}$ plasmid used in the transient transfection of undifferentiated BE(2)-M17 neuroblastoma cells
Figure taken from Feng et al., 2023.

The pDisplay-GRAB-NE2hF6.341-IRES-mCherry plasmid was a gift from Professor Yulong Li from Peking University, China. The GRAB$_{NE}$ plasmid was cloned into the pDisplay vector (Invitrogen) with an upstream IgK leader sequence and a downstream IRES-mCherry-CAAX cassette to label the cell membrane. The circular permutated EGFP was designed to be inserted into the third intracellular loop domain of the α2-AR (Feng *et al.,* 2019). The pDisplay vector contained an Ampicillin resistance gene to prevent non-transfected bacteria from growing.

The GRAB$_{NE}$ biosensor is a genetically encoded single-wavelength fluorescent GPCR activation-based NE sensor with an excitation of 488 nm and an emission of 525 nm. The binding of NE to the sensor causes a conformational change between the fifth and sixth transmembrane domains which alters the chemical environment, causing de-protonation of the cpEGFP chromophore and the resulting increase

in fluorescence. In this study, we use the $GRAB_{NE1h}$ biosensor as opposed to the $GRAB_{NE1m}$. The $GRAB_{NE1h}$ biosensor contains a single T6.34K point mutation which is close to the highly conserved E6.30 site and was shown to result in a 10-fold increase in its NE detection sensitivity (EC_{50} = ~83 nM) compared to the $GRAB_{NE1m}$ biosensors (EC_{50} = ~930 nM) sensitivity (Feng *et al.,* 2019).

The $GRAB_{NE}$ plasmid arrived in a circled area on Whatman #1 filter paper. The circle was cut out and dissolved in deionised water.

2.3.1 LB broth and agar

LB broth was prepared by adding 10 g of LB broth powder to a Duran bottle containing 500 mL of deionised water. The broth was autoclaved and left to cool until room temperature for future use.

To prepare agar plates for bacterial growth, LB agar powder was dissolved in a Duran bottle containing deionised water following the manufacturer's instructions. The LB agar was autoclaved and left to cool. Once the LB agar reached an adequate temperature to handle, a 100 µg/mL aliquot of ampicillin was added to the bottle and mixed thoroughly. Immediately, 20 mL of the LB agar was carefully pipetted into a petri dish. The plates were left to solidify at room temperature and stored at 4°C for up to two weeks.

2.3.2 High-efficiency transformation

Following the dissolving of the plasmid, the product was transformed into competent *Escherichia Coli* BL21 (*E.Coli*) cells. A 5 µL aliquot of the plasmid solution was added to 50 µL of the highly competent *E.Coli* cells, previously thawed on ice for 5 minutes. The cryogenic vial was gently mixed by hand and placed on ice for 30 minutes. The cells were heat-shocked at 42°C for 30 seconds and immediately placed back on ice for 5 minutes. After the vial had cooled, a 450 µL aliquot of Super Optimal broth containing catabolite repression outgrowth media was added to the *E.Coli* cells. The vial was placed into a 15 mL falcon tube and incubated in a shaker at 225 rpm and 37°C for one hour. During incubation, LB agar plates supplemented with 100 µg/mL ampicillin were warmed to 37°C in a separate incubator.

After one hour, the cells were mixed thoroughly by inverting the cryovial and one 50 µL aliquot and one 200 µL aliquot of the *E.Coli* culture were spread onto separate LB agar plates and incubated overnight at 37°C. The following morning, colonies were observed on the agar and two single colonies were selected and inoculated into separate universal tubes containing 20 mL of LB broth supplemented with 100 µg/mL ampicillin. The universal tubes were incubated in a shaker at 225 rpm and 37°C for 18 hours.

2.3.3 DNA extraction

For storage of bacteria, a 1.2 mL aliquot of the *E.Coli* broth and 400 µL of a 50% glycerol stock was added to a cryovial, mixed thoroughly by pipetting the liquid up and down and stored at -80°C.

After 18 hours, the universal tubes were centrifuged for 20 minutes at 2000 rpm and the supernatant was discarded. The pellet was purified using the E.Z.N.A. Plasmid DNA Mini Kit I according to the manufacturer's instructions. The concentration and purity of the acquired DNA were measured using spectrophotometry and stored in 1.5 mL Eppendorf tubes at -20°C.

2.3.4 Transient transfection of undifferentiated human BE(2)-M17 neuroblastoma cells

BE(2)-m17 cells were seeded at 1.25 x 10^4 cells per well in 200 µL of culture media in a 96-well culture plate and maintained in culture conditions for 24 hours. After 70-80% confluency, culture media was removed and 150 µL of culture media absent of antibiotics was added to each well. The cells were transiently transfected with a 50 µL aliquot containing 200 ng DNA ($GRAB_{NE}$) and 0.5 µL lipofectamine 2000 per well (diluted in 1080 µL opti-mem and pre-incubated together at room temperature for 20 min before adding to wells). After 8 hours, the media was removed and replaced with 200 µL of fresh culture media supplemented with FBS and antibiotics. Cells were then maintained in culture conditions for 48 hours.

2.4 Norepinephrine detection assays

2.4.1 High potassium Salt buffered saline

High K^+ SBS was prepared in deionised water and contained (mM): NaCl 130, KCl 80, MgCl2 1.2, CaCl2 1.5, D-Glucose 8, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10. High K^+ SBS was titrated to a pH of 7.4 at a 1 mL final volume. High K^+ SBS was used as a control to measure a neuronal response.

2.4.2 $GRAB_{NE}$ Sensitivity and selectivity assays

After 48 hours in a 96-well plate, cell media was removed, and cells were washed twice with 200 µL of SBS and then left to rest in 200 µL SBS at 37°C. A concentration range from 1-1000 nM of norepinephrine, epinephrine, or dopamine was prepared and 80 µL of each concentration was added to the corresponding wells of a 96-well U-bottomed plate. The baseline mCherry fluorescence was recorded with 3 reads per well at a 561 excitation and 600 emission wavelength.

Cells were exposed to 50 µL of neurotransmitters administered by the FlexStation III microplate reader after 20 seconds. Fluorescence readings of 3 per well were recorded at a 488 nm excitation wavelength and a 525 nm emission wavelength every 0.93 seconds over 250 seconds. All experiments were performed at 37°C.

2.4.3 Inducing Norepinephrine release through Exogenous Nucleotide exposure

After 48 hours in a 96-well plate, cell media was removed, and cells were washed twice with 200 µL of SBS and then left to rest in 200 µL SBS at 37°C. Another 96-well plate was prepared containing 80 µL of the maximum concentrations for ATP and UDP at 1 mM and 3 µM, respectively; the concentrations of ATP and UDP calculated to evoke the EC_{50} response at 100 µM and 1 µM , respectively. The well also contained 100 nM of norepinephrine as a positive control, 100 µM of ionomycin, SBS as a negative control and 80 mM K^+ SBS.

Cells were exposed to 50 µL of the nucleotides administered by the FlexStation III microplate reader after 20 seconds. Fluorescence readings of 3 per well were recorded at a 488 nm excitation wavelengths and a 525 nm emission wavelength every 0.93 seconds over 250 seconds. All experiments were performed at 37°C.

2.5 Data Analysis

Data were analysed using Origin Pro 2022 software. Data were expressed as mean ± SEM (Standard Error of the Mean). All experiments were performed in at least triplicates and the "n" number will refer to the number of experimental repeats performed on independent days using a different cell line passage number between 3 and 12 passages.. All data were tested for normal distribution using the Shaprio-wilk test and all data was tested for equal variance using Levene's test.

2.5.1 Calcium mobilisation assay analysis

Data was exported from SoftMax FlexStation III software. The background fluorescence was subtracted and the resulting F_{ratio} values were normalised to the maximal nucleotide response. Peak data represents the maximum amplitude after nucleotide exposure at 20 seconds. Nucleotide-mediated dose-response curves were fitted following the Hill1 equation and the EC_{50} (half the maximal effective concentration to give half the maximal response) values were obtained from the fitted curve. For ATP, the peak response of the lowest concentration (1 µM) was subtracted from each value to ensure a baseline response of 0% was met. Maximal response and potency were compared statistically using the Two-sample T-test.

2.5.2 $GRAB_{NE}$ Sensitivity and selectivity assays

Data was exported from SoftMax FlexStation III software. The background fluorescence was subtracted, and the resulting peak fluorescence values were normalised to the maximal response of the neurotransmitter. The peak data represents the maximum amplitude after exposure to the neurotransmitter at 20 seconds. Neurotransmitter-mediated dose-response curves were fitted following the Hill1 equation and the EC_{50} values were obtained from the fitted curve. For dopamine, the background fluorescence was not subtracted from the peak response and the peak response of

the lowest concentration (1 nM) was subtracted from each value to ensure a baseline response of 0% was met. Maximal responses and potency of catecholamines were compared statistically using the Two-sample T-test.

$$\text{Hill 1 Function y} = \text{START} + (\text{END} - \text{START})\frac{x^n}{k^n + x^n}$$

Where k is Michaelis constant, and n represents the number of cooperative sites. EC_{50} values calculated from this equation were used for statistical analysis, as well as the maximal responses.

Chapter 3: Results

3.1 Characterisation of nucleotide-evoked intracellular calcium responses in undifferentiated and differentiated human BE(2)-M17 neuroblastoma cells

3.1.1 Introduction & Aim

Immortalised neuronal cell lines can be induced to differentiate into more mature neurons by adding specific compounds or growth factors to the culture medium. This property makes neuronal cell lines attractive as *in vitro* cell models to study neuronal functions and neurotoxicity. The clonal human neuroblastoma BE(2)-M17 cell line is known to differentiate into a more prominent neuronal cell type by treatment with Staurosporine, trans-retinoic acid, and 12-O-tetradecanoylphorbol-13-acetate (Andres *et al*., 2013; Filograna *et al.,* 2015). Undifferentiated BE(2)-M17 cells had previously been shown to indicate a catecholaminergic phenotype with Staurosporine-mediated differentiation upregulating key genes including the tyrosine hydroxylase and dopamine-β-hydroxylase activity, which is specific to NE neurons (Filograna *et al.,* 2015).

In order to investigate the presence of P2 receptors in BE(2)-M17, it would be useful to establish nucleotide-evoked calcium responses in the undifferentiated and differentiated neuroblastoma cells.

3.1.2 Results

The presence of P2 receptors have not been previously explored in the BE(2)-M17 human neuroblastoma cell line. To determine the presence of P2 receptors, exogenous nucleotides were applied and changes in intracellular calcium concentrations were measured.

After 7-days of Staurosporine treatment, cells demonstrated phenotypical changes to the neurite length and ceased proliferation (Fig. 7).

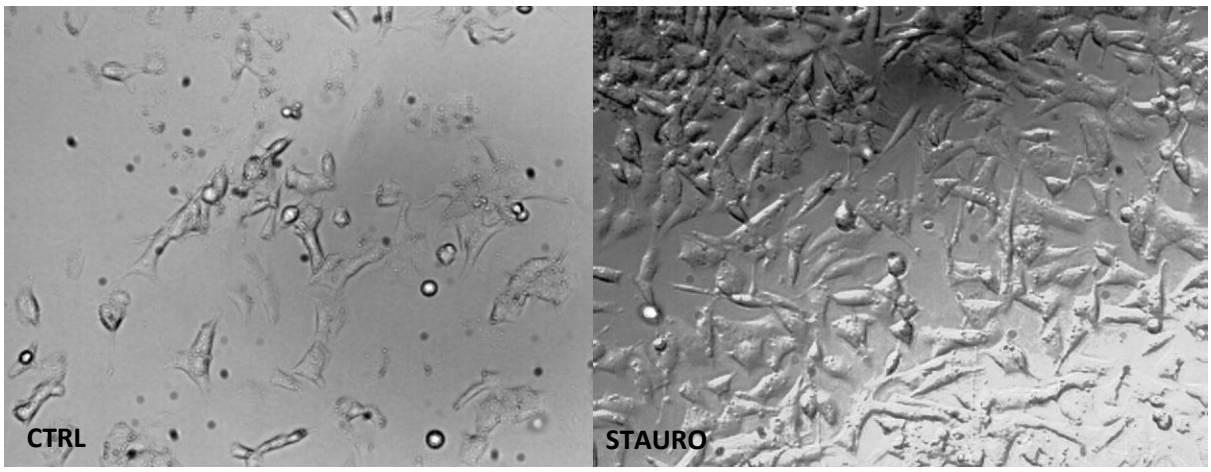

Figure 7: Cellular morphology of undifferentiated and differentiated BE(2)-M17 cells.

Representative contrast images showed that after treatment, Staurosporine promoted differentiation and neurite extensions in human BE(2)-M17 neuroblastoma cells (n = 3).

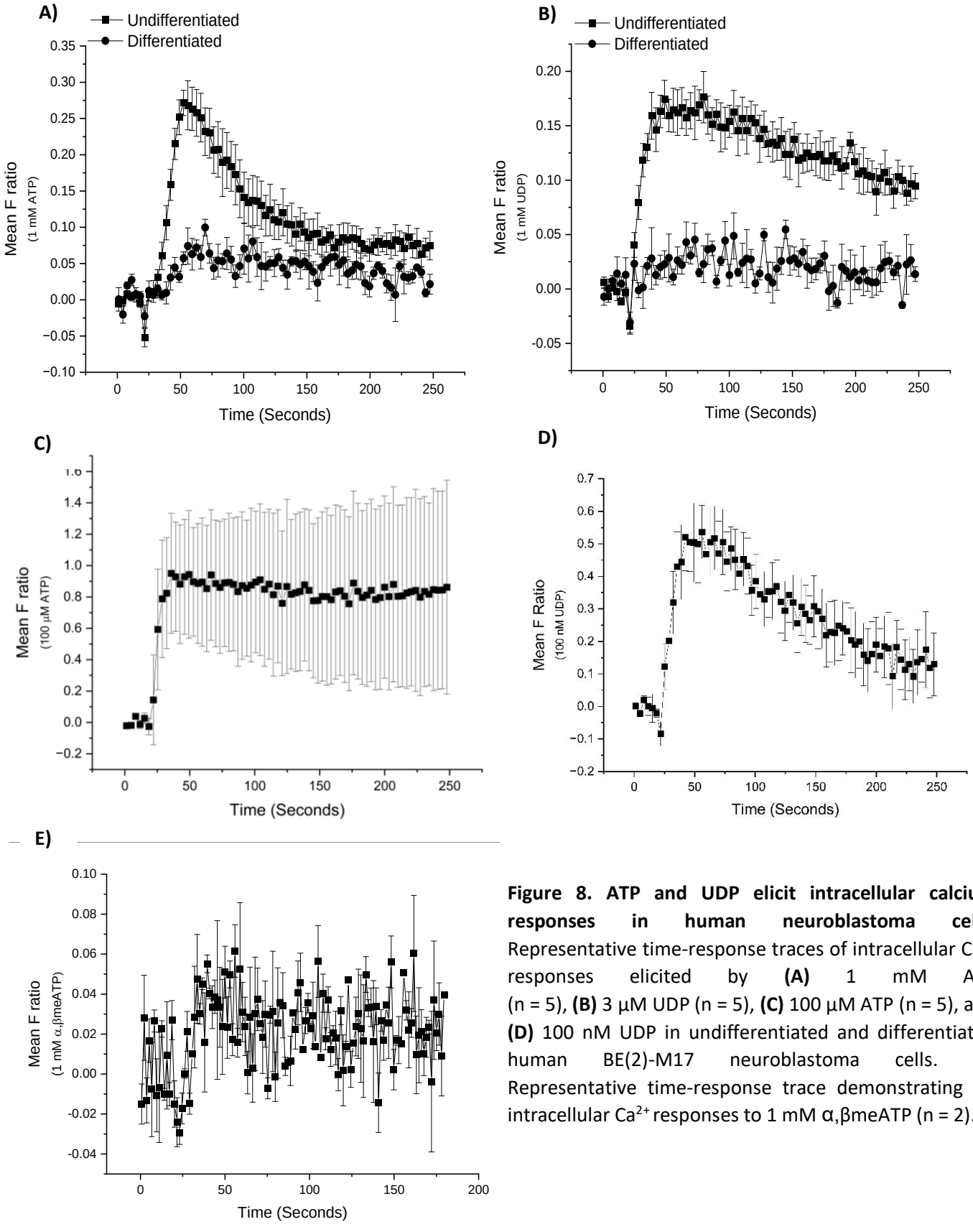

Figure 8. ATP and UDP elicit intracellular calcium responses in human neuroblastoma cells. Representative time-response traces of intracellular Ca^{2+} responses elicited by **(A)** 1 mM ATP (n = 5), **(B)** 3 µM UDP (n = 5), **(C)** 100 µM ATP (n = 5), and **(D)** 100 nM UDP in undifferentiated and differentiated human BE(2)-M17 neuroblastoma cells. **(E)** Representative time-response trace demonstrating no intracellular Ca^{2+} responses to 1 mM α,βmeATP (n = 2).

Both ATP and UDP evoked a concentration-dependent increase in $[Ca^{2+}]_i$ in undifferentiated human BE(2)-M17 neuroblastoma cells, however neither ATP nor UDP evoked an intracellular calcium response in differentiated human BE(2)-M17 cells (Fig. 8a and 8b). Responses to ATP and UDP were biphasic: an initial rapid $[Ca^{2+}]_i$ response which peaked and was followed by a decaying sustained phase.

ATP showed a steeper sustained phase almost reaching baseline levels (73.43% ± 5.57% decrease) after 250 seconds, whereas UDP demonstrated a longer sustained phase (45.63% ± 4.23% decrease) after 250 seconds. α,βmeATP did not evoke an $[Ca^{2+}]_i$ response in undifferentiated human BE(2)-M17 neuroblastoma cells at concentrations between 1 µM and 1 mM (n = 2) (Fig. 8E).

α,βmeATP is a known agonist of the three receptors is a specific agonist of P2X1, P2X3 and P2X2/P2X3 receptor (Jarvis & Khakh, 2009). Previous research has demonstrated the potential role of P2X1 and P2X3, or PX2/P2X3 receptors in the facilitation of norepinephrine release in the Rat vas deferens and Hippocampus. and determination of their presence could have been utilised in the future experiments shown hereafter (Queiroz *et al.,* 2003).

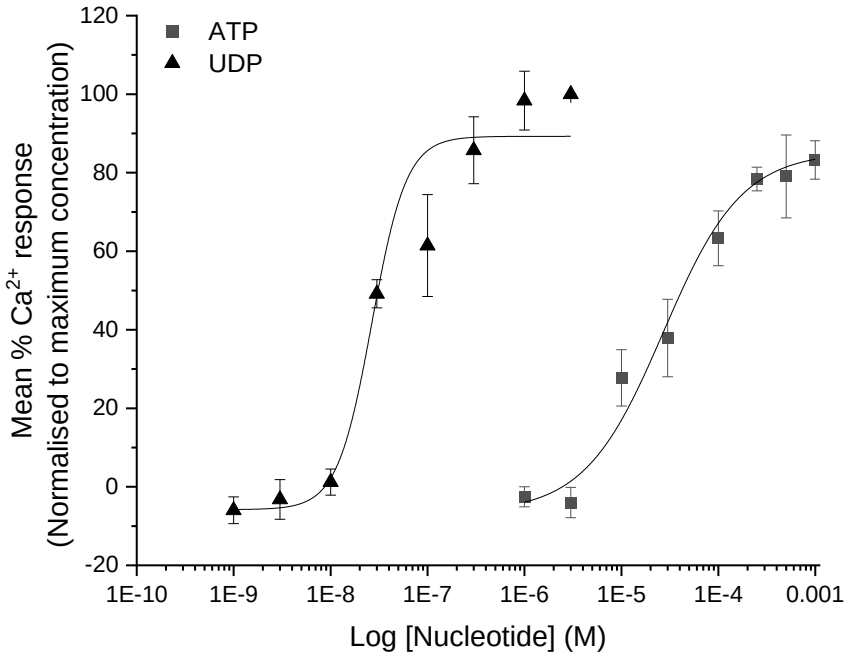

Figure 9. ATP and UDP elicit a concentration dependent calcium responses in human neuroblastoma cells.

Concentration-response curves for the peak intracellular Ca^{2+}-responses elicited by ATP (1 µM - 1 mM; n = 5) and UDP (1 nM – 3 µM; n = 5).

A concentration-response curve was measured to determine the potency and the maximal response of ATP and UDP in undifferentiated neuroblastoma cells (Fig. 9). There was a statistically significant (P=0.027) difference between the potency of ATP (EC_{50} = 120.71 ± 44.80 μM) and UDP (EC_{50} = 50.20 ± 17.14 nM) when normalised to 10 μM of ionomycin. Furthermore, there was a statistically significant difference between the maximal responses of ATP (19.00 ± 2.96) and UDP (9.84 ± 2.30) when normalised to 10 μM of ionomycin.

3.2 Characterisation of the catecholamine selectivity and sensitivity of the GRABNE biosensor in undifferentiated human BE(2)-M17 neuroblastoma cells

3.2.1 Introduction

NE is a key monoamine neurotransmitter in the central nervous systems and peripheral organs of vertebrate organisms. Despite its clear importance in a wide range of physiological processes, recording NE changes *in vivo* and *in vitro* was met with challenges. Previous methodologies such as microdialysis-coupled biochemical analysis, nano-LC-microdialysis, and fast-scan cyclic voltammetry are invasive *in vivo* techniques that require direct cranial insertion and demonstrate low spatial and temporal resolution (Feng *et al.,* 2019). Recent innovations for non-invasive real-time imaging included the cell-based neurotransmitter fluorescent engineered receptors (Muller *et al.,* 2014) which converted an extracellular NE signal into an intracellular calcium signal that can be measured using fluorescence imaging. However, these require the implantation of exogenous tumour cell lines and can report only volume transmission of NE. In this study, we used the recent innovation by Feng *et al*. $GRAB_{NE}$ is a genetically encoded single-wavelength fluorescent GPCR activation-based NE sensor with rapid kinetics, a $\Delta F/F_0$ dynamic range of ∼200%, and EGFP-comparable spectra, brightness, and photostability that can be widely applicated using several *in vitro* and *in vivo* techniques (Feng *et al.,* 2019).

3.2.2 Aims

Here we aimed to successfully express the $GRAB_{NE}$ biosensor into undifferentiated BE(2)-M17 neuroblastoma cells, determine the sensitivity and selectivity of the biosensor to catecholamines and determine the potency and maximal response of the catecholamines.

We also aimed to measure NE release in response to nucleotides to determine a relationship between ATP/UDP and NE which had previously been shown to be co-expressed in synapses.

3.2.3 Optimisation of transient transfection in undifferentiated human BE(2)-M17 neuroblastoma cells

Due to the $GRAB_{NE}$ plasmid containing an IRES-mCherry-CAAX cassette, we measured the mean peak fluorescence (Fig. 9A). Undifferentiated BE(2)-M17 cells demonstrated no statistically significant

difference in mean peak fluorescence for non-transfected and transfected cells. Therefore, we could not use mCherry as a precursor to measuring transfection efficiency.

To characterise if undifferentiated BE(2)-M17 cells were neuronal in nature, we exposed cells to 80 mM potassium concentrations to enforce depolarisation. Transfected undifferentiated BE(2)-M17 cells demonstrated no statistically significant difference between 80 mM and 5 mM potassium concentrations (Fig. 9B).

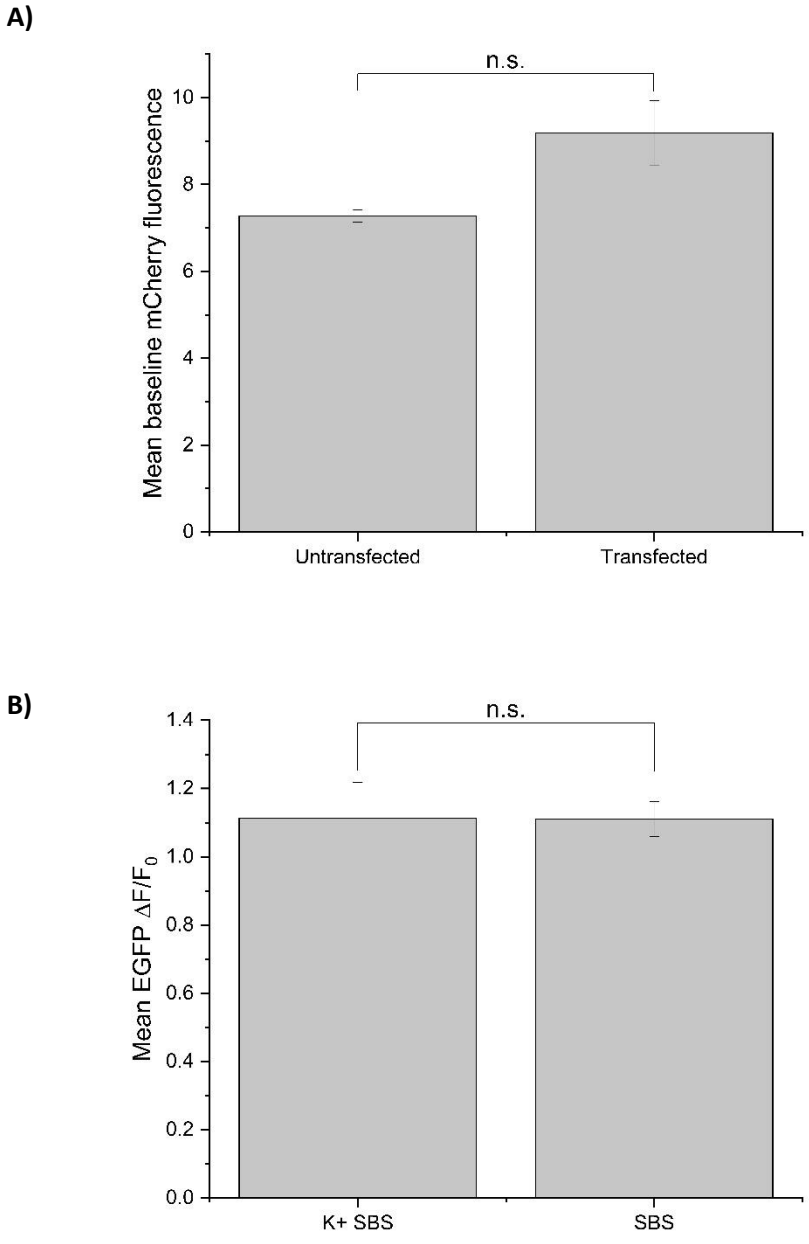

Figure 10: Transient Transfection of BE(2)-M17 with the $GRAB_{NE}$ biosensor **(A)** Comparison of the mean peak $\Delta F/F_0$ of the co-expressed mCherry Cassette in transfected and non-transfected cells (n = 5). **(B)** Mean peak $\Delta F/F_0$ response to 80 mM potassium and baseline SBS.

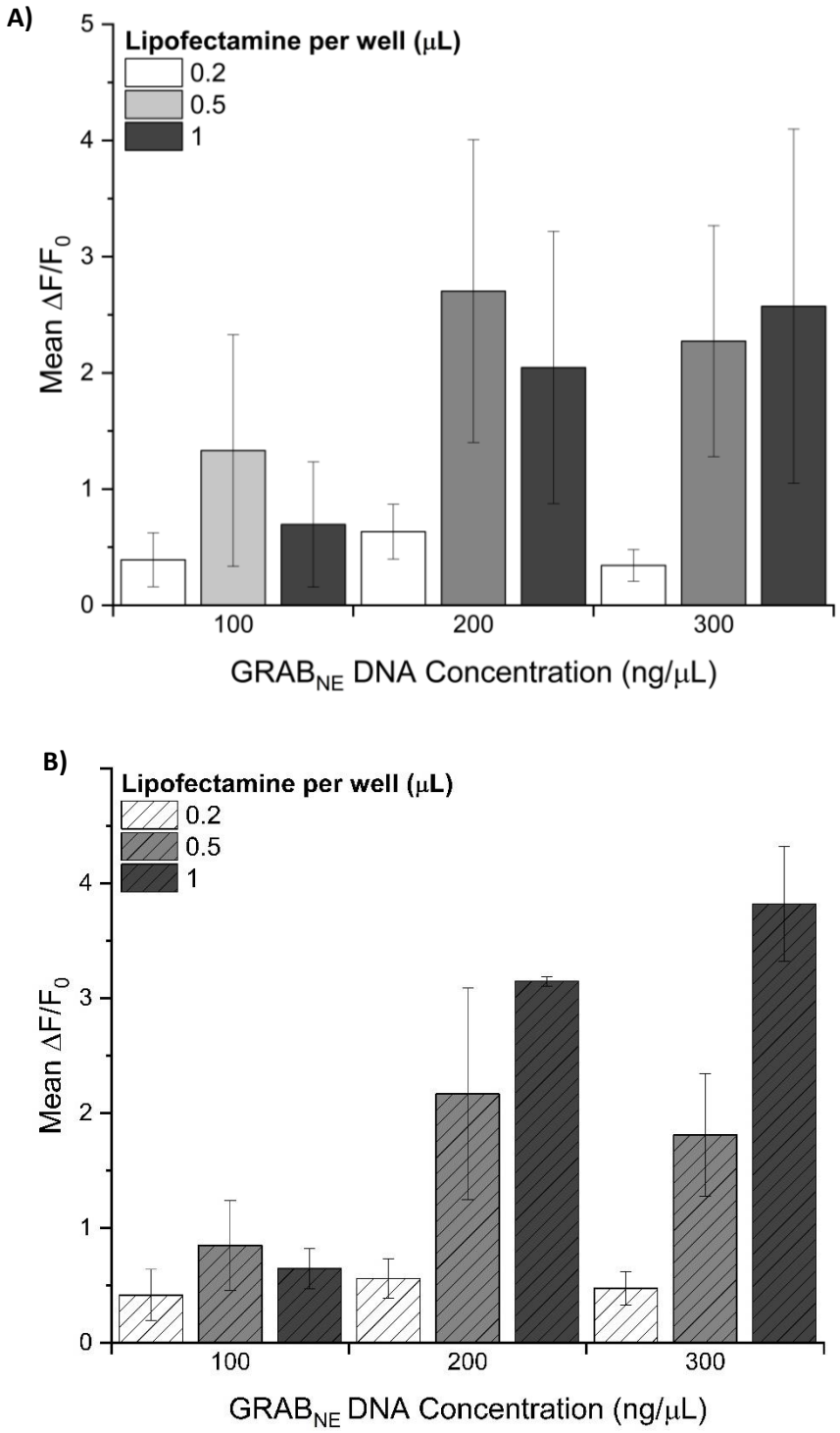

Figure 11: Optimisation of the transient transfection conditions for the $GRAB_{NE}$ biosensor. **(A)** Mean peak $\Delta F/F_0$ response to 100 µM norepinephrine after transfection with varied concentrations of DNA and Lipofectamine at 12,500 (n = 3) cells per well. **(B)** Mean peak $\Delta F/F_0$ response to 100 µM norepinephrine after transfection with varied concentrations of DNA and Lipofectamine at 25,000 (n = 3) cells per well.

We observed no baseline fluorescence at 525 nm emission; thus, we can determine the relative transfection efficiency of BE(2)-M17 cells through observing the peak magnitude of $\Delta F/F_0$ in response to the exogenous application of NE. It was important to determine the foremost conditions for transfection in order to ensure consistent responses to exogenous neurotransmitters. Therefore, we tested a variety of conditions including concentrations of Lipofectamine and $GRAB_{NE}$ cDNA, as well as cell density within the 96-well plates (Fig. 11A and 11B). From our observations, we decided to use 12,500 cells per well due to many plates suffering from overcrowding and cell death. Furthermore, we decided to use 200 ng/µL of DNA and 0.5 µL as this yielded positive, consistent results and used less resources per experiment.

3.2.4 Characterisation of the selectivity and sensitivity of the $GRAB_{NE}$ biosensor to exogenous neurotransmitters in undifferentiated human BE(2)-M17 neuroblastoma cells

Feng *et al.* established the selectivity and the sensitivity of the of the $GRAB_{NE}$ biosensor in HEK293T cells. However, the biosensor had yet to be established in undifferentiated BE(2)- M17 cells. We tested the sensitivity of the sensor to NE, and the selectivity of the sensor to NE, Epi, and DA to ensure future responses were due to NE release. Responses to exogenous NE were monophasic with very little decrease in response after the initial peak at 20 seconds. Exogenous NE elicited a concentration-dependent increase in transfected BE(2)-M17 cells and demonstrated high sensitivity to concentrations above 30 nM. The $GRAB_{NE}$ biosensor was shown to be very sensitive to norepinephrine, eliciting an EGFP signal from as low as 30 nM NE (Fig. 12C). We also observed desenstiation of the receptor at greater concentrations of norepinephrine (3 µM) (Fig. 12A and 12B).

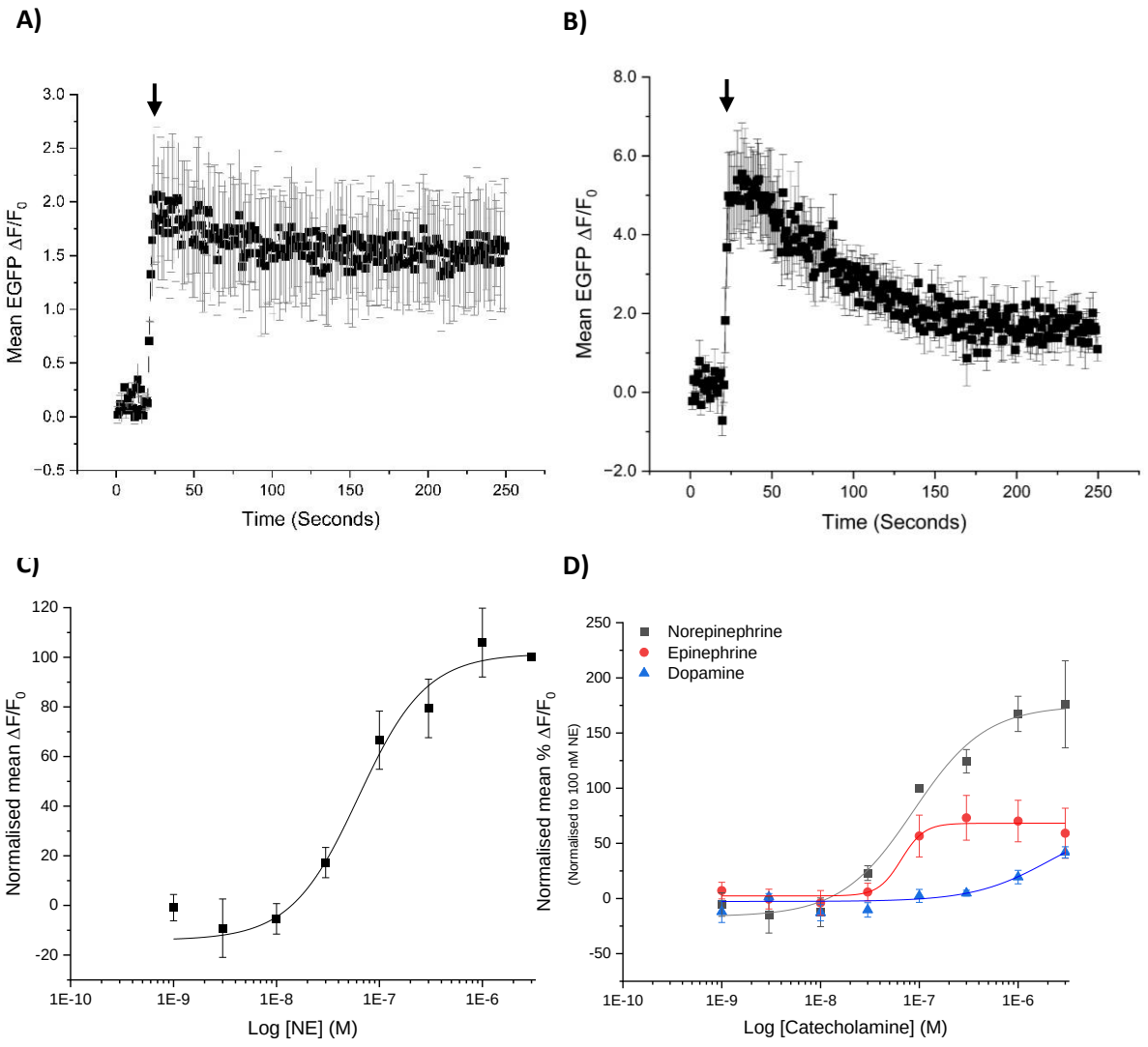

Figure 12: Exogenous neurotransmitters activate the α2-AR $GRAB_{NE}$ biosensor **(A)** Representative mean trace for 3 µM exogenous norepinephrine in undifferentiated human BE(2)-M17 neuroblastoma cells stably expressing the $GRAB_{NE}$ biosensor (n = 5). **(B)** Representative mean trace for 100 nM exogenous norepinephrine in undifferentiated human BE(2)-M17 neuroblastoma cells stably expressing the $GRAB_{NE}$ biosensor (n = 5). The arrow represents the injection of norepinephrine **(C)** Mean concentration-response curve (1 nM – 3 µM) for peak $\Delta F/F_0$ elicited by norepinephrine in undifferentiated human BE(2)-M17 neuroblastoma cells represented as a percentage of the 3 µM response (n = 5). **(D)** comparative mean concentration-response curves (1 nM – 3 µM) for peak $\Delta F/F_0$ elicited by norepinephrine (n = 5), epinephrine (n = 5), and

A concentration-response curve was measured to determine the selectivity of the receptor by measuring the potency and the maximal response of NE, Epi, and DA in transfected BE(2)-M17 cells. The potency did not statistically differ between catecholamines (Norepinephrine: 91.34 ± 27.01 nM; n = 5, Epinephrine: 59.54 ± 7.92 nM; n = 5, Dopamine: 1.19 ± 0.59 µM; n = 5), however, NE elicited a significantly greater maximal response than EPI and DOPA.

	Potency (nM)	Norepinephrine	Epinephrine	Dopamine
Norepinephrine	91.34 ± 27.01		n.s	n.s
Epinephrine	59.54 ± 7.92			n.s
Dopamine	1.19 ± 0.59			

Table 2: Statistical analysis between the potency of catecholamines acting on the $GRAB_{NE}$ biosensor in undifferentiated BE(2)-M17 neuroblastoma cells.

	Maximal Response	Norepinephrine	Epinephrine	Dopamine
Norepinephrine	176.07 ± 39.46		P = 0.033	P = 0.0097
Epinephrine	59.15 ± 22.67			n.s
Dopamine	41.84 ± 5.06			

Table 3: Statistical analysis between the Maximal responses of catecholamines acting on the $GRAB_{NE}$ biosensor in undifferentiated BE(2)-M17 neuroblastoma cells.

3.3.5 The effect of exogenous nucleotides on neurotransmitter release in undifferentiated human BE(2)-M17 neuroblastoma cells

ATP and NE are co-stored in synaptic vesicles in sympathetic nerves and when co-released act post-junctionally to evoke the contraction of visceral and vascular smooth muscle (Kennedy *et al.,* 2007). However, little is known about the effect of ATP on NE release pre-junctionally. To determine the effects of Nucleotides on NE release, we measured the mean peak $\Delta F/F_0$ with the maximal and EC_{50} concentrations of nucleotides. However, there was no statistically significant difference between the mean peak $\Delta F/F_0$ of SBS and the nucleotides (Fig. 13).

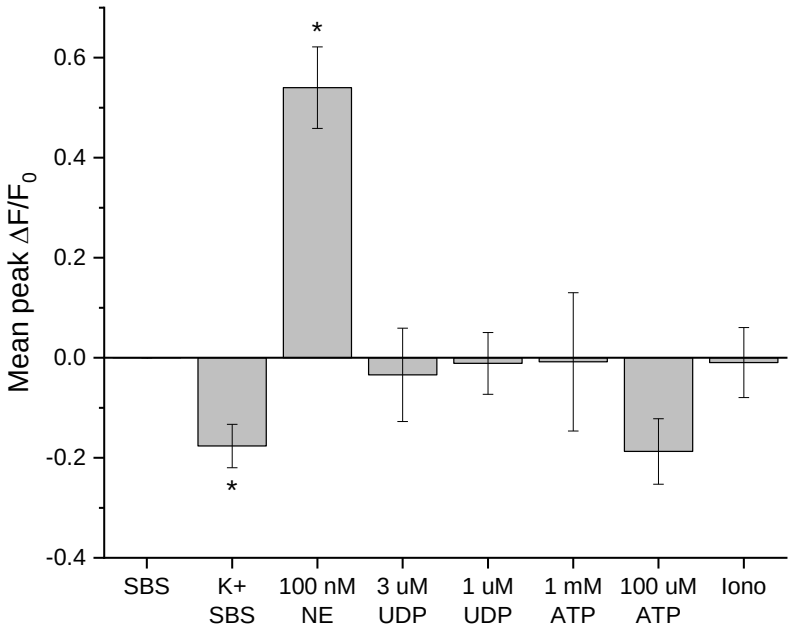

Figure 13: Exogenous nucleotides do not trigger norepinephrine release in human neuroblastoma cells.

Mean peak $\Delta F/F_0$ of transfected BE(2)-M17 cells exposed to the calculated maximal and EC_{50} concentrations of ATP (n = 5) and UDP (n = 5).

Chapter 4: Discussion and Conclusions

4.1 Identification of P2 receptors in human BE(2)-M17 neuroblastoma cells

Demonstrated in this study are novel findings identifying the presence of P2 receptors in undifferentiated human BE(2)-M17 neuroblastoma cells. Measured intracellular calcium responses to exogenous ATP suggest an overall presence of P2X receptors and a significant greater potency and maximal response to UDP greatly suggests the presence of P2Y6.

Early studies have demonstrated P2X7 receptor expression on the mouse NG108-15 line and the parent N18TG-2 cell line (Watano *et al.,* 2002). IP3 assay studies have shown mouse neuroblastoma lines N1E 115, Neuro 2a, and NB4 1A3 and the rat glioma/mouse neuroblastoma hybrid line NG108-15 to give robust responses to both UTP and UDP suggesting the presence of P2Y receptors (Sak *et al.,* 1999). Following this, RT-PCR studies have revealed the presence of P2Y6 and P2Y4 receptor expression in NG108-15 cells (Sak *et al*., 2001)

P2 expression has been extensively evaluated in other human neuroblastoma cell lines. Moore *et al*. screened more than 40 cell lines by Taq-man PCR. In the study, high P2Y4 (SK-N-BE and differentiated SH-SY-5Y) and P2Y11 (NT-2, IMR32 and SH-SY-5Y) expression was noted suggesting a possible neuronal localisation in the human brain; P2Y1, P2Y2, P2Y6 and P2Y14 were absent (Moore *et al.,* 2001; Puchałowicz *et al.,* 2005). However, Western blot analysis of SH-SY5Y cells detected the presence of P2X1,2,4,5,6,7 and P2Y1,2,4,6, but not the P2X3 and P2Y12 receptors (Cavaliere *et al.,* 2005). Fura-2 imaging also confirmed the presence and exhibited high expression of P2X7 in SH-SY5Y cells (Larsson *et al.,* 2002; Raffaghello *et al.,* 2006). Certain neuroblastoma cell lines have appeared devoid of any P2Y receptor expression including SK-N-SH and SK-N-MC neuroblastoma cells, thus potentially offering alternative null cell lines for heterologous expression of P2Y receptor subtypes (Moore *et al.,* 2001).

In contrast, Staurosporine-mediated differentiation of human BE(2)-M17 neuroblastoma cells demonstrated a loss of calcium response to exogenous ATP and UDP suggesting the differentiation process involved the loss of expression of P2 receptors on the cell surface.

Previous research has shown that the expression of certain P2 receptors can be rapidly modulated during retinoic acid differentiation. Barely detectable mRNA levels of P2Y4 and no P2Y6 mRNA were observed in undifferentiated SH-SY5Y neuroblastoma cells. However, Moore *et al.* observed small increases in levels of P2Y4 and P2Y6 mRNA in differentiated SH-SY5Y cells (Moore *et al.,* 2001).

Furthermore, Northern blot analysis in SK-N-BE(2) cells showed that the expression of the dopamine β-hydroxylase gene was dramatically decreased by retinoic acid as previously described in sympathetic neuronal cells. However, there was no difference in the intensity of P2Y6 receptor mRNA signals between the control and retinoic acid-treated cells (Lee *et al*. 2003) However, P2Y4 has been linked to up-regulating mRNA transcription of genes associated with retinoic acid differentiation of SH-SY5Y cells (Cavaliere *et al.,* 2005). In murine models, Retinoic acid-induced differentiated Neuro2a cells expressed P2X7 at significantly lower levels. Wu *et al*. conclude that P2X7 receptors may play a critical role in maintaining Ca^{2+} homeostasis and cell survival of neuroblastoma cells (Wu *et al.,* 2009; Puchałowicz *et al.,* 2005).

Little research has shown the expression and modulation of P2 receptors in staurosporine-mediated differentiation. Therefore, we briefly suggest that P2 receptors are down-regulated during staurosporine-mediated differentiation of BE(2)-M17 neuroblastoma cells.

α,βmeATP, a P2X1, P2X3, and P2X2/3 agonist (Illes *et al.,* 2020), did not elicit a response in undifferentiated BE(2)-m17 cells. As stated previously, prior research has shown the presence of P2X1 and P2X2 in SH-SY5Y cells, with an absence of P2X3 (Cavaliere *et al.,* 2005). This suggests undifferentiated BE(2)-m17 cells do not contain P2X1, P2X3, and P2X2/3 receptors elucidating the presence of P2X2,4,5,6 or P2X7 receptors.

Undifferentiated BE(2)-m17 cells elicited a concentration-dependent increase in intracellular calcium when exposed to exogenous ATP and UDP. We observed a statistically significant difference (P=0.027) in the potency between the two nucleotides (ATP = 120.71 ± 44.80 μM; UDP = 50.20 ± 17.14 nM), furthermore, we did observe a significant difference in the maximal response. Previous research in neuroblastoma cells showed similar responses to ATP. Larsson *et al.* observed a dose-response relationship in SH-SY5Y cells with a potency of 185 ± 8 μM for ATP^{4-} and a similar maximal response at 1 mM (Larsson *et al.,* 2002). Similarly, in hP2Y6 transfected SH-SY5Y cells demonstrated a dose-dependent intracellular calcium response to UDP with a lower potency of 20 nM. Furthermore, Apolloni *et al.* observed a decrease in maximal response but the same potency in the absence of extracellular Ca^{2+} suggesting UDP-dependent intracellular Ca^{2+} elevation was mainly driven by intracellular store release. Maximal response in the transfected SH-SY5Y cells was similar to undifferentiated BE(2)-m17 cells further elucidating the presence of P2Y6 (Apolloni *et al.* 2010).

SK-N-BE(2)C cells also elicited a concentration-dependent increase in intracellular calcium when exposed to ATP, ADP, UTP, and UDP. SK-N-BE(2)C cells demonstrated a greater maximal concentration of 10 ± 0.3 μM and a greater potency of 100 ± 20 nM to UDP than our undifferentiated BE(2)-m17 cells, suggesting undifferentiated BE(2)-m17 cells are more sensitive to UDP. ATP was observed to

have little effect on cytosolic Ca^{2+} even at the supramaximal concentration (1 mM) suggesting undifferentiated BE(2)-m17 cells are also more sensitive to ATP (Lee *et al.* 2003). Collectively, this demonstrates a high likelihood of P2Y6 expression in BE(2)-M17 cells. Further RT-PCR and Western blot analysis would have allowed absolute confirmation of the molecular presence of P2Y6.

To conclude, both ATP and UDP elicited a pharmacological response in undifferentiated BE(2)-M17 neuroblastoma cells. Although we can conclusively suggest the presence of purinergic receptors in these neuroblastoma cells, further research would be required to provide a definitive conclusion. This could be explored through the use of RT-PCR and Western blot techniques to demonstrate a presence of receptor mRNA and protein or the use of known receptor antagonists could be used to demonstrate the molecular and pharmacological expression of the cells.

Furthermore, it would have been interesting to explore the differences of the presence of P2 receptors in the undifferentiated and Staurosporine-mediated differentiated cells. This would have given a clear explanation to the almost complete abolition of response when exposed to UDP and ATP.

4.2 Transient transfection of undifferentiated human BE(2)-M17 neuroblastoma cells

4.2.1 Transient transfection optimisation

Following the identification of P2 receptors in undifferentiated BE(2)-m17 cells, it was crucial to determine the optimal conditions for transient transfection of the $GRAB_{NE}$ plasmid into undifferentiated BE(2)-m17 cells. To optimise, concentrations of lipofectamine 2000 and cDNA were tested. Lipofectamine 2000 is a cationic liposome-based reagent that provides high transfection efficiency and high levels of transgene expression in a range of mammalian cell types *in vitro* using a simple protocol (Dalby *et al.,* 2004). Previous literature had shown that 0.5-1 µL/well of Lipofectamine has a greater transfection efficiency, with lower toxicity than cells transfected with higher concentrations of Lipofectamine (2 µL/well) in SK-N-BE(2) cells (Meng *et al.,* 2020). We, therefore, used concentrations between 0.2-1 µL of Lipofectamine. We also tested various concentrations (100-300 ng/µL) of the extracted cDNA and the initial seeding density to ensure high transfection efficiency and low cell death. Measuring the peak $\Delta F/F_0$ and observing the cells under an optical microscope, we decided to use 200 ng/µL of cDNA and 0.5 µL of Lipofectamine at 12,500 cells per well. These conditions produced a high peak when exposed to exogenous NE, demonstrated low cell death, and demonstrated little overcrowding in the wells of the plate.

To further optimise the study, we aimed to measure the differences in peak $\Delta F/F_0$ for the mCherry cassette as a way to accurately measure the transfection efficiency. However, there was no statistically

significant difference between the peak $\Delta F/F_0$ for transfected and non-transfected cells. Therefore, we could not use mCherry emission to measure transfection efficiency.

Differentiation of BE(2)-M17 cells by staurosporine and retinoic acid had previously been shown to include a consistent neuron-like appearance, showing a significant increase in β-tubulin III and neurofilament expression compared to undifferentiated cells (Filograna *et al.,* 2015). To determine whether undifferentiated BE(2)-m17 were neuronal in nature, we exposed transfected cells to high concentrations of potassium to manually depolarise the membrane. However, there was no statistically significant difference in the peak $\Delta F/F_0$ for NE in non-transfected and transfected cells when exposed to high concentrations of potassium, suggesting the undifferentiated BE(2)-m17 cells may not be neuronal in nature. We could therefore not use high potassium as a positive control for natural NE release.

4.2.2 The selectivity and sensitivity of the $GRAB_{NE}$ biosensor

High ligand specificity is an essential requirement for tools designed to detect structurally similar monoamine-based molecules. The $GRAB_{NE}$ biosensor is based on the α_2AR which responds to both NE and Epi, but not other neurotransmitters around physiological conditions due to their similarity in structure. Furthermore, because NE and DA are structurally similar yet functionally distinct (Feng *et al.,* 2019), we characterised the selectivity of the $GRAB_{NE}$ biosensor to the three catecholamines, as well as characterise the sensitivity of the biosensor to decreasing concentrations of NE to ensure future responses were due to NE release.

Demonstrated in this study are findings that exogenous NE activated the $GRAB_{NE}$ biosensor and induced a concentration-dependent increase in fluorescence in undifferentiated BE(2)-m17) cells. NE induced a maximal response at 3 µM and had a potency of 91.34 ± 27.01 nM. Previous literature has shown human blood plasma concentrations of NE to be approximately 0.5 nM (Denfield *et al.,* 2018), and 21.55 nM. Furthermore, Goldstein *et al.* estimated a 3.3 nM concentration of NE in the average neuroeffector junction(Goldstein *et al.,* 1986).

To measure the selectivity of the $GRAB_{NE}$ biosensor, we measured the responses to two similar catecholamines to NE, Epi and DA. NE exhibited a significantly greater maximal response than Epi and DA suggesting the NE had a greater efficacy on the $GRAB_{NE}$ biosensor. However, there was no statistical difference between the EC_{50} concentrations for NE compared to Epi and DA suggesting all three catecholamines are equally potent to the $GRAB_{NE}$ biosensor. We found the potency of NE to be very similar to that detailed in Feng *et al's* paper (0.1 µM). However, we found that DA was more potent to $GRAN_{NE}$ in undifferentiated BE(2)-m17 cells (1.19 ± 0.59 µM) than HEK293T (3.7 µM).

Previous literature has shown DA to interact with α2-ARs. The activation of adrenergic receptors by DA can trigger intracellular cascades involving changes in calcium and/or cAMP concentrations or influence neuronal excitability (Cornil & Ball. 2008; Zhang *et al.,* 1999). Furthermore, it has also been suggested that DA might activate different intracellular cascades than NE or that the system in which the receptor is expressed influences the response measured (Cornil & Ball. 2008; Zhang *et al.,* 2004) suggesting why DA may enact a response on the $GRAB_{NE}$ biosensor.

4.3 Exogenous nucleotides do not induce Norepinephrine release.

ATP and NE are co-stored in synaptic vesicles in sympathetic nerves and when co-released act post-junctionally to evoke the contraction of visceral and vascular smooth muscle (Kennedy *et al.,* 2007). Extracellular ATP is rapidly degraded by ectophosphatases to AMP, which is subsequently dephosphorylated by 5'-nucleotidase to adenosine. Then endogenous adenosine is rapidly cleared from the interstitium, at least in part by cellular uptake through specific nucleoside transporters. In a variety of *in vitro* models, adenosine has been shown to inhibit NE release from sympathetic nerve endings (Rongen *et al.,* 1996; Von Kügelgen *et al.,* 1992; Wennmalm *et al.,* 1988). Although pre-junctional interactions between P1 receptors and NE release have been widely explored, interactions between P2 receptors and pre-synaptic NE release have not been explored.

Here we demonstrated no significant difference in NE release between varying nucleotide concentrations and blank control. We found the minimum threshold for the $GRAB_{NE}$ biosensor to be 30 nM. When exposed to nucleotides, undifferentiated BE(2)-m17 cells may release below 30 nM concentrations of NE. Filograna *et al.* demonstrated low expression levels of the proteins involved in NE synthesis (5.4 ± 1.7 nmol/g) which rapidly increased following staurosporine-mediated differentiation (45 ± 14 nmol/g) which may explain a non-significant result.

Although not significant, we did notice a decrease in the mean peak $\Delta F/F_0$ when exposed to 100 µM ATP. This may have been due to the degradation of ATP by the ectophosphatases and activation of P1 receptors in undifferentiated BE(2)-m17 cells. However, UDP did not elicit a response suggesting P2Y6 does not activate a NE response but may inhibit NE release. Interestingly, we found a significant difference in mean peak $\Delta F/F_0$ between the control and high potassium concentrations. The inability to naturally induce NE from these cells using high potassium SBS became an issue the undifferentiated cells may have not been neuronal. Future experiments using transfected differentiated cells may have yielded a more appropriate model system and yielded more detailed data on ATP induced NE release.

Previously when directly testing high potassium, we found no difference. Furthermore, ionomycin, a calcium ionophore, did not elicit NE release suggesting that the underlying calcium response of P2

receptors does not evoke NE release, however, future research using known NE excitatory molecules may show inhibition of NE release.

To summise, ATP and UDP both elicit a concentration dependent calcium response in undifferentiated BE(2)-M17 neuroblastoma cells. UDP elicited a siginficantly greater maximal response and greater potency than ATP suggesting the presence of P2 receptors in these neuroblastoma cells with a high likelihood of P2Y6 expression. Staurosporine-mediated differentiation of BE(2)-M17 neuroblastoma cells resulted in the abolition of ATP- and UDP-evoked calcium responses.

In this study, we successfully transiently transfected undifferentiated BE(2)-M17 neuroblastoma cells with the pDisplay-GRAB-NE2hF6.341-IRES-mCherry plasmid and demonstrated successful EGFP fluorescence responses to norepinephrine, epinephrine, and dopamine. Norepinephrine demonstrated a significantly greater maximal response than epinephrine and dopamine. Finaly, we demonstrated ATP and UDP both do not excite NE release in undifferentiated BE(2)-M17 neuroblastoma cells.

Chapter 5: Glossary

NE	Norepinephrine
Epi	Epinephrine
DA	Dopamine
DAG	diacylglycerol
IP3	Inositol trisphosphate
IP3R	Inositol trisphosphate Receptor
cAMP	Cyclic adenosine monophosphate
ATP	Adenosine triphosphate
CNS	Central Nervous System
AR	Adrenergic Receptor

Chapter 6: References

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