Studies on the Role of Cannabinoids on Dopamine Transporter Function: An optical Method

By

Dilichukwu Elobi

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SUPERVISORS: Dr Keith Brain

Professor Janice Marshall

Pharmacology
School of Clinical & Experimental Medicine
University of Birmingham
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ABSTRACT:

Dopamine transporters (DAT) as well as other monoamine transporters are targets for several antipsychotic drugs of abuse thereby making them a potential target for regulation of neurotransmitters in the body. *Cannabis Sativa* is a known psycostimulant which contains compounds called cannabinoids (CB). CB exerts their effect in the body by mainly activating the CB_1 receptor which is expressed predominantly in the central nervous system. Both dopamine (DA) and CB are linked to the reward system and enforcement of addictive substances. This study aims to investigate the effect cannabinoid have on DA transmission in differentiated PC12 cells using a fluorescence based assay called neurotransmitter transporter uptake assay (NTUA). This homogenous assay uses a fluorescent false transmitter which mimics either NAd, DA and serotonin transporter substrate. An increase in fluorescence in cells indicates possible transporter activity, thereby making it possible to assess transporter activity by quantitative fluorescence technique. Differentiated PC12 cells showed increased fluorescence signal when compared to the undifferentiated cells. Fluorescent uptake was significantly reduced in the presence of the DAT blocker, GBR (100nM) and the noradrenaline transporter blocker, desipramine (1µM). Suggesting the fluorescent false transmitter isn't specific. The cannabinoids also showed no significant effect on the fluorescent uptake of the cells. While PCR results showed that differentiated PC12 cells had low expressions of the cannabinoid receptor 1 gene (CNR1), making this cell line unsuitable for this cannabinoid study, however further testing is required.

Acknowledgements

I would like to thank my Supervisor Dr Keith Brain for being understanding and patient throughout this research.

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LIST OF ABBRECIATION

ACEA – Arachidonyl-2’-chloroethylamide

Ad - Adrenaline

AM281 – N-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide

CA - Catecholamines

CB – Cannabinoids

Cnr1- CB_1 receptor gene

CNS – Central nervous system

DA – Dopamine

DAG - 1,2-diacylglycerol

DAT- Dopamine transporter

GBR 12909 – Vanoxerine

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

LED – light emmiting diode

N – Number of experiment

NAd – Noradrenaline

Nc – Number of cells

NE – Serotonin

NET – Serotonin transporter

NTUA – Neurotransmitter transporter uptake assay

q-PCR – Quantitative Polymerase chain reaction

UDG - uracil-*N*-glycosylase

UNG - uracil-*N*-glycosylase

VMAT2 - vesicular monoamine transporter 2

VTA - ventral tegmental area

INTRODUCTION

Noradrenergic Transmission

Catecholamines (CAs) include compounds with a catechol moiety and an amine group. The three pharmacologically important and widely distributed CAs in the brain are noradrenaline (NAd), adrenaline (Ad), and dopamine (DA). CAs are important neurotransmitters the brain and peripheral nervous system (Tsunoda, 2006). Compared to NAd, the distribution of DA in the brain is more restricted. It is most abundant in the corpus striatum, the part of the brain concerned with the coordination of movement. Ad on the other hand occurs in smaller amounts in the brain, much less than 10% of the NAd (Carlsson, 1959).

In the late 1950s,3-hydroxytyramine (DA) was identified as a neuromodulator and it was also recognised as the most prominent catecholamine in the brain (Carlsson, 1959; Missale et al., 1998). DA's importance in neuropharmacology is due to its involvement in variety of functions such as neuroendocrine secretion, locomotor activity, feeding, emotions, learning and reward (Jaber et al., 1996). The major dopaminergic pathways in the brain are mesocorticolimbic, nigrostriatal and mesolimbic systems (Missale et al., 1998; Beaulieu and Gainetdinov, 2011). Dopaminergic neurons have important physiological roles in immune and cardiovascular functions (Jaber et al., 1996). Many disorders result from dopaminergic dysfunction, which is not surprising based on the various critical processes DA is involved in the body. Studies show that most drugs of abuse act on the reward circuitry, leading to a prolonged increase in extracellular DA in the nucleus accumbens (NA) and other forebrain areas (Herkenham et al., 1990; Lupica and Riegel, 2005).

Most drugs used to treat neurological disorder and addiction affect DA signalling in the brain. An important example is the treatment of the neurodegenerative disorder Parkinson's diseases, which results from a loss of dopaminergic neurons in the substantia nigra (Kopin, 1993). L-dopa, a metabolic precursor of DA is used to improve symptoms of the disease, by helping with the local synthesis of DA and hence compensating for the DA lost during degeneration in the substantial nigra (Girault & Greengard, 2004).Other abnormal DA signalling related disorders include

Huntington disease, schizophrenia, depression, bipolar disorder and attention deficit disorder (Beaulieu and Gainetdinov, 2011). The key steps in normal catecholaminergic neurotransmission are shown in figure 1.

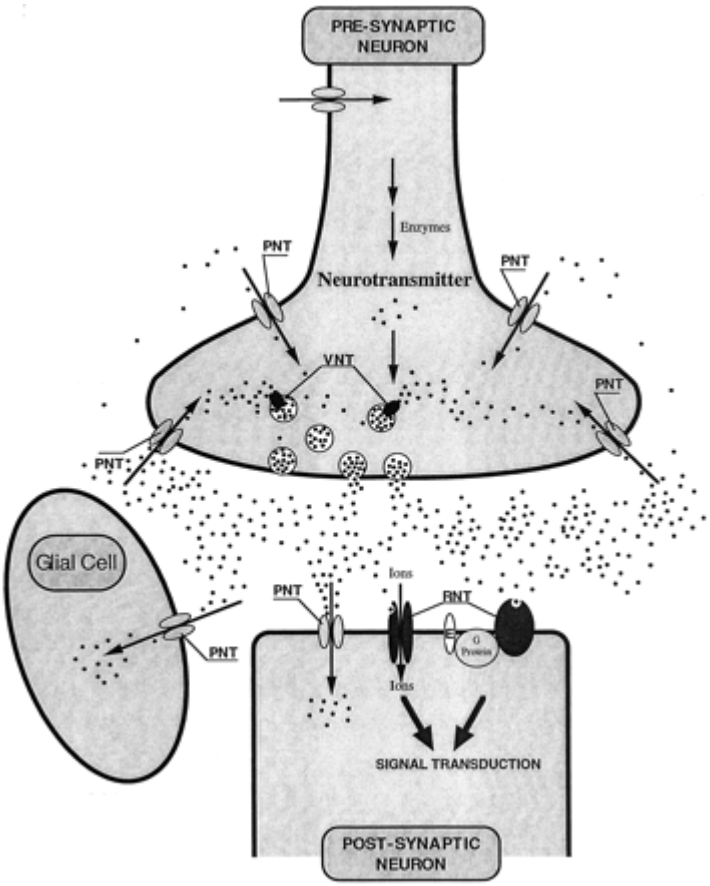

Figure 1: Main Steps involved in Neurotransmission. Neurotransmitter is synthesized then stored (by vesicular neurotransmitter transporters; VNT) in the synaptic vesicles, and released through exocytosis. The neurotransmitter transporter (PNT) then removes the neurotransmitter from the synaptic cleft hence aiding uptake in pre- and post- synaptic neurons (Masson et al., 1999).

DA is a metabolite of the amino acid tyrosine (Tyr) and its signalling begins by the conversion of tyrosine to L-3-4-dihydroxyphenylalanine (L-DOPA) in the cytoplasm by tyrosine hydroxylase (TH, a rate limiting step), followed by decarboxylation of L-DOPA to DA by DOPA decarboxylase (Beaulieu and Gainetdinov, 2011). Dopaminergic neurons lack dopamine β -hydroxylase and thus do not produce noradrenaline (Masson et al., 1999). DA is then transported and stored in storage synaptic vesicles. The proper transport and packaging of monoamines into specialized secretory vesicles is required to maintain homeostasis in the central

nervous system. The process is mediated by specific vesicular transporter: particularly the vesicular monoamine transporter 2 (VMAT2). VMAT2 is predominantly localized to the central nervous system in monoaminergic brain regions, where it packages free monoamines from the cytosol into small synaptic and dense core vesicles (Nirenberg et al., 1996).

Dopaminergic Receptors

After DA is released from presynaptic terminals, it interacts with and activates DA receptors on the pre- and postsynaptic neuron. The various actions of DA are mediated by specific receptors named D_1 to D_5. The first evidence of DA receptors existence in the brain was in 1972, when it was revealed that dopamine stimulates adenylyl cyclase (AC) (Kebabian and Calne, 1979). All DA receptors belong to a large G-protein-coupled transmembrane receptor family (Jaber et al., 1996). Initially, the two recognised classes of DA receptors were the D_1 and D_2 receptors, which respectively stimulate and inhibit AC. Gene cloning later revealed further subtypes, D_1 to D_5 (Girault & Greengard, 2004).The D_1-like receptor family includes D_5, this is mainly due to similarities in their homology and pharmacology. The D_2 family includes D_3 and D_4 ; which are pharmacologically more important. Activation of D_2 receptors at a molecular level, opposes the effect of D_1 receptor activation, but has a more synergistic action with more complex output (Missale et al., 1998).

DA receptors are expressed in the brain in distinct but overlapping areas (Jaber et al., 1996). They are enriched in distinct populations of striatal neurons, although there is a degree of co-expression. D_1 receptors are abundant and widespread in areas receiving a dopaminergic innervation (namely the striatum, limbic system, thalamus and hypothalamus), as are D_2 receptors, which also occur in the pituitary gland (Undieh, 2010). D_2 receptors are found not only on dopaminergic neurones, where they function as inhibitory auto-receptors, but also on non-dopaminergic neurons. D_3 receptors occur in the limbic system but not in the striatum. Although D_3 receptors seem to play a minor role in normal circumstances, they appear to be up-regulated in pathologic conditions, making them prime targets for drug development. The D_4 receptor is much more weakly expressed, mainly in the cortex and limbic system (Jackson and Danielsson, 1994).

Dopamine Transporter (DAT)

Monoamine transporters are localized on the plasma membrane of nerve terminals, where they are crucial for the termination of monoamine transmission and maintenance of presynaptic monoamine storage (Jorgensen et al., 2007). The neurotransmission of DA begins with its presynaptic release and interaction with postsynaptic receptors. DA's reuptake is largely terminated via a sodium- chloride (Na^+-/Cl^-) dependent DAT (Gulley and Zahniser, 2003). The synaptic concentration of DA in the body is regulated by DAT activity; hence it plays an important role in the;

(i) clearance and maintenance of DA homeostasis in the CNS (Ciliax et al., 1995; Chen and Reith, 2000)
(ii) Reduction of potential neurotoxicity from excitatory neurotransmitters (Nelson, 1998).

The importance of DAT in the brain has been appreciated for some time also because of the intense psychomotor stimulation produced by drugsthat target and block DAT activity (Zahniser and Sorkin, 2004).

Monoamine transporters are major targets for psychostimulant, anti-depressant and addictive drugs (Amara and Sonders, 1998). Interacting and/or binding with DAT protein can regulate its function. Studies carried out with cocaine suggest that its effect in the brain is due to its action on dopamine transporters. Cocaine, when in contact with the transporter, binds and blocks dopamine transport. The psycholostimulant, amphetamine structurally resemble DA and thus can easily be transported into the cell by DAT. The regulation of the transporter by these drugs can have profound pharmacological and neurobiological consequences (Chen and Reith, 2000)

These drugs also have affinity for other Na^+/Cl^- dependent neurotransmitter transporters, which include; NA, serotonin, GABA, and amino acid transporters (Motenson and Amara, 2003; Zhang et al., 2009). In mammals these transporters have similar structure (figure 2), possessing a 12- transmembrane helices, connected by a single intracellular and extracellular loop, with the N- and C- termini located in the cytosol (Nelson, 1998; Chen and Reith, 2000).

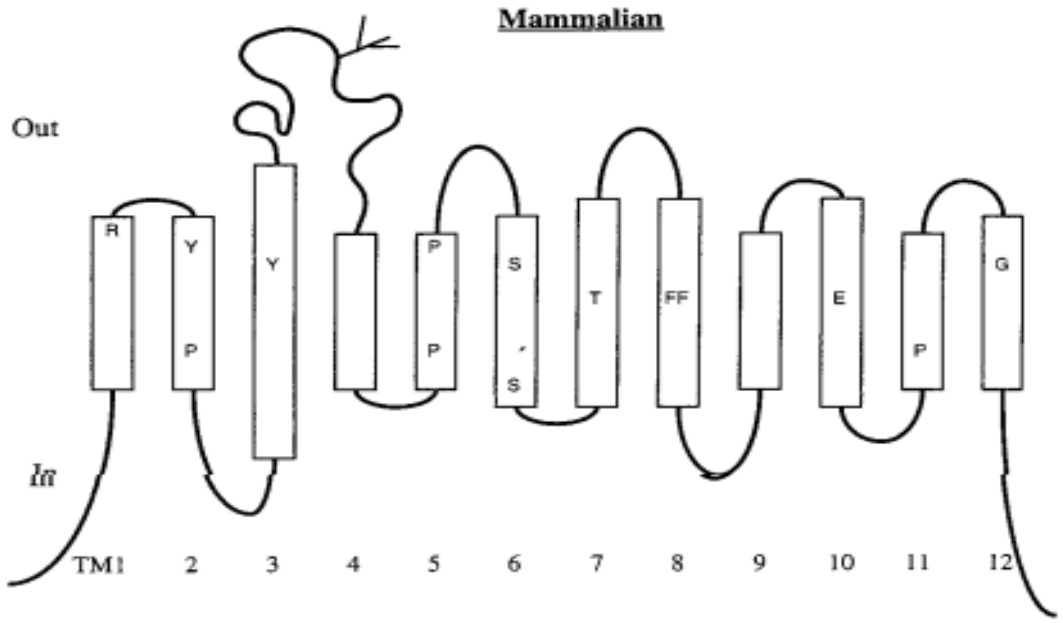

Figure 2: Proposed diagram of mammalian monoamine transporters (Nelson, 1998)

Like DAT, most members of the Na^+/Cl^- dependent carrier family are expressed at the plasma membrane, where they catalyse the accumulation of their respective substrates within the cell. In order to move substrate against a concentration gradient, dopamine transporters co-transport $2Na^+$ and $1Cl^-$ions along with substrate; the ions are needed to provide the thermodynamic driving force for inward flux. Thus, transport requires sodium and chloride and, depending on the net charge of substrates and ions, the process is potentially electrogenic (Motenson and Amara, 2003). Noradrenaline is said to be co-transported with $1Na^+$ and $1Cl^-$ (Nelson, 1998).

In the past few years it has been shown that DAT can be regulated by exposure to DAT substrates and inhibitors, and by ligands interacting with dopamine receptors (Motenson and Amara, 2003; Zahniser and Sorkin, 2004). Although deletion of DAT has offered a powerful tool to study the transporter role in dopaminergic neurotransmission, more transient regulation of transporter activity is an important mechanism by which functional changes in the transporter can occur in the system. Recent studies have shown that transporter activity can be altered more persistently after exposure to and removal of transporter substrates and blockers. Resulting changes in transporter conformation and effect is either due to change in affinity of the transporter for dopamine, or change in the number of transporters expressed at

the cell surface. Interestingly, the bulk of the evidence supports the latter (Gulley and Zahniser, 2003).

Given that DAT function can be modified, I wish to explore whether cannabinoids might do so. Such regulation could have important implications that would improve treatment for addiction and neurological disorders.

What are cannabinoids?

Cannabis sativa is a plant known for its medicinal and recreational purposes (Casu et al., 2003). They are bushy plants which grows wild in tropical climate. Cannabis plants contain many chemical compounds that differ in their pharmacological properties. Consumption of cannabis in different quantities either by smoking, eating or drinking are known to induce relaxation, euphoria, anxiety, depression and hallucination (Manzanares, et al., 2006). In the 1960s, the active component of this plant and the primary substance responsible for its psychoactive effectwas discovered and named (−)-trans-Δ^9-tetrahydrocannabinol (Δ^9-THC) (Pacher, et. al, 2006). Δ^9-THC is present in the female plant (Freund, Katona and Piomelli, 2003). Cannabis contains other metabolites that are potentially of biological interest, including flavonoids, terpens and carotenoids (Guy, Whittle, Robson, 2004). Presently *C. sativa*is recognized to contain more than 60 terpenoid compounds called cannabinoids (Pertwee, 2001).

Cannabinoids are natural or synthetic compounds exerting their pharmacological effect by activating cannabinoid CB_1 and/or CB_2 receptors (Romero et al., 1998). The different types of cannabinoid are shown in figure 3. The discovery of an endogenous cannabinoid system in the 1990 restored interest in cannabinoid as medicines (Guy et al., 2004). The United States made a law allowing cannabis to be prescribed for medical use (Manzanares et al, 2006). It is mainly used to relieve pain in cancer and AIDS patients. The principal cannabinoids found in cannabis are; Δ^9-THC, Δ^8-THC, cannabinol, cannabicyclol and cannabidiol (Alexander et al., 2009).

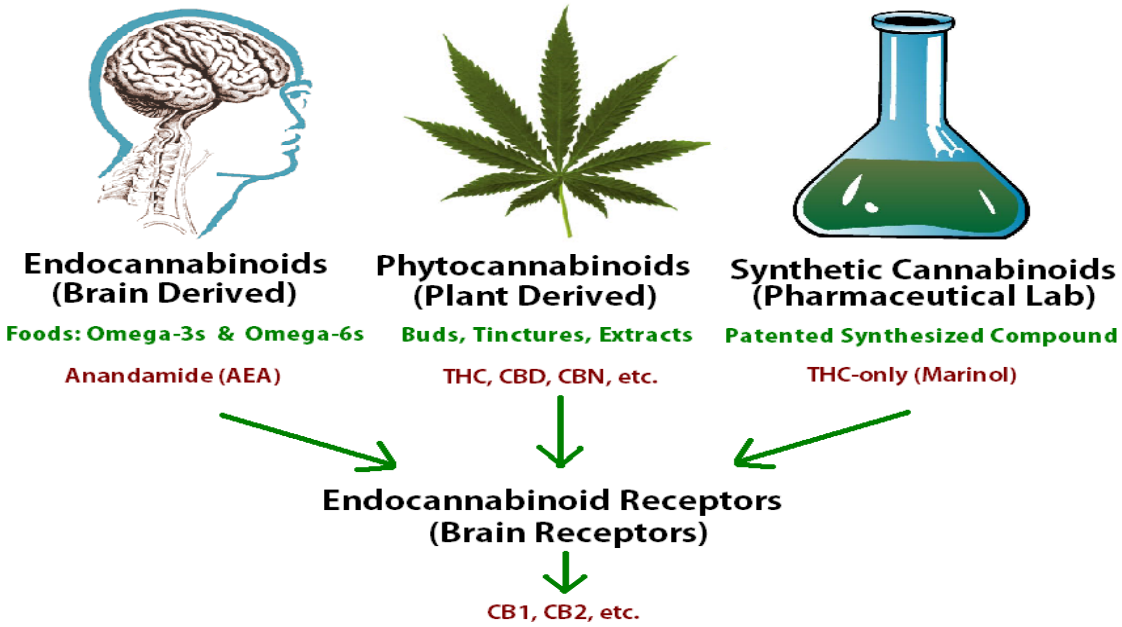

Figure 3: Showing types of cannabinoids (Marder et al., 2007).

Endogenous cannabinoid system

Endocannabinoids are lipid functional ligands of cannabinoid (CB) receptors that are synthesized by the body. All endocannabinoids are derivatives of poly-unsaturated fatty acids (Pertwee 2006; Di Marzo, *et al*. 1997). They are particularly well-known for retrograde signalling in the nervous system (i.e the endocannabinoid travels from postsynaptic neurons to presynaptic neurons). They are present in the brain and most tissues, where they regulate functions such as appetite, memory and mood. Howlett in the 1980s discovered the presence of G-protein coupled cannabinoid receptor in the brain (Manzanares et al., 2006; Howlett et al*.,* 1988). When activated, they regulate most brain and body processes. This property makes them the ideal target for understanding and treating many diseases (Marder et al., 2007).

In the early ninety's, the cannabinoids type 1(CB_1) and 2(CB_2) receptors were cloned from the brain (Matsuda et. al., 1990; Munro et al., 1993). Both receptor types are coupled through G proteins to adenylyl cyclase and mitogen-activated protein (MAP) kinase. CB_1 receptors which have been the focus of most studies, are found predominantly in central and peripheral nerve terminals (CNS) where they mediate inhibition of transmitter release (Pertwee, 2006). CB_2 is mainly located on tissue and immune cells are likely to be involved in regulating immune responses (Storr, et al., 2004); one of its roles is to mediate cytokine release (Lutz, 2002). CB_1 receptors

have been shown to be abundant in brain areas such as cortex, cerebellum and hippocampus (Manzanares et al., 2006). Dense concentrations of the CB_1 receptors have been detected in structures involved in pain signalling (Tsou et al., 1998). Activation of the CB_1 receptor subtype leads to inhibition of adenylyl cyclase, an increase in the activities of mitogen activated protein kinase (MAPK), and regulates ion channels (increases and decreases K^+ and Ca^{2+} conductance respectively). Cannabinoid receptors are frequently located presynaptically and when activated induce suppression/inhibition of neurotransmitter release (Howlett, 2004; Pertwee, 1999). Cannabinoid inhibits the release of many neurotransmitters including DA, NA, GABA, acetylcholine and L-glutamate (Manzanares et al., 2006). These endocannabinoids along with their receptors constitute the 'endocannabinoid system' (Pertwee 2006).

Endogenous ligands for cannabinoid receptors have also been identified: anandamide (arachidonylethanolamide, AEA) and 2-arachidonyl glycerol (2-AG). Anandamide was the first endocannabinoid to be discovered and was isolated from porcine brain (Devane et al., 1992). It is enzymatically synthesized in the brain areas involved in movement, thoughts and memory (Manzanares et al., 2006). It binds primarily to CB_1 receptors, and it has the same pharmacological activity as Δ^9 –THC, but is less potent. Both *in vivo and in vitro* tests have confirmed the similarity of actions between anandamide and cannabinoids in the CNS and peripheral tissues (Di Marzo, 1997; Freund et al., 2003). All endocannabinoid including anandamide are highly lipophilic and are known to be membrane bound. Hence they can diffuse and interact with membrane bound enzymes and receptors. They are also known to diffuse into the cytoplasm, where they are transported by a saturable, high affinity anandamide transporter. This process which is facilitated by fatty acid hydrolase, leads to an action on the presynaptic cannabinoid receptor. Anandamide is synthesized on demand in cell membranes from phospholipid precursors in response to a rise in increase in the cytoplasmic calcium store (Christie, 2011).

2-arachidonyl glycerol is more abundant and binds to both CB_1 and CB_2 receptors. 2-AG is synthesized, mainly in the neurons, in two possible ways. Firstly a hydrolysis reaction of membrane phospholipids mediated by Phospholipase C (PLC) to produce 1,2-diacylglycerol (DAG) which is converted to 2-AG by diacylglycerol lipase, figure 4. Secondly, 2-AG can be synthesized by the hydrolysis of lysophospholipid to 2-AG

by a lyso-PLC activity (Freund et al., 2003). In the body these endocannabinoids are "produced only on demand" rather than stored in vesicles (Di Marzo et al., 1997). Once in the cell these substances are hydrolysed through a reaction is catalysed by fatty acid amide (FAAH), which is found in both neurones and in some tissues (Pertwee, 2001).

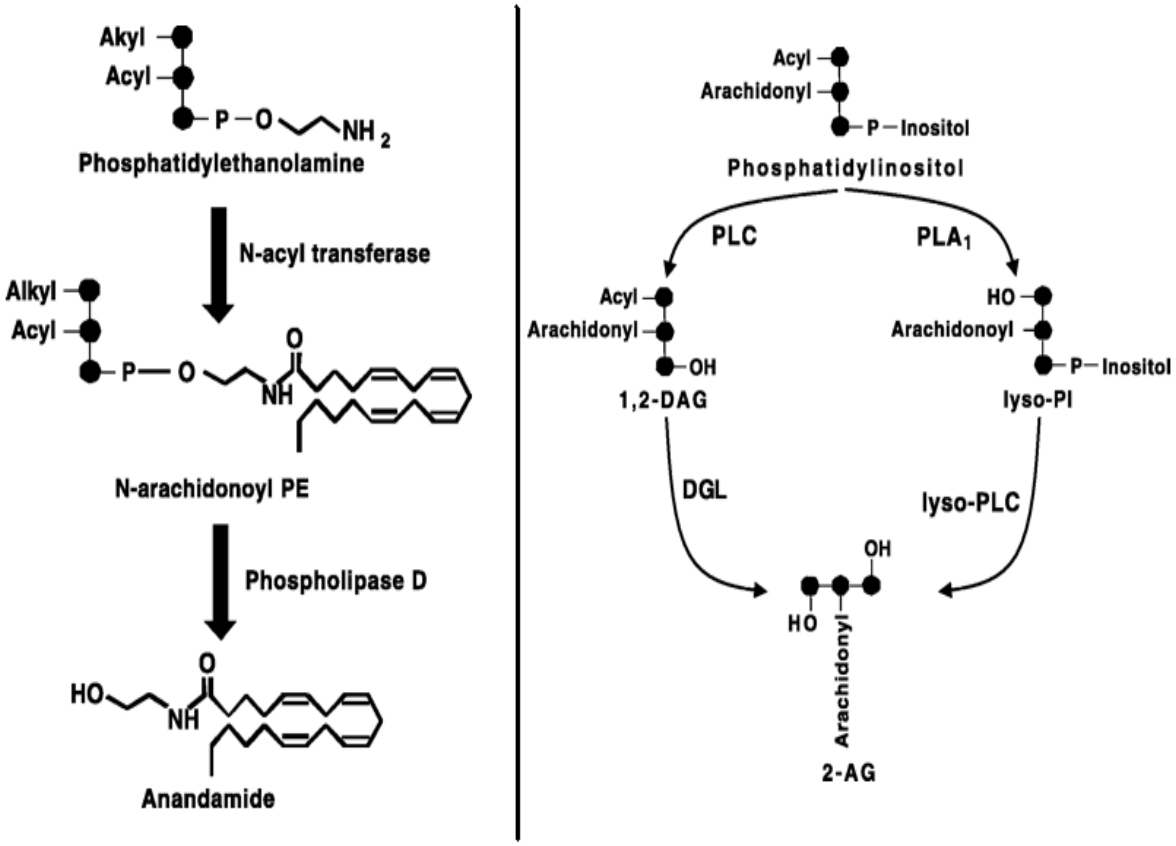

Figure 4: illustration of the possible mechanism of anadamide and 2-arachidonoylglycerol formation (2-AG formation. PE, phosphatidylethanolamine, 1,2-diacylglycerol lipase; DGL, 1,2-diacylglycerol lipase; PI, phosphatidylinositol; PLA_1, phospholipase A_1 PLC, phospholipase C;DAG, 1,2-diacylglycerol. (Freund et al., 2003).

Cannabinoids and Dopamine

Dopaminergic neurotransmission is critical in the reinforcing properties of addictive substances. Most drugs of abuse increase extracellular DA levels through an action on axon terminals of the DA neurons, located in an area of the limbic region known as the ventral tegmental area (VTA). The dopaminergic neurons in the VTA are the primary source of DA in the forebrain (Wise, 2004; Lupica and Riegel, 2005).

Cannabis is one of the most frequently abused drugs and its consumption affects a variety of neurotransmitter release (French, 1997). DA happens to be one of the neurotransmitters implicated in the central nervous system effect that follow cannabinoid administration. Such behavioural effects seen after cannabinoid administration are particularly associated with the actions ofΔ^9-THC (French, 1997).

Psychostimulants as well as cannabinoids increase DA function in the nucleus accumbens (NAc, located in the VTA) through the inhibition of DAT that is located on the axon terminal (Wise and Bozarth, 1987). The magnitude of DA increase depends on the strain and genetics of the rats assessed (Gardner, 2005; Chen et al., 1991). Studies have shown that rats pre-treated with the CB_1 receptor agonist, WIN55, 212-2 showed an increased mesolimbic DA concentration (Oleson and Cheer, 2012). The same result was recorded with small doses of cocaine and nicotine in the nucleus accumbens of a rat. Cannabinoid receptor antagonists such as Rimonabant can reduce the self-administration of, and craving for, several commonly addictive drugs by blocking the effects of endogenously released cannabinoid molecules (endocannabinoids) that are released in an activity- and Ca^{2+}-dependent manner from mesencephalic dopamine neurons. Despite the mechanism of their actions, rimonabant inhibited the DA in each case (Cheer, et al., 2007; Herkenham et al 1990). It is hypothesized that, through the antagonism of cannabinoid CB_1 receptors located on inhibitory and excitatory axon terminals targeting the midbrain DA neurons, the effects of the endocannabinoids are blocked (Romero et al., 1998).

Given that cannabinoids and dopaminergic function are both important for reward, it is useful to explore how these two systems interact presynaptically and to investigate the effect cannabinoid would have on DAT function through a cellular imaging method. Given this, we need to have an efficient method to monitor DA transporter function. The methods mostly used for measuring transporter function are biochemical uptake assays, micro-dialysis and electrophysiological recording of transport associated current. In this study a Neurotransmitter transporter uptake assay was the method used to monitor DAT activity.

Neurotransmitter Transport Uptake Assay (NTUA):

This assay uses a fluorescent transporter substrate to detect and measure neurotransmitter transporter rate. The fluorescent compound mimics serotonin (NE), NAd and DAT substrates and hence can be transported into the cells through such transporters, resulting in an increased fluorescence signal, figure 5. The increased fluorescence offers the possibility to assess transport activity by quantitative fluorescence techniques (Jorgensen et al., 2007). It can also be used to:

- label catecholamine-containing vesicles
- screen for live-cell kinetic uptake of neurotransmitters, hence being able to monitor real time changes in transport function and regulation (Moleculardevices.com)

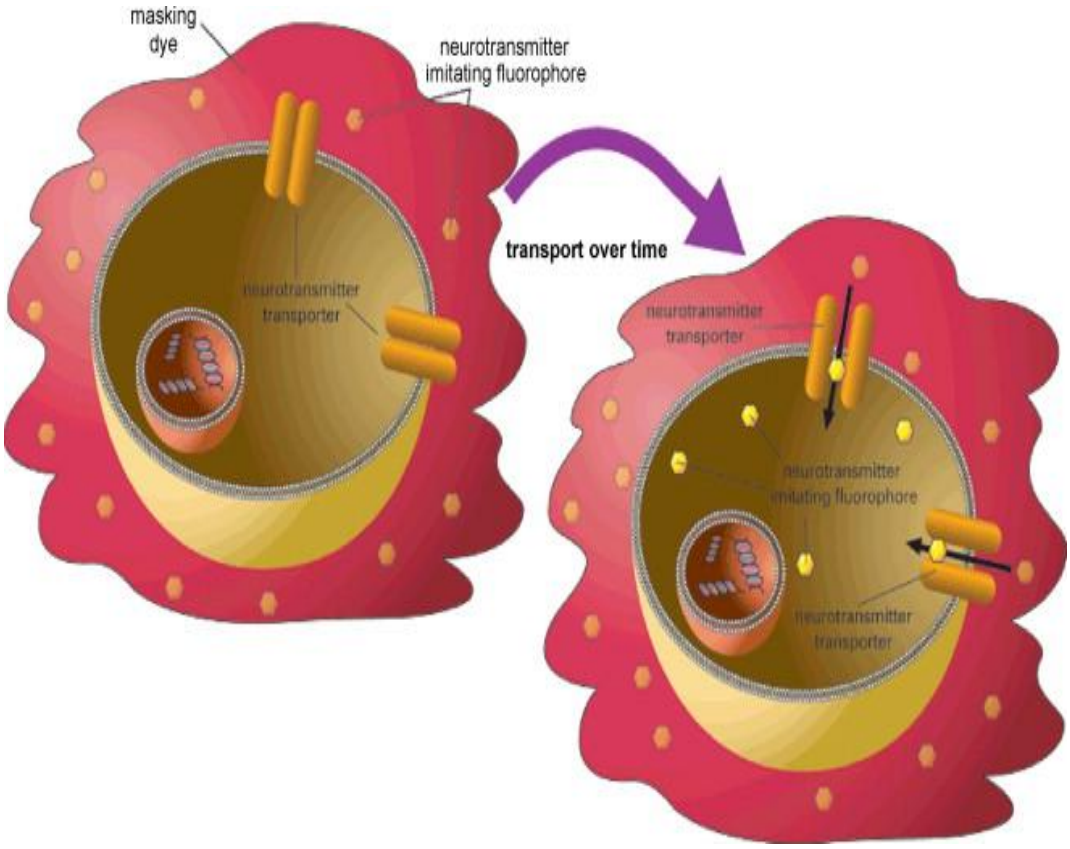

Figure 5: Schematic illustration of the Principle behind NTUA (Jorgensen et al., 2007).

The ability to monitor the uptake of these neurotransmitters is important to better understand diseases that affect brain function. The results from this study may help further development of pharmacological agents to improve patients with disorders linked to dopaminergic transmission such as Parkinson's disease, depression and even give us insight on a cellular level of addictive effect of cannabis.

Aims and objective

2.1 Aims

To investigate the effect of cannabinoids on DAT function

2.2 Objectives

1. Culture cells – To optimise the cell culture conditions to maximise DAT function in a cell type that expresses CB_1 receptors.

2. To establish a viable and reproducible method for monitoring NTUA under a conventional fluorescence microscope.

3. To test the NTUA specificity– to determine which transporters actively transport the fluorescent substrate into the cell lines.

4. To test and characterize the effect of cannabinoids on DAT function.

HYPOTHESIS

Cannabinoids are known to inhibit the release of variety neurotransmitters and also inhibit electrical activity by a depolarisation. With knowledge of this, it's safe to hypothesize that in the presence of CB_1 agonist there should be a noticeable drop in the cells fluorescence signal when compared to the control.

3. Materials and Method

For the purpose of this study, this assay has been carried out using cell lines which synthesise catecholamine (specifically DA) and express catecholamine transporters. For this study BE-(2)-M17 cells and PC12 cells were used.

3.1. Cell Culture

Culture methods for these cell lines, and methods to induce their differentiation, are already established for both of the cell lines used in this study. However my initial effort is to investigate optimum methodology for culturing of each of the cell lines given the aims of the study.

3.1.1. BE-(2)-M17

Cells were maintained in Optimem cell culture media (Invitrogen), diluted with 10% foetal bovine serum (FBS; Invitrogen) and 50µg/ml of penicillin/streptomycin (Sigma-Aldrich) in the final volume. Frozen aliquots of cells were thawed and their cryogenic medium replaced with fresh warm media. Thereafter, medium was replaced 2-3 times per week at even intervals. More frequent media changes allowed faster growth.

Cells were kept in 25cm^2flasks and were stored at 37^{o}C in a humified environment consisting of 5%CO_2 and 95% air. Cells were passaged when they become more than 70% confluent. Cells were maintained at a density no greater than 500,000cells.ml^{-1}, and media was changed a minimum of 3 times per week. When passaging BE-(2)-ME17 cells, old media was removed and replaced and gently washed with warm Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich). DPBS was then removed and replaced with 1ml of trypsin (Invitrogen). After a few minutes cells were checked under the microscope to ensure that they had been separated from the culture surface. Cells were then transferred to a falcon tube and centrifuged, spinning the cells at 1000-1200 rpm for about 30s. A pellet formed at the bottom and the old medium was discarded without disrupting the pallet. Cells were then re-suspended with fresh medium, pipetting vigorously until no clumps or cluster of cells was observed. The cells were then transferred to a new culture flask and returned to the incubator.

3.1.2. PC12 CELLS:

Rat adrenal phaeochromocytoma (PC12) cells were grown in 25cm^2 tissue culture flask in RPMI 1640 (+glutamax) cell culture media (Invitrogen) supplemented with 10% horse serum (HS, Invitrogen), 5% FBS and 50µg/ml penicillin/streptomycin (Invitrogen) .The cells grew quickly, with the number doubling every two days and passaged twice a week. PC12 cells were passaged by breaking clumps of cells in the culture flask and transferring to a falcon tube for centrifuge. Cells were spun at 800RPM for 5mins. After the pellet formed, the old medium was discarded without disturbing the pellet. The new medium was then added, before transferring the cells to a new flask. They grew satisfactory in suspension in the culture flask.

For experiments, cells were cultured on poly-l-lysine (Sigma-Aldrich, 0.1mg/ml) coated 13mm sterilized glass coverslips at a density of 10,000 cells per coverslip. Coverslips were sterilized by dipping in 70% ethanol and allowed to dry. Poly-l-lysine or collagen (Sigma-Aldrich, 40µg/ml) solution was prepared using with filtered distilled water (1:5dilution). Coverslips were coated in the appropriate cell adherent reagent and left to dry. Once the coverslip had dried, cells were pipetted (approx. 50,000cells) onto the surface of the coverslip and placed in the incubator for a few hrs. Once cells were settled, the coverslips were flooded with warm medium to a volume of 1-1.5mls. The cultures were ready to use the day after culturing. Only coverslips on which the cells appeared flattened and adherent to the coverslip were used for experiment.

For the differentiation protocol, PC12 cells were plated down onto a coverslip coated with human collagen 40µg/ml and completely removed from RPMI culture media. RPMI media was replaced with serum-free Delbecco's Modified Eagle Medium (DMEM; Invitrogen) with the addition of 1% HS, 1% Penicillin/Streptomycin and 100ng/mL of nerve growth factor (NGF; from Sigma-Aldrich). Cells were fed this every day for 7 days until the day of experiment (following a protocol from hopkinsmedicine.org). All cell culture procedures were carried out in a sterile culture hood (Courtesy of Prof Ann Logan's laboratory). On the day of each experiment, a cover slip with cells was taken to the imaging setup and perfused with physiological saline solution (PSS; refer to table 1). Before commencing any experiment, perfusion

time was noted before the addition of any drug (based on a protocol used by Dr P. Sidaway (personal communication).

3.2. Physiological Saline Solution (PSS)

Table 1: The salt composition of PSS in 1litres

Salt	Molecular weight(g/mol)	Concentration (mM)	Volume or mass required to make 1L of solution
$CaCl_2$	110.98	1.8	1.8mL
$MgCl_2$	95.211	1.3	1.3mL
Glucose	180.16	5	0.901g
NaCl	58.44	130	7.592g
KCl	74.55	5	0.373g
HEPES	238.3	20	4.766g
EDTA	368.4	1	0.368g

***pH was adjusted to 7.4 by the addition of NaOH. Molarity of $CaCl_2$ and $MgCl_2$ is 1M.**

3.3. Live cell Imaging

Cells were mounted on a Sylgard-lined organ bath and were perfused with warm (32-37^{0}C) physiological solution (Table 1). Using a Cairn Opto-LED light source (Cairn imaging, Kent UK), using the NTUA cells were excited at 405nm and emitted at 520nm. Cells were picked at random and images were taken with an Olympus BX51W1 epifluorescence microscope (Olympus, southern UK) attached to an Orca CCD camera (Hamamatsu photonics, Welwyn Garden city, UK). Specific dilutions of the NTUA solution were prepared with PSS and loaded onto the cells; images taken prior to the addition of NTUA fluorescence substrate were used as control.

3.4. Drugs Preparation

NTUA Kit (MDS Analytical technologies, Berkshire UK) was diluted in PSS (10mL per unit) to make stock solutions and stored in 200µl aliquotsat -20^{o}C.Stock was further diluted on the day of experiment to 1:100 or 1:10 with PSS. GBR 12909 hydrochloride was dissolved in dimethyl sulfoxide (DMSO; Tocris Bioscience, Bristol UK) to a stock concentration of 1mM and stored in the -20^{0}C freezer. On the day of experiments, dilutions were made using PSS to 100nM., Desipramine was diluted

with PSS to 10mM aliquots and stored in the -20^0 freezer. On the day of experiments PSS was further used to dilute desipramine to 1µM for studies. Ethanol was used to dilute CB1 agonist ACEA to 13.6mM and stored in the -20^{0}freezers. On the day of studies, it ACEA was further diluted to a working concentration of 100nM.

3.5. Image analysis

Image analysis from digital fluorescence micrographs were performed using Image J version 1.44 analysis software (http://rsb.info.nih.gov/ij/). Cells were randomly selected, number of cells (n) = 4-13 cells. For NTUA uptake measurement's a region of interest was drawn around the cells from the bright field image stack. For subsequent images stacks, the slice on which this segment was in focus was identified. The region of interest was restored and automatically fitted to the terminal (using an algorithm based on highest local fluorescent signal), then the mean fluorescent signal from this ROI was measured.

3.6. Total RNA extraction and quantitative real-time PCR (q-PCR)

To determine if the PC12 cells as well fresh brain tissue from rat expresses the gene encoding the CB_1 receptor (Cnr1), q-PCR was carried out: PC12 cells were differentiated for 7days and total ribonucleic acid (RNA) was extracted on the 7th day from frozen tissue samples (rat cortex and brain stem) and differentiated PC12 cells then stored in -20^{0}C until use. RNA was extracted from the samples as follows; each sample was incubated in 1ml of Trizol reagent for 5mins at room temperature. 0.2ml of chloroform was added to each samples, then mixed carefully and centrifuged for 5mins at 4^{0}C at 12,000 rpm. Afterwards the colourless aqueous phase (~60% of TRIzol) formed after centrifuge was transferred to a fresh tube. 500µl of 70% ethanol was added to the lysate (colourless aqueous phase) and mixed properly. The samples were then transferred to RNeasy mini column and centrifuged for 15s at 800 rpm. The flow through was discarded, before adding 700µl of Buffer RW1 to the RNeasy column and proceeded to centrifuge to wash the RNA in the column. 500µl of buffer RPE, whose main composition is ethanol and used for washing membrane bound RNA. Was added to the RNeasy column and centrifuged (this step was repeated twice). After the second repeat, the RNeasy silic-gel membrane was dry, and then transferred to a new tube where 40µl of RNase free water was added directly into the silica gel membrane was dried, and centrifuged for 1min at 800 rpm to elute. The eluted RNA (about 2µg) was stored at -20^{0}Cuntil used (White, 2005).

cDNA was transcribed from the total RNA isolated from each sample (differentiated PC12 cells, slice of rat cortex and brain stem) using the manufacture's instruction on the kit (high capacity cDNA kit, Life technologies). The amount of cDNA in each sample was calculated using a Nano Drop ND 1000 spectrophotometer. The Rattus norvegics cannabinoid receptor 1 [Cnr1, containing TaqmanProbe (FAM) primer sequences used were as follows:

- forward 5'-ggacatggagtgctttatgattc-3'
- Reverse 5'gagggacagtacagcgatgg-3' (Pubmed - NM_012784.4).

The expression of housekeeping gene Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) containing TaqmanProbe (VIC) [Catalogue No 4352338E] was used as control. qPCR was performed with the 7500 fast real time PCR system (Applied Biosytems CA, USA).The PCR reaction mixtures were 20µl final volume in each well. Each reaction well contained 16µl of Taqman PCR (Applied Bisoystems CA, USA) master mix containing either Cnr1 or rat GAPDH (for control wells), 4µl of sample or water (for blank wells) in the desired wells. qPCR was repeated in triplicate for each sample.Thermal cycling conditions were 2mins at 50^0C, required for optimal uracil-*N*-glycosylase (UDG) enzyme activation; 10mins at 95^0C was to activate the AmpliTaq Gold DNA polymerase. Further incubated for 40cycles at 95^0C for 15s, this is to denature the DNA and 60^0C for 1min to anneal/extend the DNA. The thermal cycler was set with the following incubation conditions: 2 min at 50 °C to allow the activation of the uracil-*N*-glycosylase (UNG) enzyme, an incubation step for 10 min at 95 °C to denature the UNG enzyme and activate AmpliTaq Gold polymerase, and 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 60 °C for 30 s. All 3 runs were conducted on different days using the same qPCR machine. Each condition was repeated in triplicate.

3.7. Plate reader

This was carried out using the Synergy HT Multi Detection Reader (BioTek, Winooski, VT). Fluorescent false transmitter uptake by PC12 cells was investigated using a fluorescence plate reader. PC12 cells were differentiated in a 96 well plate for 7days. On the 7^{th}day cells in wells were incubated in either NTUA (1:10) , NTUA (1:10) in the presence of GBR(100nM), NTUA(1:10) + ACEA(100nM), NTUA(1:10) + ACEA(100nM) +AM281(100nM) or NTUA(1:10) + AM281(100nM).Fluorescence

readings were taken every 5mins for 30mins. There were hence 5 separate conditions and 5wells for each condition. Set to 485/20 excitation filter and 525/20 emission filter, with sensitivity setting of 70. Parameters were set using the KC4 Data Analysis software (BioTek, Winooski, VT).

3.8. Statistical analysis

Each value is the mean value of the fluorescence signal in independent experiments 5 -7 independent experiments were carried out. Normality was tested using D'Agustino-Pearson omnibus test. Statistical significance for comparisons among groups was determined using unpaired student *t*-test for sets of data which passed the normality test. While a Mann-Whitney test was carried out for sets of data which failed the normality test. All statistical analysis were carried out using the software Graphpad Prism6 (GraphPad Software, San Diego, CA, USA).For multiple groups one way ANOVA was carried out. Where $P< 0.05$ is considered statistically significant. See Appendix for more details.

NOTE from now on NTUA kit content would be referred to as 'florescent false transmitter

4. Result

4.1. NTUA labelling

In order to optically monitor DAT rate, NTUA kit was used. The kit contains a fluorescent dye which serves as substrate for either DA, NAd and NE transporters. This assay was used in cells expressing human DAT. Studies were initially carried out with fluorescent false transmitter (1:100 dilution) on BE-(2)-ME17 cells. These cells were found to have weak uptake of the fluorescent false transmitter. Due to this the concentration of NTUA was then increased to 1:10 dilution, but still the cell showed weak uptake of the fluorescence substrate (fig. 9). This resulted in a decision to change to a different cell line. The PC12 cell line was picked for this study not only due to its availability but also it richly expresses DAT (Melikian and Buckley, 1999). These cells grew in small clusters, much smaller than the BE-(2)-ME17 cells. Figure 6 below shows a typical bright field microscopy image of differentiated PC12. Cells are clearly visible with processors and visible vesicles.

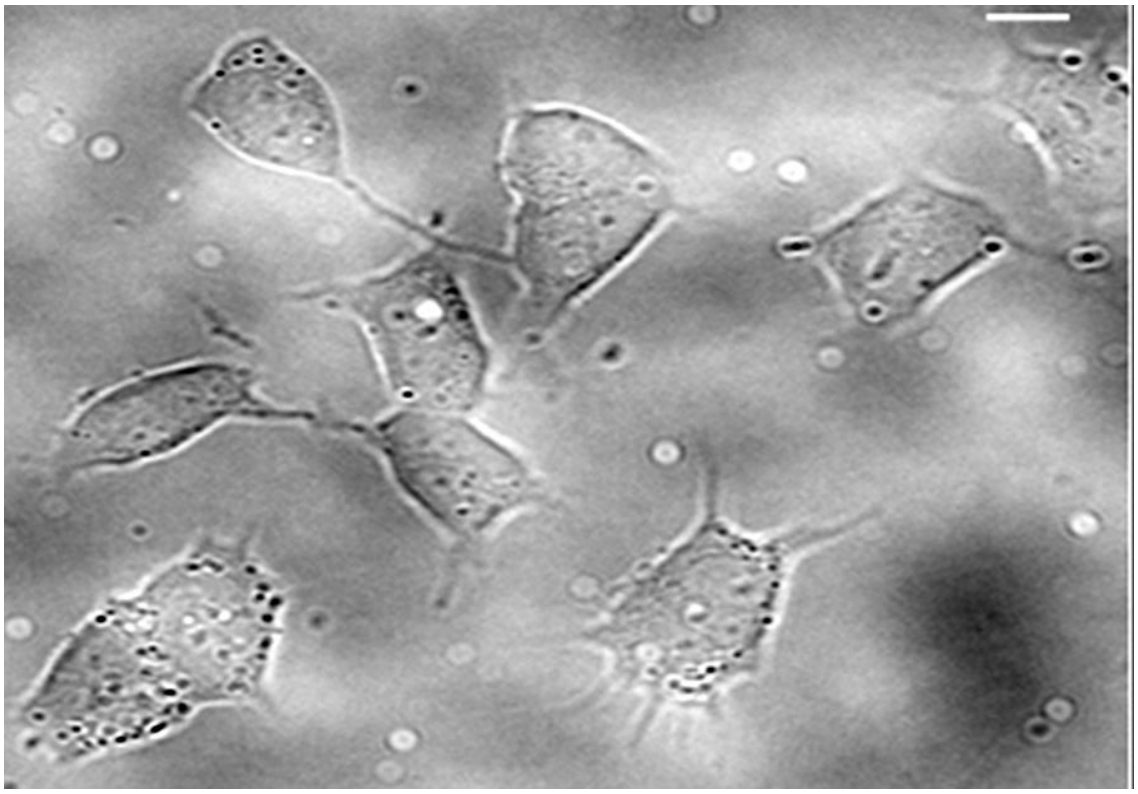

Figure 6: A typical bright field image. (Scale bar = 10µm).

4.2. BE-(2)-M17 cells

M17 cells were plated down on coverslip coated with poly-l-lysine and then kept in culture media in the incubator a day before the actual day of study. On the day of study, each coverslip was taken out of the culture media and placed in water bath where cells would be perfused with PSS.

Bright field images were always taken every 10minutes before fluorescent images were taken (Fig. 7). This was to enable focusing and analysing using ImageJ software. The content, shape and location of the cells were very visible in the bright field images.

4.2.1. Fluorescent false transmitter Uptake in BE-(2)-ME17 cells: After perfusing cells with PSS for 10mins, cells were exposed to the fluoresent false transmitter (1:10) for 60mins; images were taken every 10mins. Images taken with the light emmiting diode (LED) prior to the addition of the fluorescent false transmitter, control images was taken, 0mins. Figure 7 shows LED images taken at 0mins, 30mins and 60mins. After addition of the fluorescent false transmitter(1:10) cells are observed to exibit an increase in fluorescence at this stage when compared to the control image(0mins) At 30mins and 60mins cells show increase in fluoresence signal, 60mins showing more fluorescence than other times.

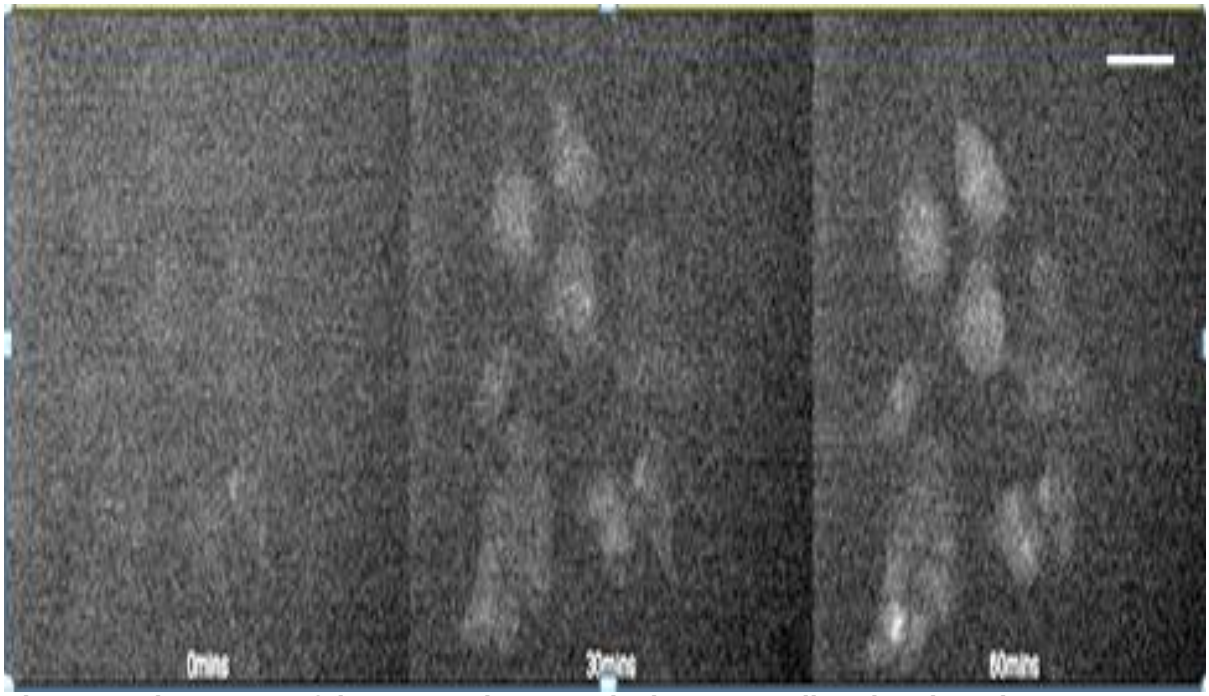

Figure 7: Fluorescent false transmitter uptake in ME17 cells. Showing Fluorecence signal at 0mins, 30mins and 60mins. (Scale bar = 10µm).

4.2.2. Fluorescent false transmitter Uptake in BE-(2)-ME17 cells in presence of GBR 12909 (100nM): GBR 12909, a dopamine transporter blocker was diluted with PSS to 100nM and cells were perfused with the solution for 20mins. After perfusion cells were exposed to the fluorescent false transmitter (1:10), diluted with PSS and GBR (100nM) solution. Images were taken every 10mins for 60mins (Fig. 8).

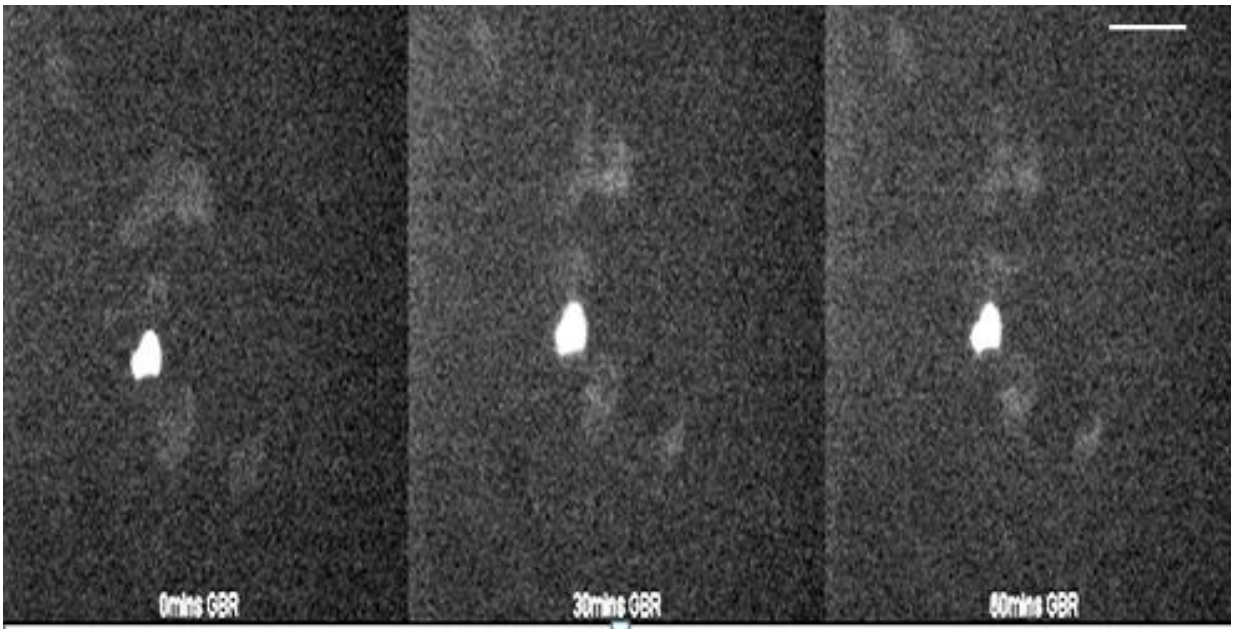

Figure 8: ME17 cell uptake of fluorescent false transmitter in presence of dopamine transporter blocker GBR (100nM). Showing fluorescence images at 0mins, 30mins and 60mins respectively. The bright object seen here is debris which was close to the cells, but it remained constant throughout the experiment and it wasn't included in the analysis. Scale bar = 10µm.

In the presence of GBR, cells show steady, but weak uptake of the fluorescence false transmitter. An increase in fluorescence signal is detected from the start to end point (fig. 8 & 9). Below is the graphical representation after analysing the above experiment showing fluorescence false transmitter in the presence and absence of GBR (100nM) in BE-2-M17 cells. Figure 9, shows a time dependent increase in uptake of fluorescence transmitter. However in the presence of GBR, cells showed a reduced uptake of transmitter, when compared to the transmitter alone. At 10mins there was a drop in fluorescence of the cells exposed to the fluorescent false transmitter alone. No significant reduction in fluorescence signal in the presence of GBR when compared to its absence was observed.

If the fluorescence false transmitter is a DAT substrate, GBR 12909 should inhibit its uptake, hence little or no fluorescence should be detected in this case. However M17 cells in general during the course of this study, showed weak and inconsistent uptake of the fluorescence false transmitter.

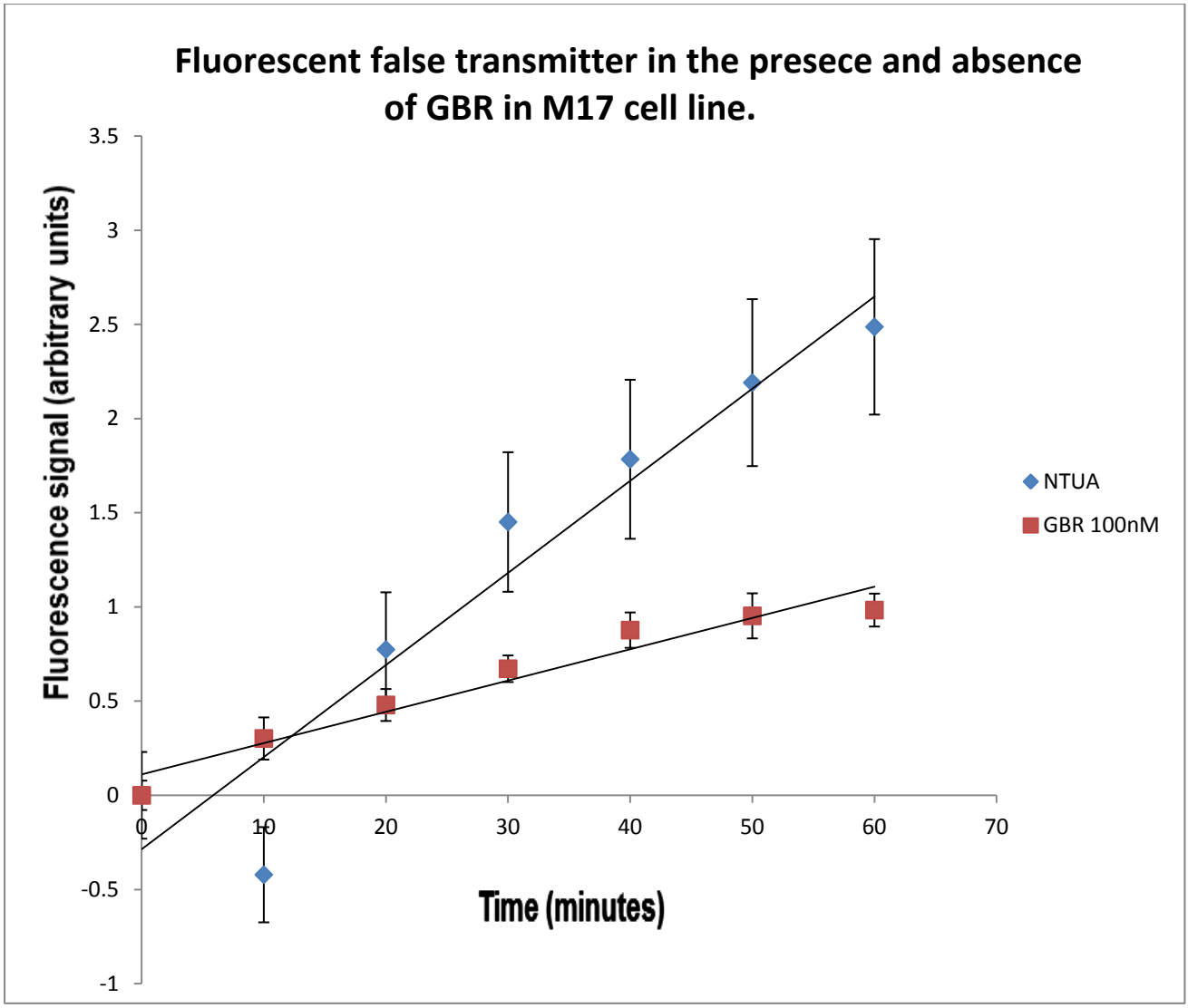

Figure 9: Graph showing fluorescent false transmitter uptake in BE-(2)-ME17 cell line and also its uptake in the presence of the GBR 100nM. Unpaired Student *t*-test, *P*>0.05, measuring the change in end point. Data points are presented as mean ± S.E. Nc= 6-8 cells.

4.3. PC12 Cells

It was observed that PC12 cells adhered poorly to plastics, therefore cells needed higher concentration of poly-l-lysine. Once perfusion was turned on during experiments cells were easily washed away. This shows that concentration level of poly-l-lysine used was not adhesive enough. Although the concentration of poly-l-lysine was increased a number of times, the result remained the same. Therefore collagen was used to coat coverslips to enable cells to adhere to coverslips.

4.3.1. Differentiation

Undifferentiated cells appeared small and rounded. The first set of PC12 cells differentiated showed no neuritis out growth during the differentiation period. It became necessary to purchase a new NGF for the study. Thereafter 7days of incubating PC12 cells in DMEM containing 100ng/mL NGF, cells were seen to stop proliferating, they appeared flattened, larger body mass with continuous extension of neuritis, (Fig 10).

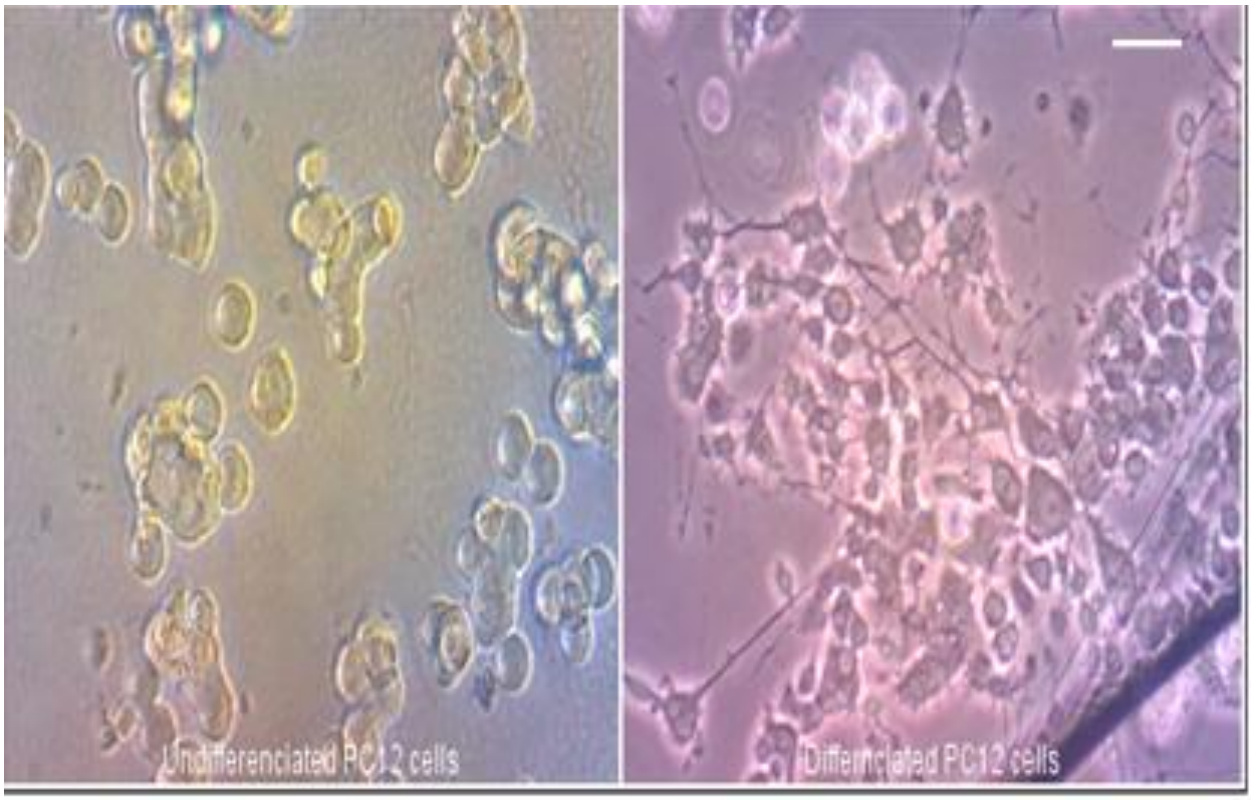

Figure 10: Differentiated cell image. Cells in the absent of NGF appear rounded, small and no visible neuritis or processors. Second image shows cells on the 7th day after the addition of NGF. Cells appear to have larger cell bodies with extended processors. (Scale bar = 50µm)

4.3.2. Fluorescent false transmitter Uptake in PC12cells:

On the day of experiments, cells were perfused in PSS for 20mins before directly adding the fluorescent false transmitter (1:10). In initial experiments, PC 12 cells showed weak uptake of NTUA, so the cells were differentiated and their uptake was compared. Control images were taken prior (all images at 0mins are control) to addition of the false transmitter, whilst on addition of the drug readings were taken every 5mins for duration of 60mins before washing out with PSS for 30minutes. Before addition of the fluorescence transmitter (0mins) the nucleus and vesicles

were visible, after the addition of false transmitter, the vesicles of the cells became more prominent and punctuate (Figure 11). It was observed that in the differentiated cells the processors do not take up the fluorescent false transmitter (figure 12).

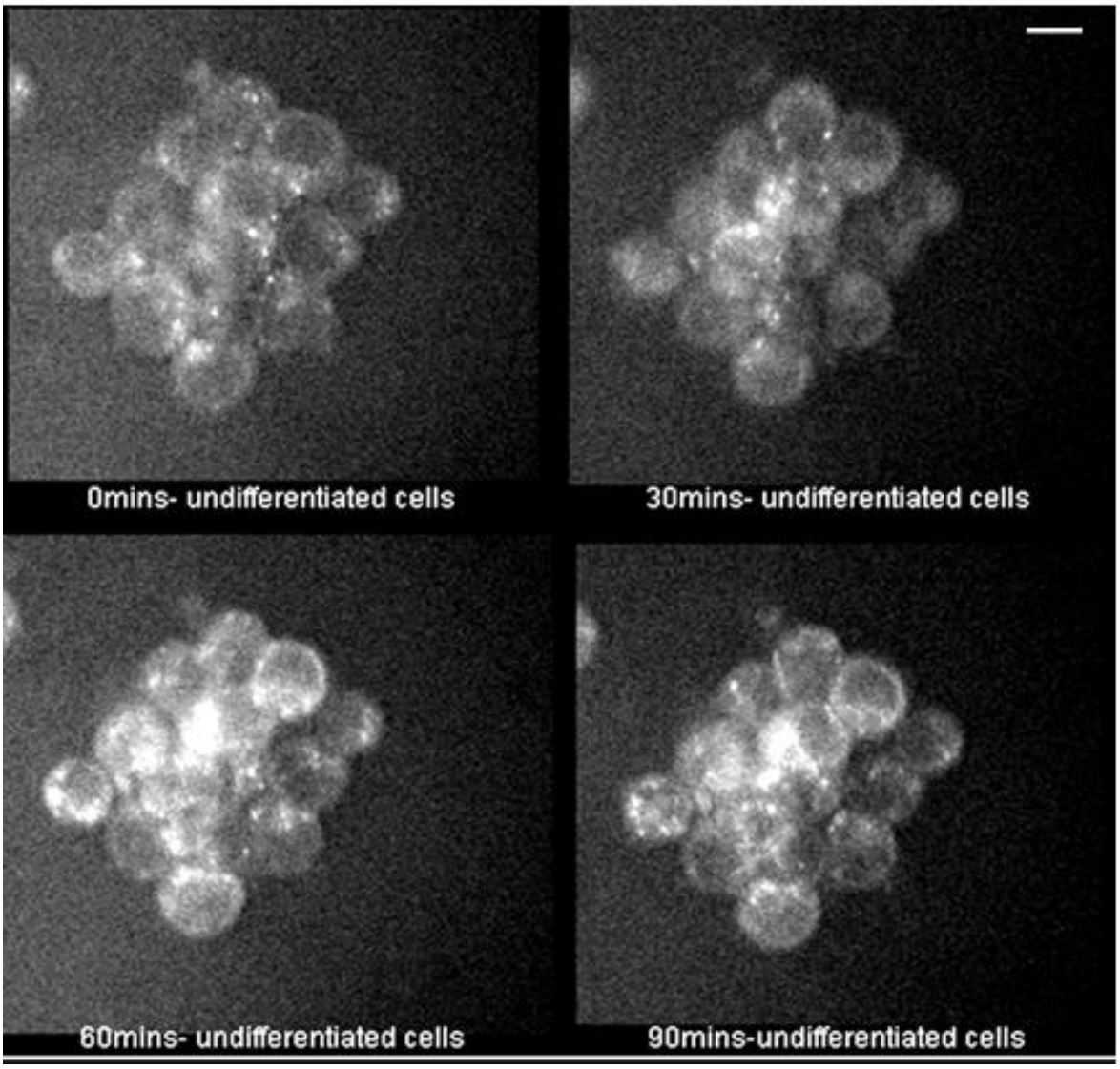

Figure 11: images of NTUA in undifferentiated PC12 cells at different times ; 0mins – before addition of NTUA to the cells, 30mins, 60mins after addition of fluorescent false transmitter ; 90mins after perfusing the cells with PSS(washout). Scale bar = 10µm.

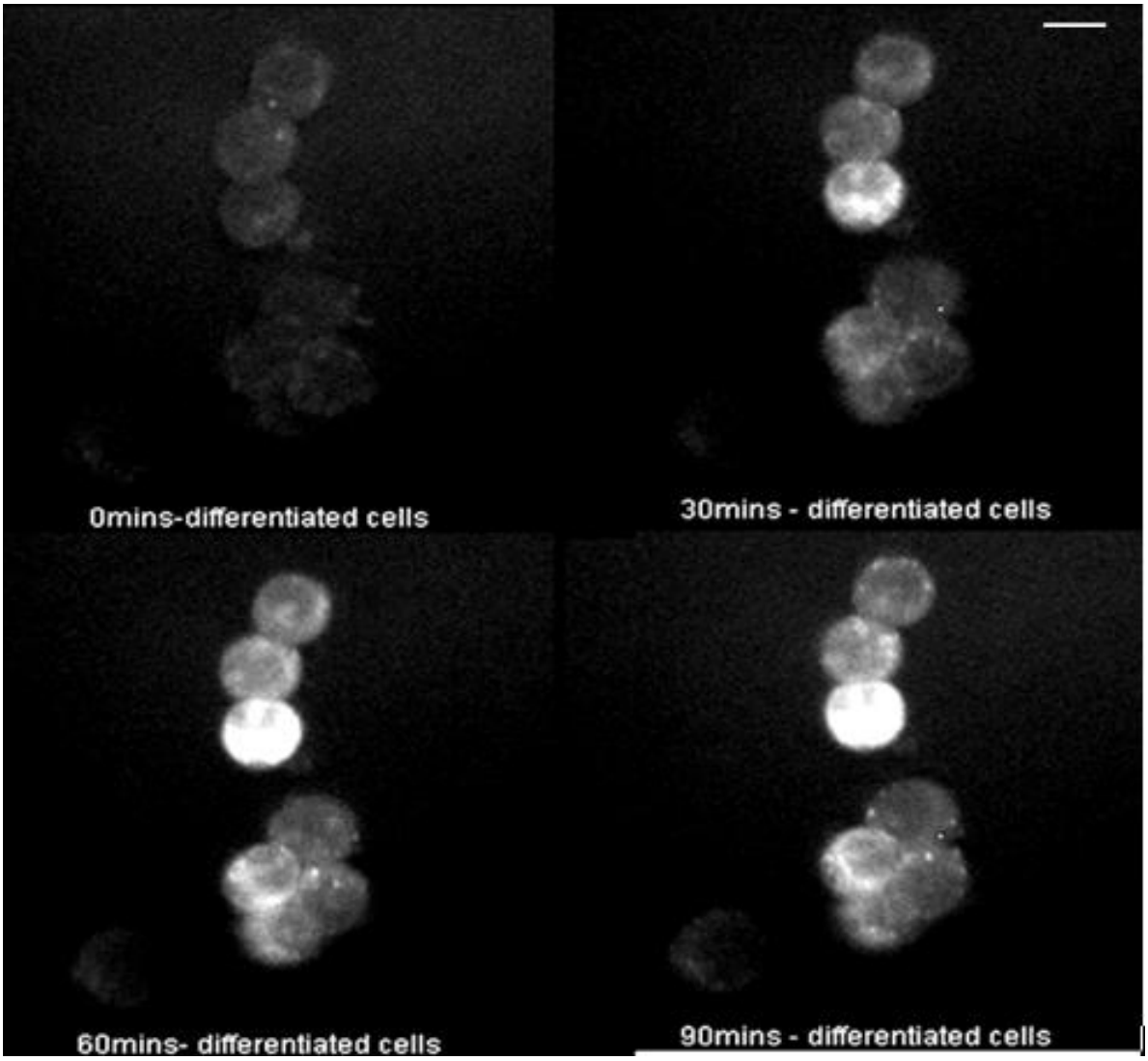

Figure 12: Showing images of uptake of the fluorescent false transmitter in differentiated PC12 cells at different time; 0mins (control), 30mins, 60mins after addition of the false transmitter; 90mins after washing the drug out of the cells with PSS (Scale bar = 10µm).

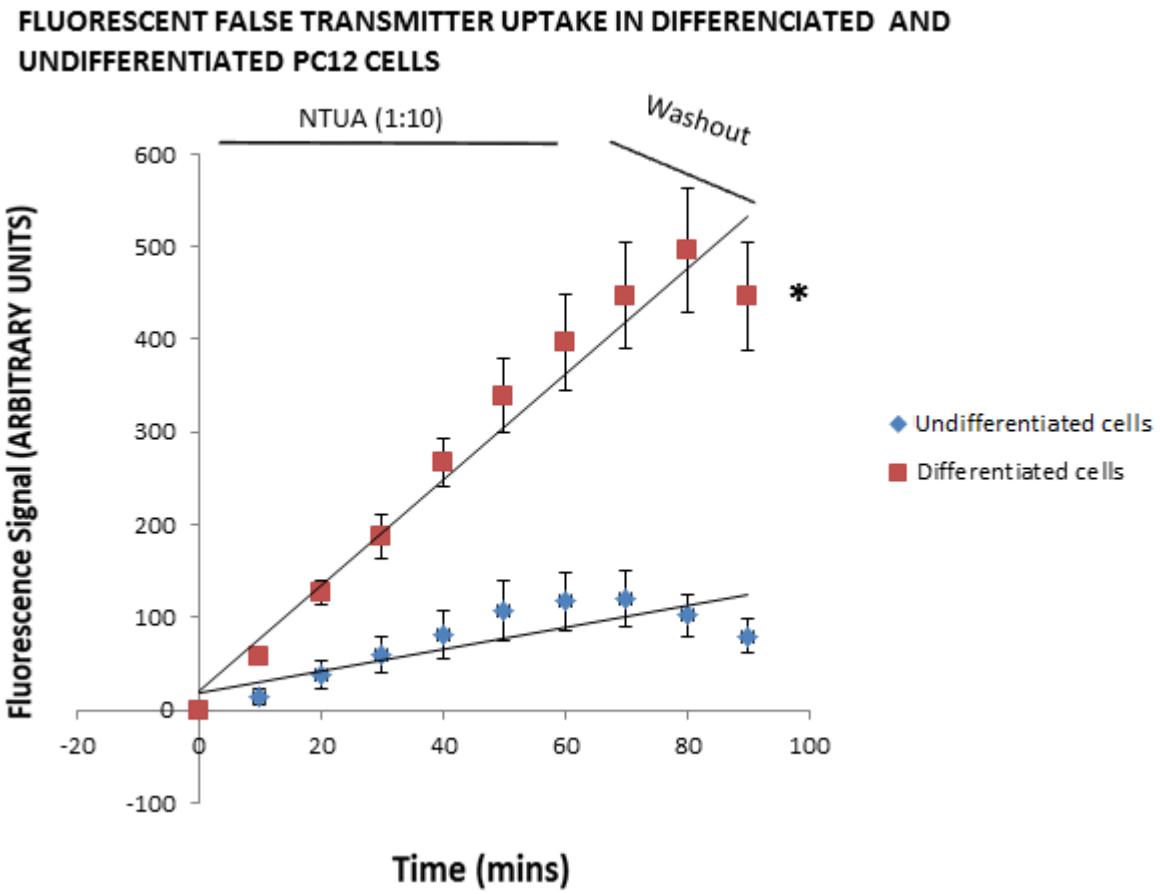

Figure 13: Graph showing effect of the fluorescent false transmitter (1:10) over time on undifferentiated and differentiated PC12 cells. A time dependent uptake of the fluorescence neurotransmitter transporter substrate was observed in both the differentiated and undifferentiated cells. After 60mins, the cells were perused with PSS (washout) for 30mins, the fluorescent labelling gradually reduced. *P<0.05; Nc= 6-12cells.

There was an increase in fluorescence signal in both differentiated and undifferentiated PC12 cells upon addition of the fluorescent false transmitter (1:10). However, the differentiated cell type showed a greater fluorescent signal; hence it had a greater uptake of the fluorescent transmitter. A significant increase in fluorescence signal was detected in all experiments carried out on the differentiated cells when compared to the fluorescence signal in the undifferentiated cells ($P< 0.05$, *Fig. 13 and 14).* Fluorescence signal in both cells dropped during washout with PSS.

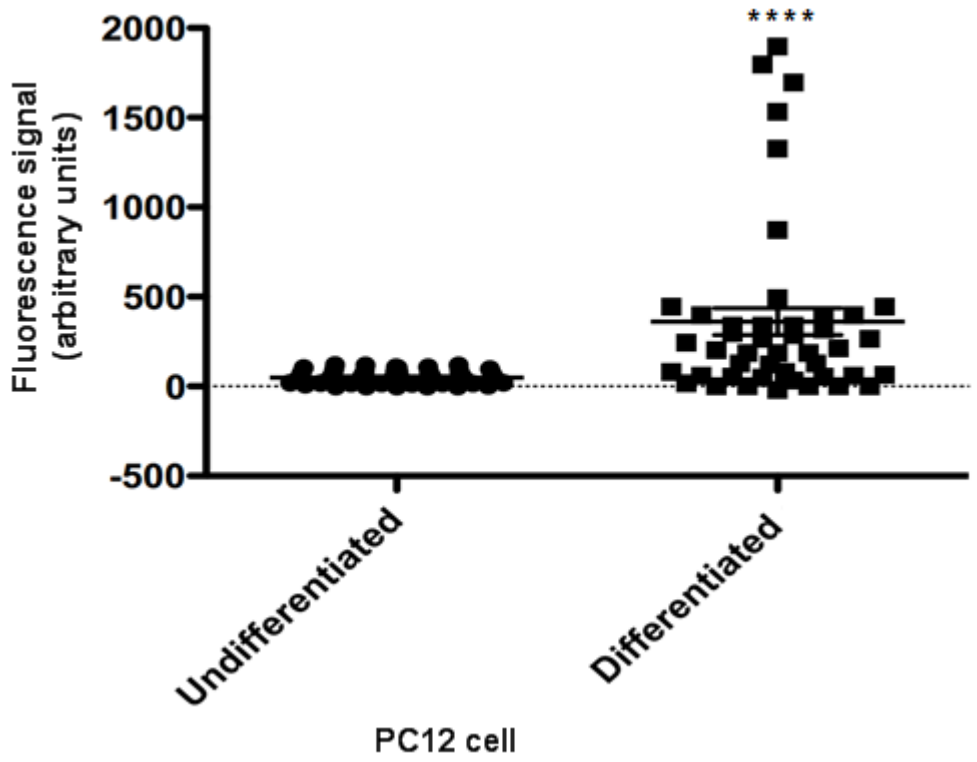

Figure 14: Result of all experiments carried out on undifferentiated and differentiated PC12 cells. This shows and compares the fluorescence signal after the addition of fluorescence false transmitter (1:10) in undifferentiated and differentiated PC12 cells (Mann-Whitney test ****P<0.0001, N=6).

4.3.3. Fluorescent false transmitter uptake in differentiated PC12 cells in the presence of DAT blocker:

To determine if the increase in fluorescence was due to uptake through DAT, the cells were pre incubated with GBR 12909, a DAT blocker (100nM). Figure 15 shows the image of the uptake in differentiated cell without the blocker GBR 12909. Figure 16 shows uptake of the fluorescent false transmitter by differentiated cells in the presence of GBR 12909. After incubation of PC12 cells in GBR for 20mins, perfusion was stopped and a control image was taken (0mins). Thereafter, the cells were exposed to the fluorescent false transmitter (1:10) and images were taken every 10mins. Both conditions showed increased fluorescent signal when compared to the control. However when compared, there was reduced signal in the presence of GBR (Figure 16). Upon return to PSS for 30mins, fluorescence signal dropped gradually, (figure 17).

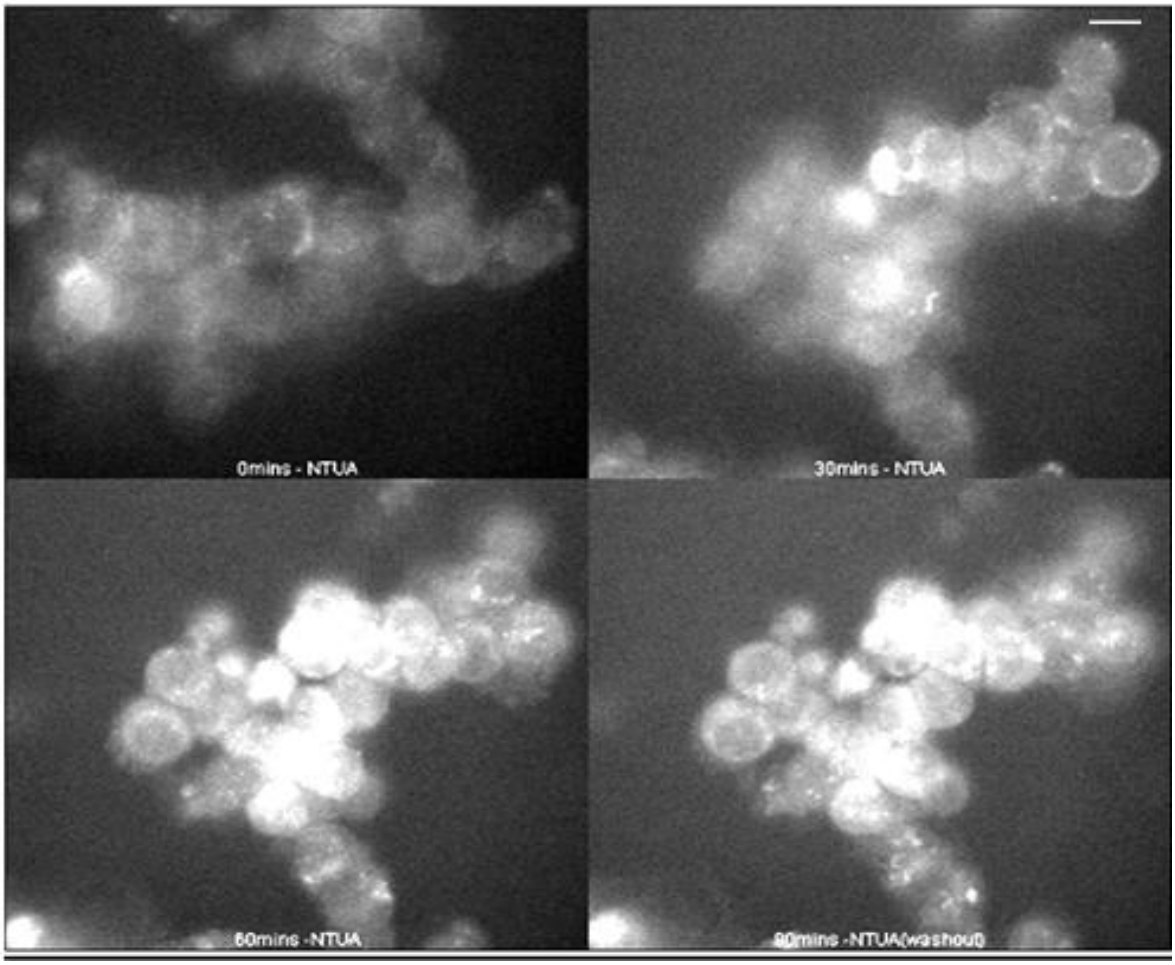

Figure 15: Showing images of fluorescent uptake in differentiated PC12 cells at different times 0mins, 30mins, 60mins and at 90mins after perfusing the cells with PSS (washout). (Scale bar = 10µm).

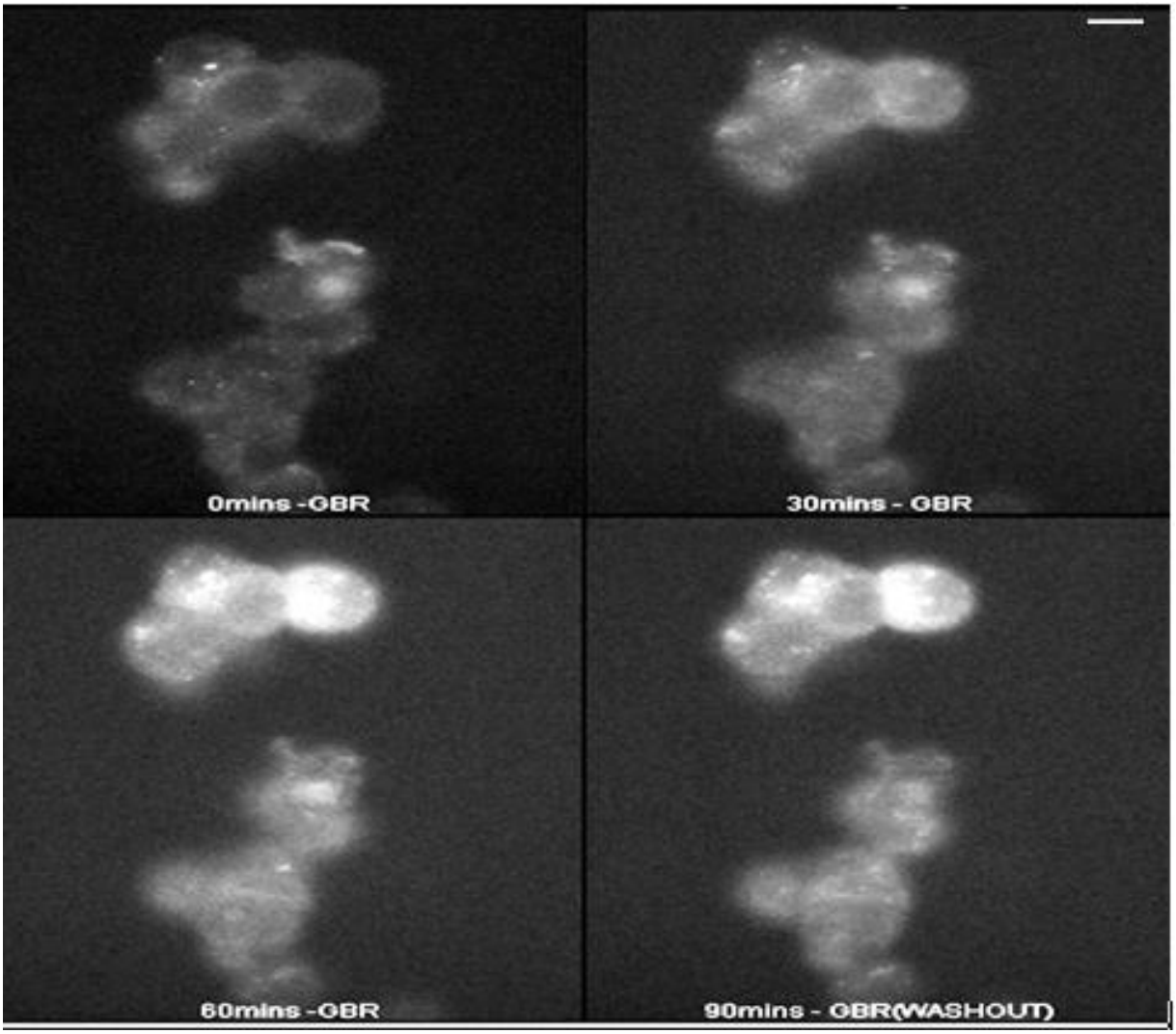

Figure 16: Fluorescence image showing change in fluorescent signal in the presence of GBR (100nM). Image shows cells prior to exposure of fluorescent false transmitter (0mins), after addition of fluorescent transmitter at 30mins and 60mins. The drug was washed off the cells with PSS for 30mins. The last frame was taken 90mins after the start of the experiment. (Scale bar = 10µm).

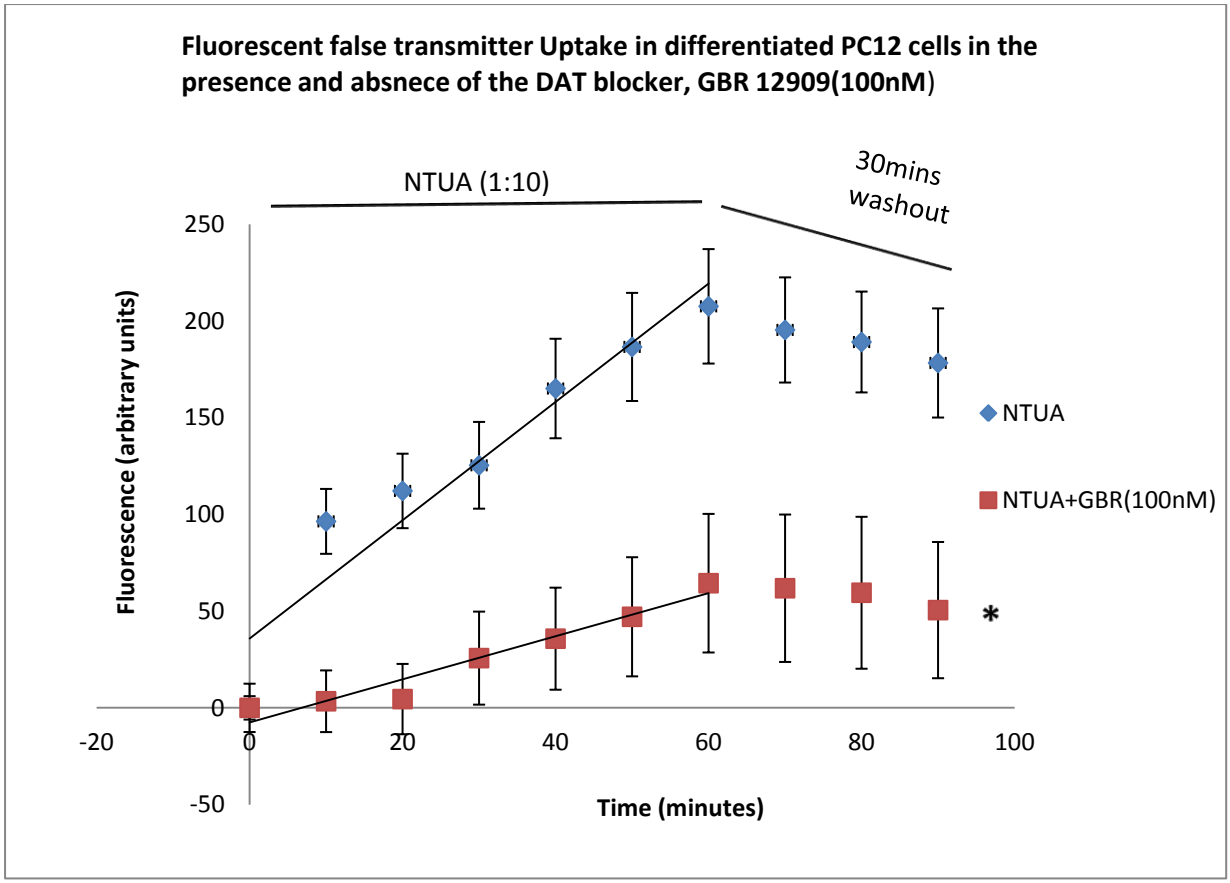

Figure 17: Graph demonstrating the increase in fluorescence signal in differentiated PC12 cells in the presence of GBR (100nM). Indicating the 60mins incubation period and 30mins washout periods. Data points are presented as mean ± S.E.M, P= <0.05, Nc = 5-6 cells (see Appendix for raw data).

GBR 100nM significantly reduced the uptake of fluorescent transmitter in the differentiated cells when compared to GBR absence (Fig. 17 & 18). GBR (100nM) didn't completely block fluorescent uptake in the cells. Fluorescent intensity was seen to increase with time but having low signals when compared to control. Result of all studies carried out showed GBR significantly lowered the uptake of NTUA in PC12 cells (Fig. 18).

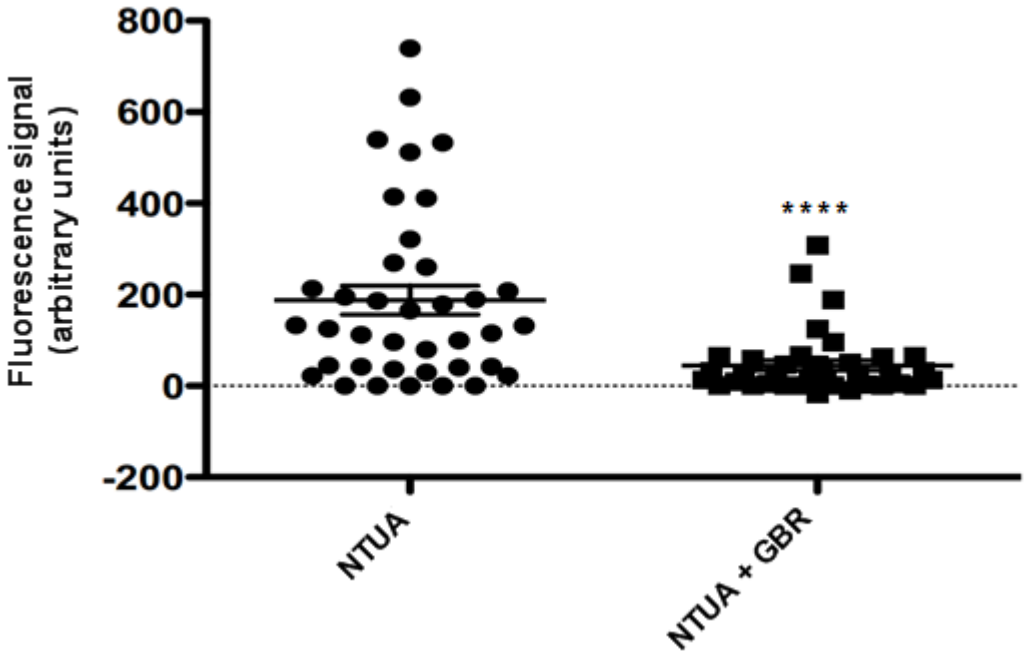

Figure 18: Plot of fluorescent transmitter uptake for all individual studies carried out on PC12 cells in the presence and absence of GBR (100nM). For the GBR study cells were incubated in GBR for 20mins before proceeding to expose the cells to the fluorescent transmitter (NTUA) (Mann-Whitney test ****P<0.0001; N=5).

4.3.4. SPECIFICITY: fluorescent false transmitter Uptake in Differentiated PC12 cells in the presence of DESIPRAMINE (1µM)

To determine if the increase in fluorescence was due to uptake of fluorescent false transmitter by other catecholamine transporter, studies were carried out on differentiated PC12 cells in the presence of noradrenaline transporter inhibitor, desipramine (IµM). Cells were pre-incubated in desipramine (1µM) for 20mins. After which control images were taken (0mins) before cells were exposed to the false transmitter (1:10), pictures were taken every 10mins. Figure 19 below is the images taken of cells in the exposed to the fluorescent false transmitter alone. While figure 20 shows the uptake in the presence of desipramine.

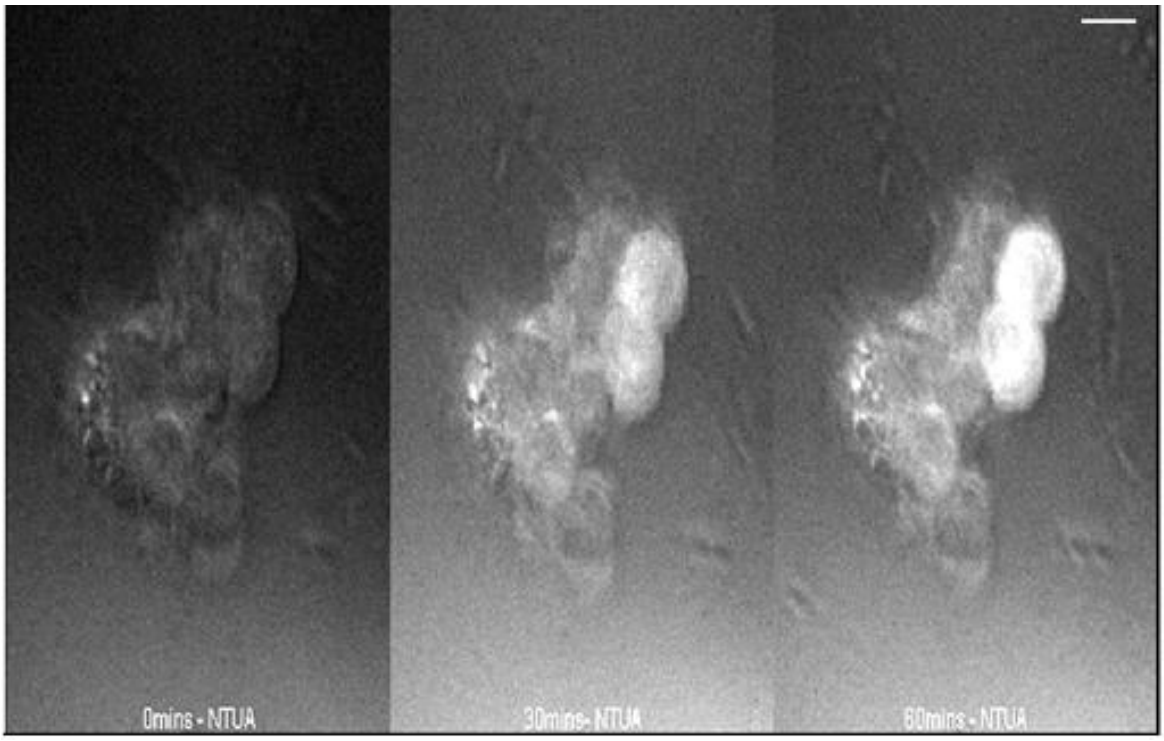

Figure 19: Images showing fluorescence uptake in differentiated PC12 cells. It shows theres an increase in fluorescence signal in the PC12 cells from 0mins (before exposure NTUA) to 30mins and at 60mins. (Scale bar = 10µm).

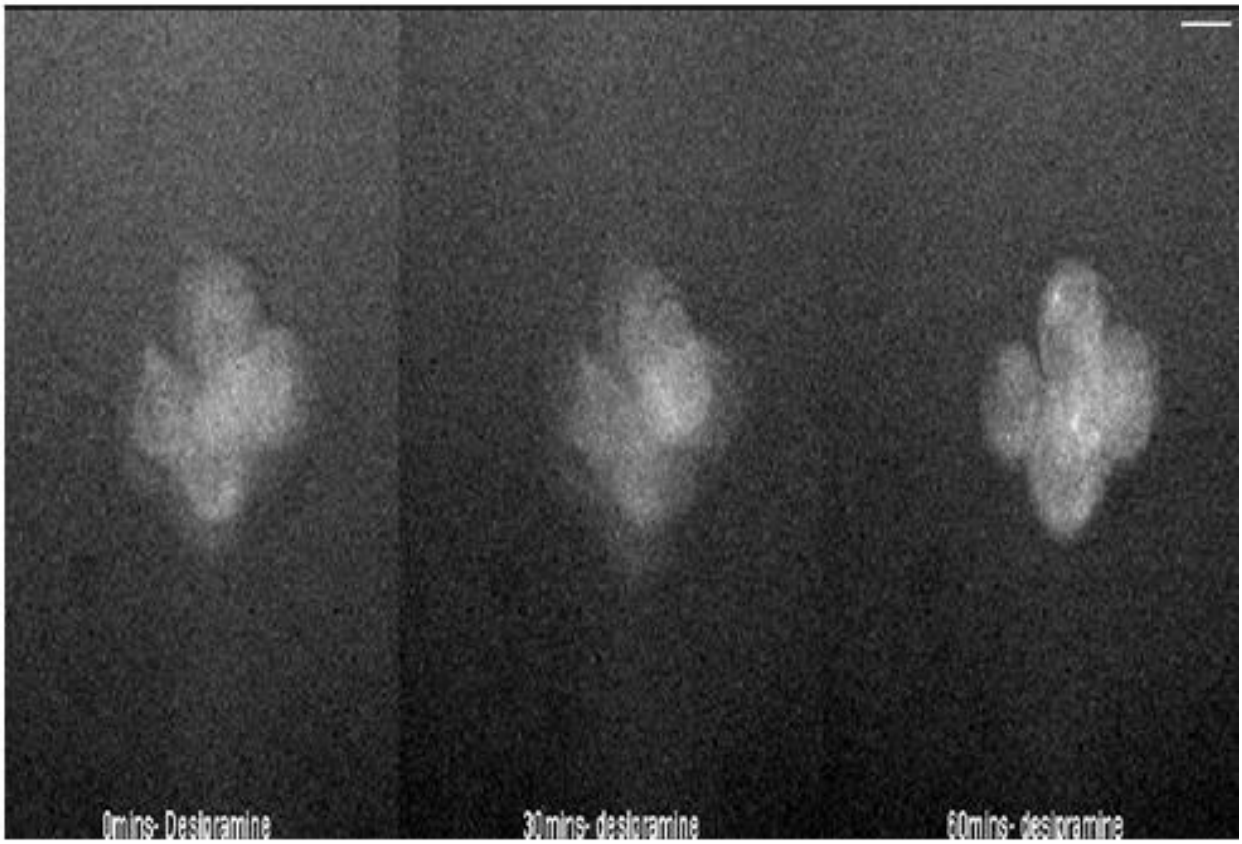

Figure 20: Showing increase in fluorescence signal (NTUA uptake) in PC12 cells in the presence of desipramine (1μM) at different times; 0mins, at 30mins and at 60mins. Scale bar = 10μm.

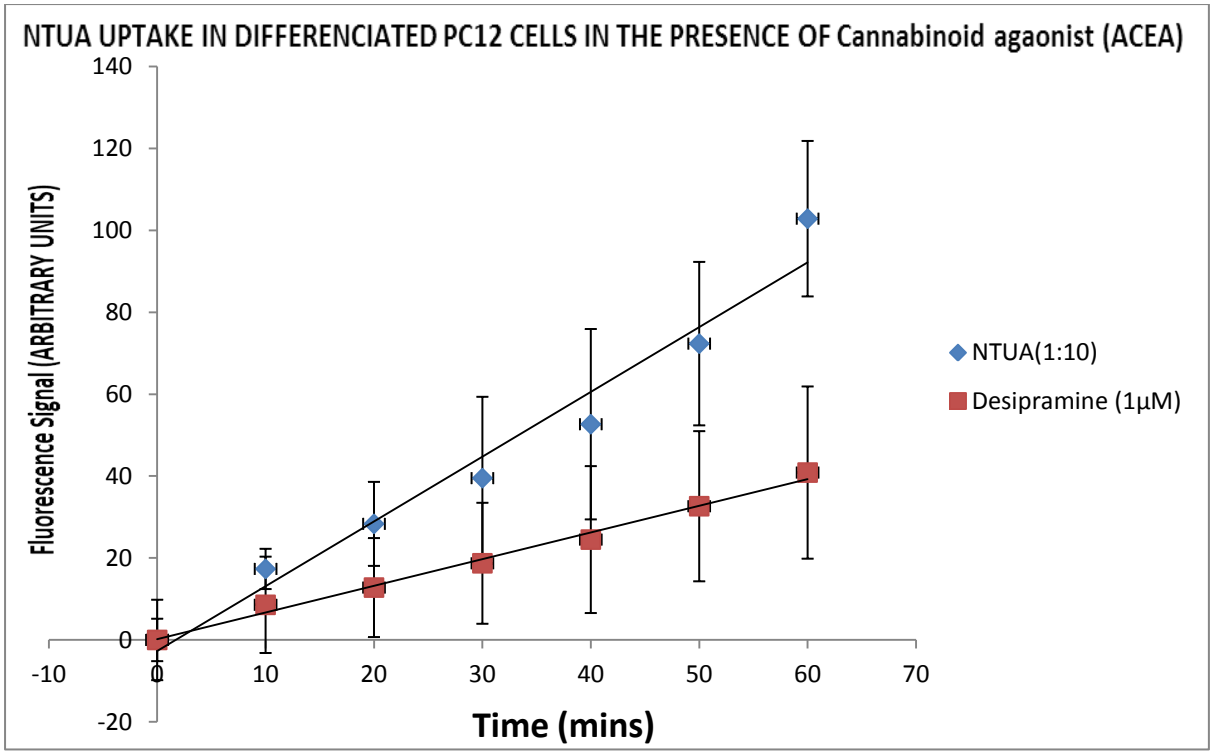

Figure 21:Graph showing fluorescence signal in differentiated PC12 cells during exposure NTUA and NTUA in the presence of desipramine. Following exposure to NTUA (1:10) a time dependent uptake of the fluorescence neurotransmitter transporter substrate was observed in both conditions. Data points are presented as mean ± S.E. (Student's unpaired t-test P<0.05. Nc=4-6)

Cells were seen to gradually increase in fluorescence after exposure to NTUA (1:10) in the presence and absence of desipramine. However, cells that were pre-incubated with desipramine showed a lower fluorescence uptake. Figure 22, shows a compilation of fluorescence uptake in all experiments. A non-significant reduction in fluorescence signal in presence of desipramine when compared to the control was observed (*P>0.05).

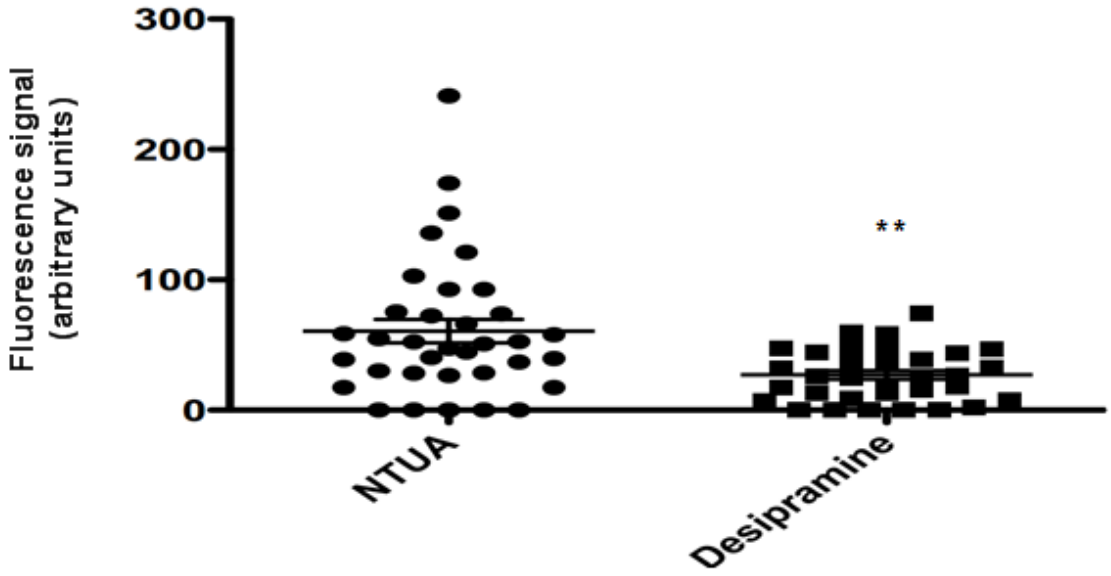

Figure 22: Result of all experiments carried differentiated PC12 cells in presence of desipramine. This shows and compared the relative increase in fluorescence signal (NTUA, 1:10) in differentiated cells, in the presence and absence of desipramine. (Mann-Whitney test **P = 0.00014; N= 5).

4.3.5. Effect of cannabinoid (CB1) receptor agonist ACEA, on the uptake of the fluorescent false transmitter in differentiated PC12 cells: In other to determine the role cannabinoids play in DA transport (fluorescence signal of the cells), CB1 selective agonist ACEA (100nm) was tested in cells. Control studies were carried, fluorescent uptake in differentiated cells. Followed by studies in the presence of the agonist, ACEA (100nM); cells were pre incubated in ACEA (100nM) for 20mins, afterwards the fluorescent false transmitter was added to the cells for 60mins. Images were taken every 10mins, see figure 23 and 24.

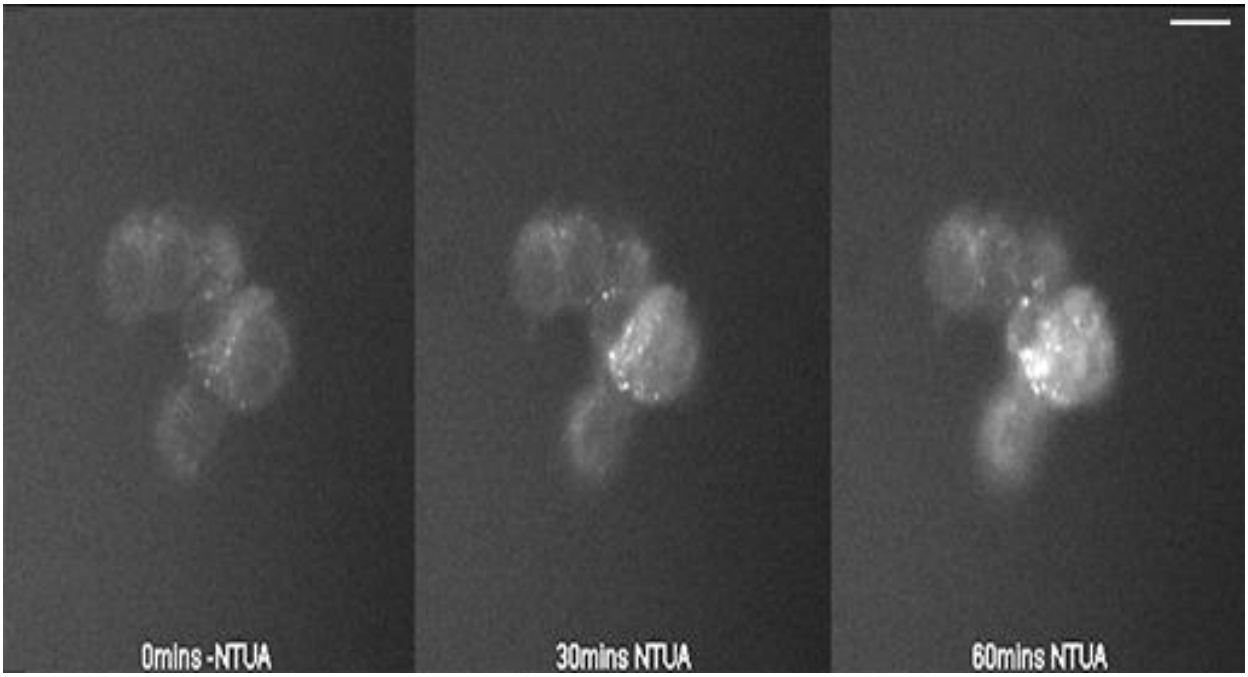

Figure 23: Shows fluorescence signal in the differentiated PC12cells at different time intervals; 0mins, 30mins and at 60mins. Scale bar = 10µm.

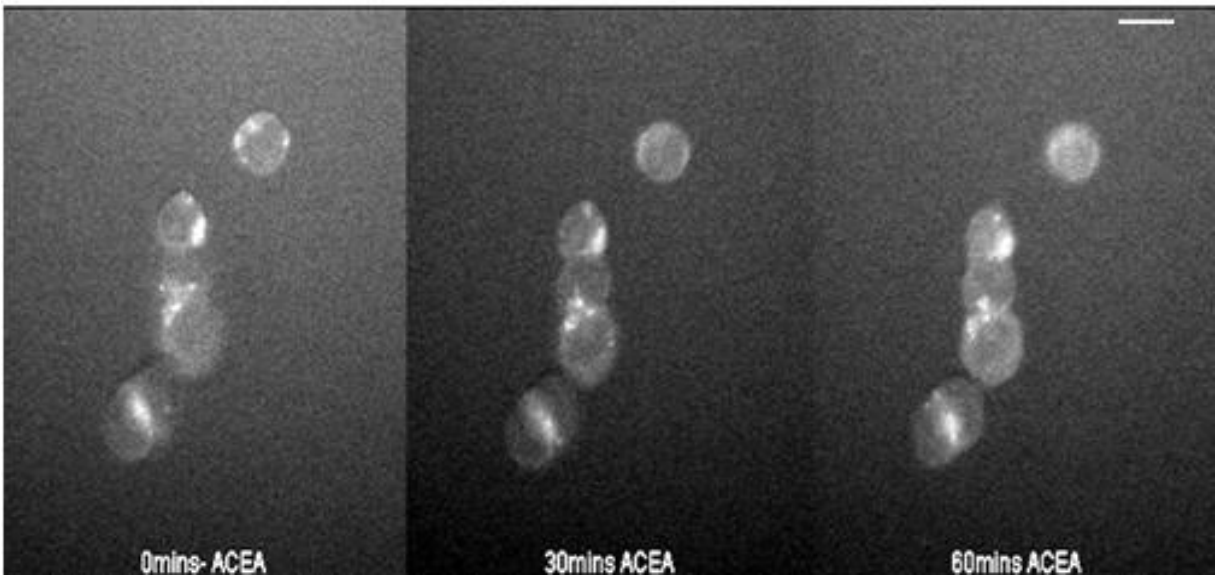

Figure 24: Shows fluorescence signal in the presence of ACEA (100nM) in the differentiated PC12 cells at different time intervals 0mins, 30mins and at 60mins. Scale bar = 10µm Scale bar = 10µm.

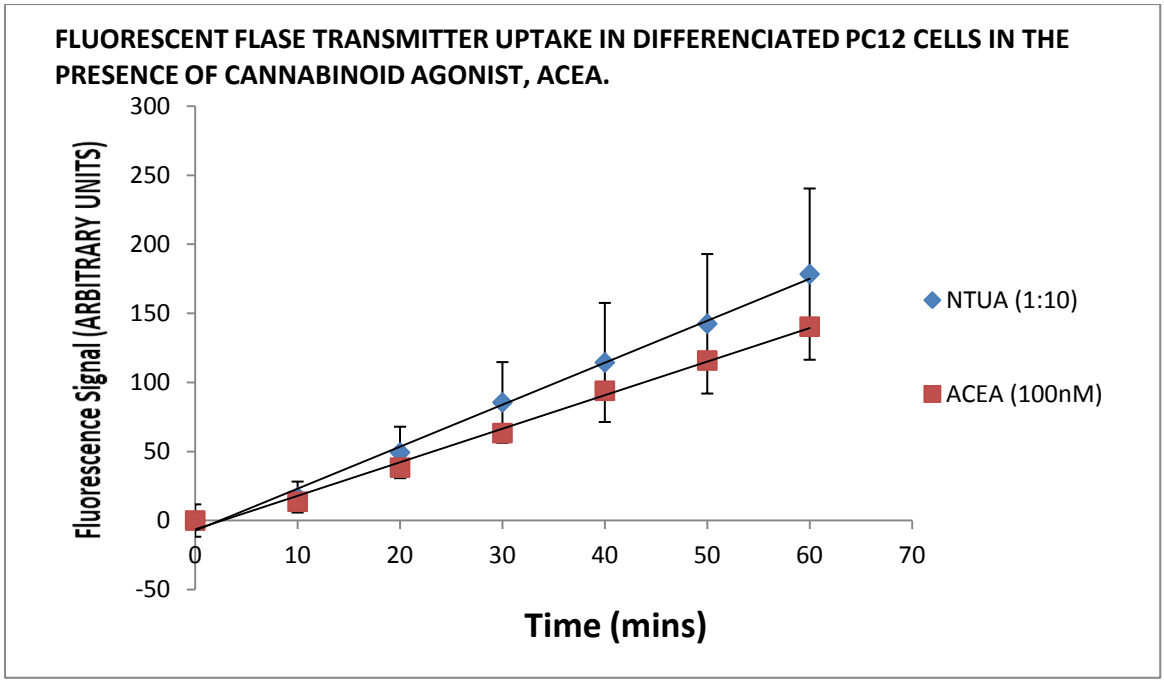

Figure 25; Graph showing fluorescence signal in differentiated PC12 cells during exposure NTUA and in presence of the CB1 agonist ACEA. Data points are presented as mean ± S.E. (Student's unpaired t-test P>0.05; Nc = 5-6)

Following exposure to the fluorescent flase transmitter (1:10) a time dependent uptake of the fluorescence neurotransmitter transporter substrate was observed in the cells (fig. 25). There was no significant difference in fluorescence signal in the cells pre-treated with ACEA compared to the control (N= 6; Fig, 26).

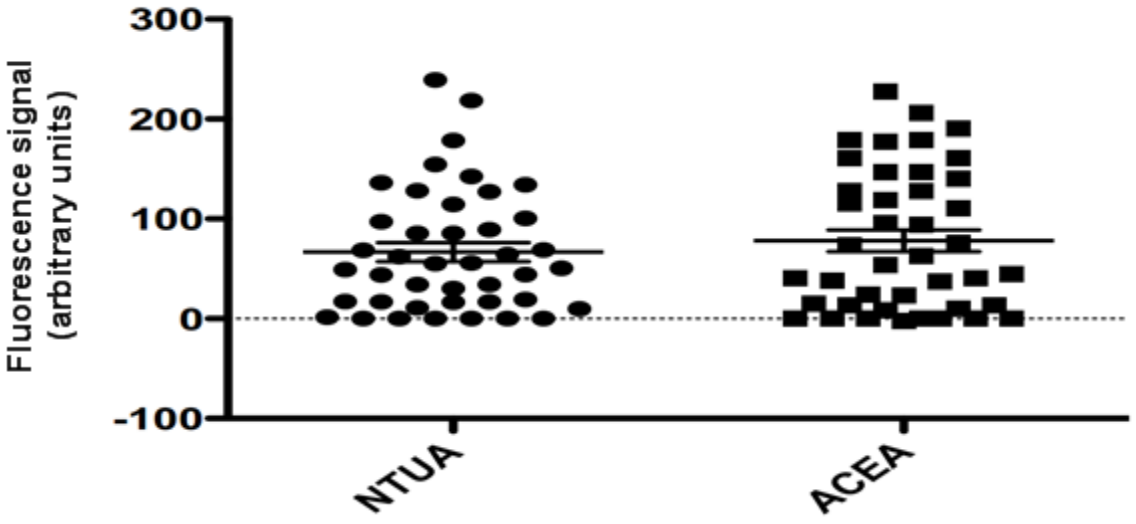

Figure 26: Result of all experiments carried out on the uptake of fluorescent false transmitter (1:10) in the presence of ACEA in differentiated PC12 cells. It compared the relative increase in fluorescence signal in the presence and absence of CB1

receptor agonist ACEA. ACEA had no significant effect on fluorescent transmitter uptake (N= 6, Mann-Whitney test *P*>0.05, *P*= 0.6192; N=6).

4.3.6. Effect of cannabinoid (CB_1) receptor antagonist AM281, on NTUA uptake in differentiated PC12 cells

Cells were pre-incubated with the CB_1 receptor agonist ACEA (100nm) for 10mins, before exposing cells to the fluorescent false transmitter (1:10) for 60mins. Images were taken every 10miutes for 60mins.Vesicles in cells become more as time increase (Fig. 27).

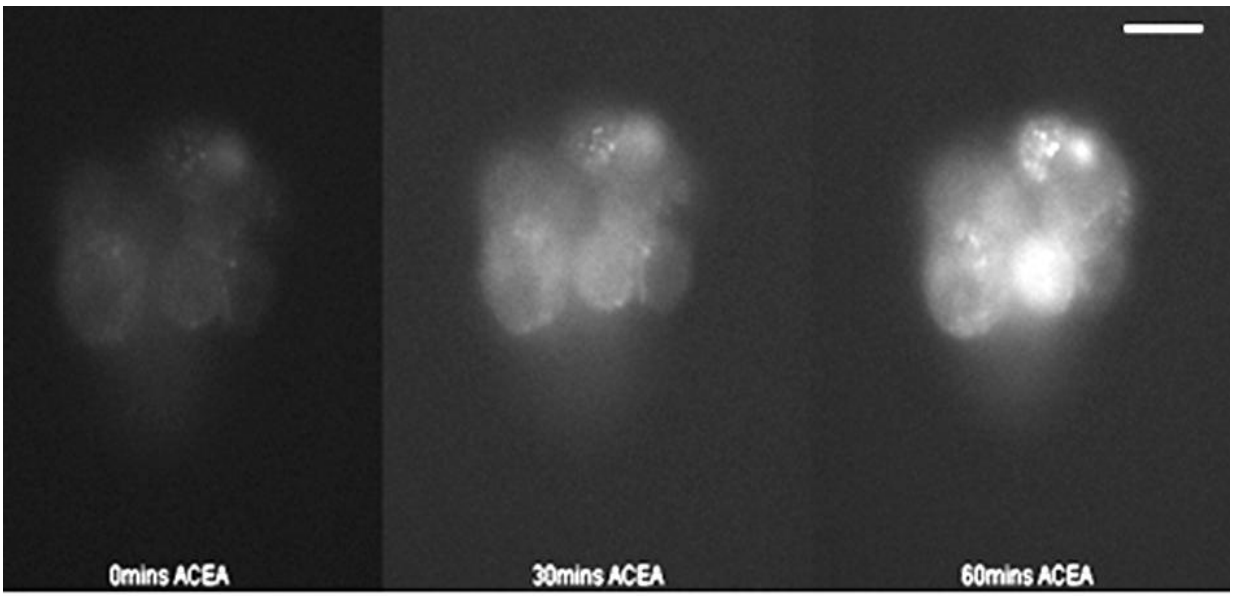

Figure 27: Shows fluorescence false transmitter uptake in the differentiated PC12 cells at different time intervals in the presence of CB_1 agonist ACEA (0mins, 30mins and at 60mins). Scale bar = 10µm.

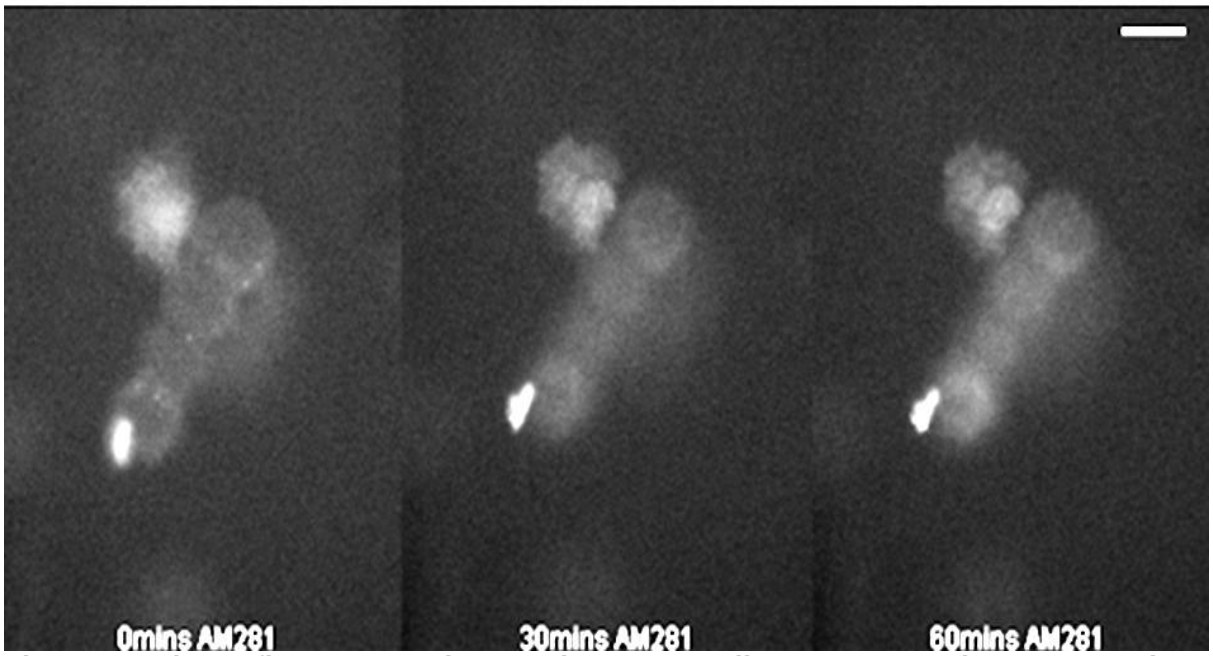

Figure 28: Shows fluorescence image when PC12 cells were exposed to ACEA and AM281 cells at at different time intervals 0mins, 30mins and at 60mins. Scale bar = 10µm Scale bar = 10µm.

Cells were pre-incubated with the CB_1 receptor agonist ACEA (100nm) plus the cannabinoid antagonist AM281 (100nM) for 10mins, before exposing cells to fluorescent false transmitter (1:10) for 60mins. Images were taken every 10miutes for 60mins (fig. 28). An increase fluorescence signal was detected in both conditions, however no significant change was observed in the presence of the CB_1 antagonist AM281 (fig. 29).

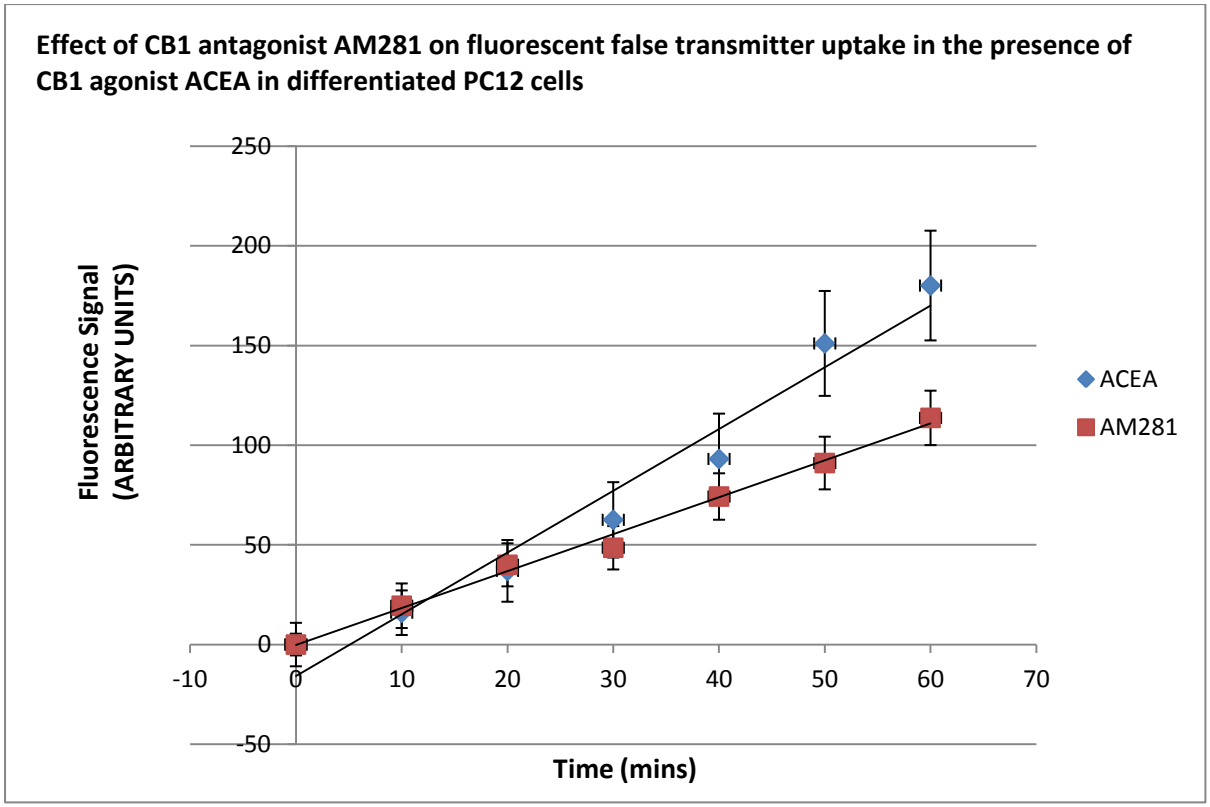

Figure 29: Graph showing fluorescence signal in differentiated PC12 cells during exposure to ACEA and AM281. Data points are presented as mean ± S.E. Student's unpaired t-test P>0.05; Nc= 5-7 cells.

4.4. Plate reader

. Cells were plated down in and differentiated in a 96 well plate. On the day of experiment, cells were exposed to 5 conditions the fluorescent false transmitter (1:10) was added to each well with a cells, either in the presence of, GBR (100nM), ACEA (100nM), AM281 (100nM) or AM281 + ACEA. The well with just the fluorescent false transmitter (1:10) acted as the control and each condition was repeated in 5wells. After analysis, a gradual fall in signal was observed for all wells. Figure 30 shows the graph representation for the average reading gotten from the control wells. Result showed that, uptake of the fluorescent false transmitter wasn't achieved by the cells using the plate reader technique. All conditions showed no increase in fluorescence signal with time and reading weren't consistent (See Appendix).

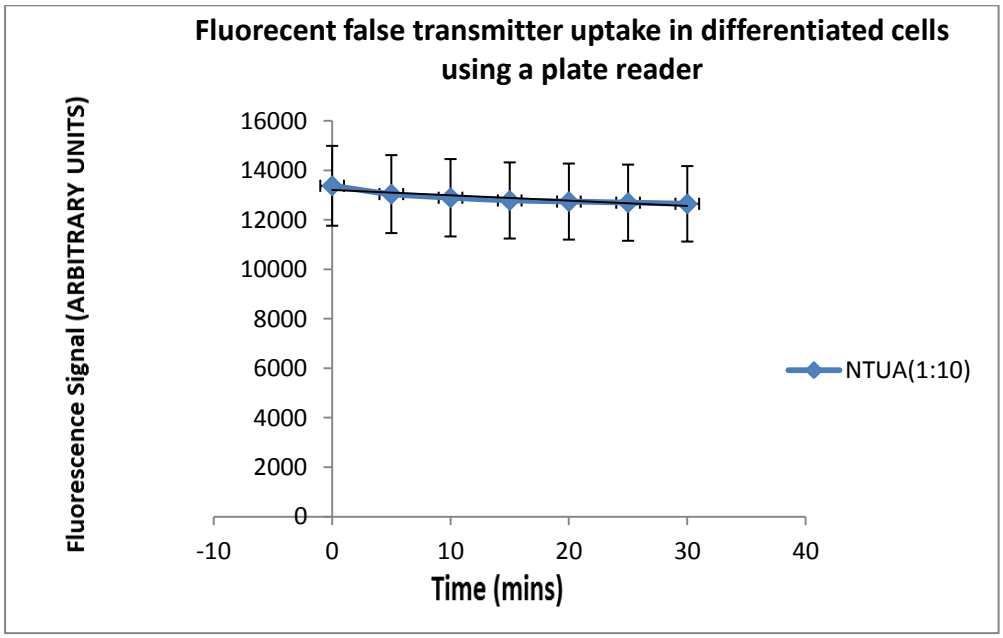

Figure 30: Shows fluorescence signal of PC12cells in the presence of the false transmitter using a fluorescence plate reader

4.5. Quantitative real time PCR (q-PCR):

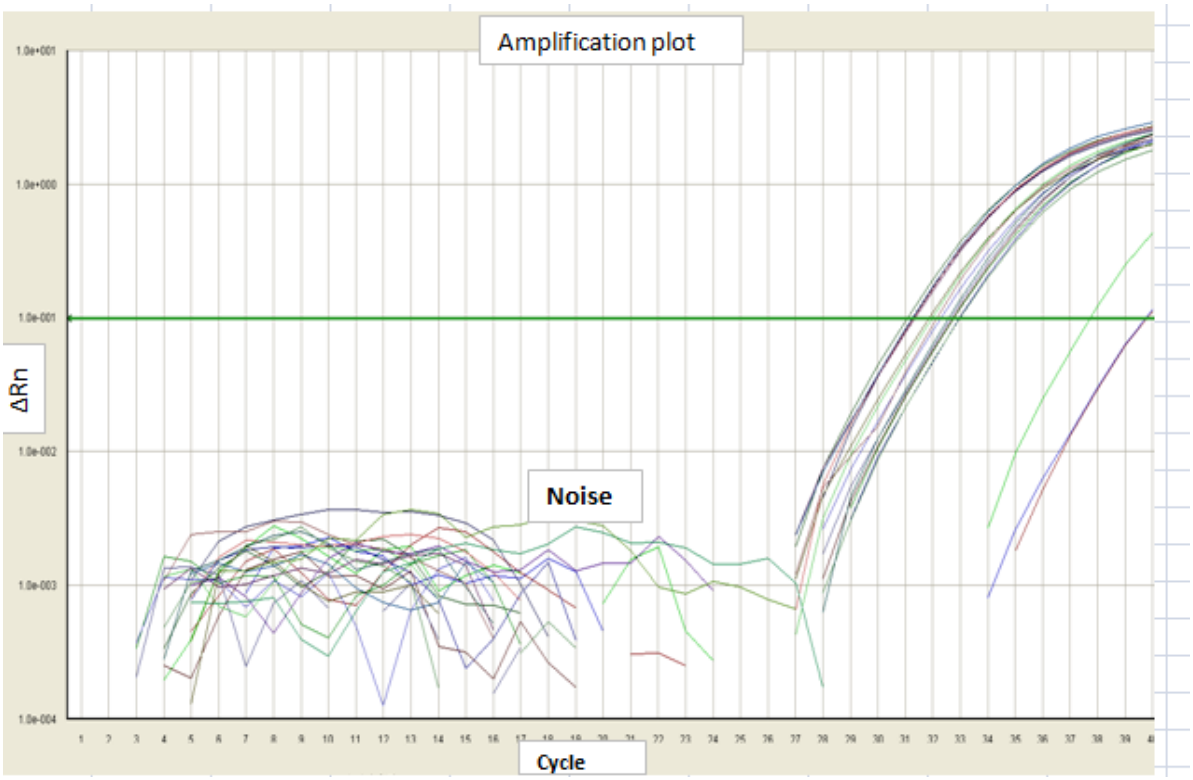

Figure 31: Graphical representation of q-PCR analysis carried out showing PCR cycle number for each sample. Log fold dilution of Cnr1 in triplicate wells for each sample. The green line running across indicates the threshold which remained the same in all q-PCR runs.

The quantitative level of CB_1 receptor gene (Cnr1) expression was measured in PC12 cells which were differentiated in vitro for seven days and also as a positive control in slice of rat brain (both cortex and brain stem). Each sample was measured in triplicate wells and for ensuring reproducibility q-PCR was performed on three different days. Figure 31 shows the average relative expression of the CB1 receptor (Cnr1) gene in each sample.Cnr1 was detected in all brain samples. In comparison it was detected in a low quantity in PC12 cells. This shows the relative quantity in all samples when compared to the control (GAPDH) are approximately equal for example sample 2 is 100 folds higher than sample 1 and sample C1 is 10 fold more than sample 2 in both cases. Both samples of PC12 cells tested showed insignificant difference in the mRNA expression of the CB1 receptor gene.

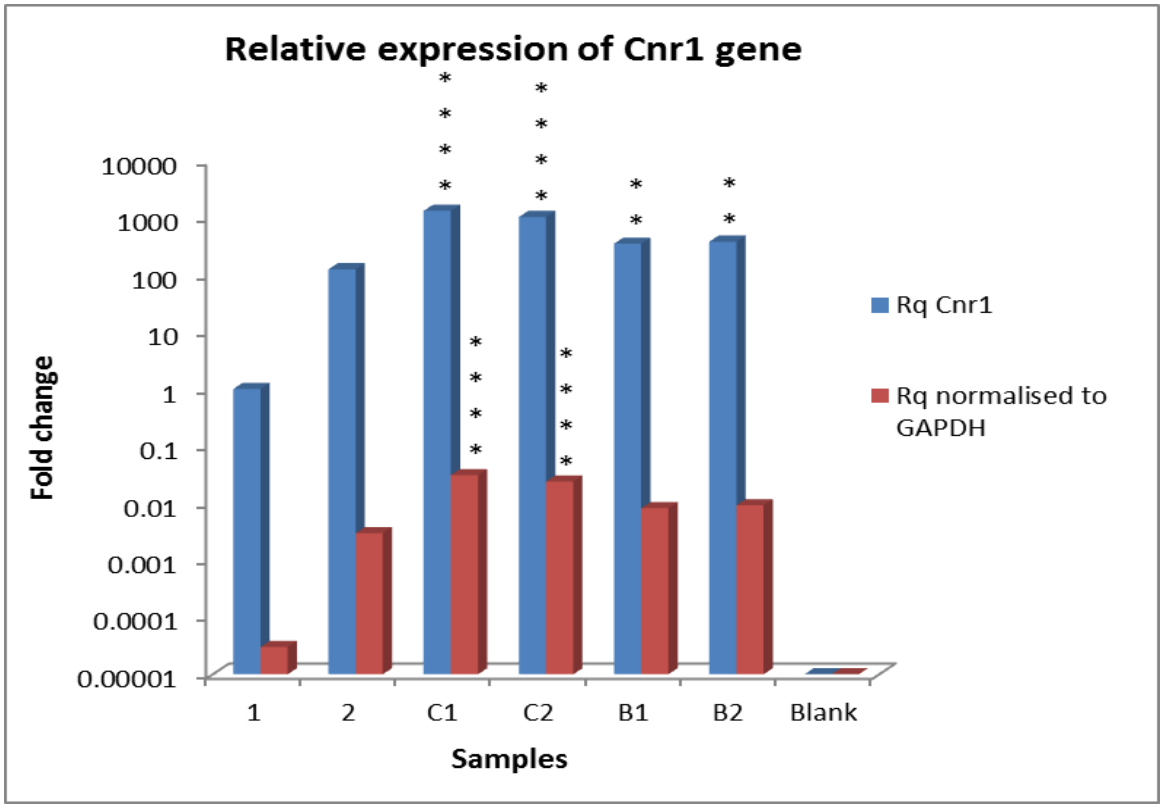

Figure 32: Chart showing fold change comparing the average relative expression of three qPCR runs carried out on the samples. cDNA levels of Cnr1 measured in differentiated PC12 cells and rat brain tissue. (Samples: 1- : 8th day differentiated PC12 cells; 2:- 7th day differentiated PC12 cells; C1-2:- cortex; B1-2 - Brain stem). Also shows fold change in gene relative expression normalized to the control gene (GAPDH). Fold change refers to the change in expression level of the Crn1 gene, from the final to initial value in each sample. Bars with star (*) are statistically significantly (**P<0.0001, **P<0.01 using one way ANOVA) different compared to sample 1.**

5.0. Discussion

A link between cannabinoid signalling and the DAT was initially suggested by an incidental observation that tetrahydrocannabinol seems to inhibit noradrenaline uptake into sympathetic terminals in the mouse vas deferens (Kennard and Brain, personal communication). If such a link occurs for the noradrenaline transporter, then the question might naturally be asked as to whether the dopamine transporter is similarly regulated.

Abnormalities in brain monoamine transmission are associated with a number of neurodegenerative and neuropsychiatric diseases including drug addiction, depression and Parkinson's disease (Manson et al., 2005). DA is linked to most neuropsychiatric disorders. DAT is localized at presynaptic sites, where it is vital for maintenance of DA storage and for the termination of DA transmission (Jorgensen, Nielsen, Peters and Dyhring, 2008). Studying changes in this transporter function may facilitate to gain new insights into the mechanisms regulating neurotransmission in neuropsychiatric diseases (Petal et al., 2003). Presynaptically, Na^+/Cl^--dependent transporters play a crucial role in limiting the actions of catecholamine at target receptors in both the central and peripheral nervous system (Manson et al., 2005). Studies show contribution of these transporters and their regulation to a wide range of physiological activities, e.g. cognitive behaviour and autonomic function (Wu et. al., 2002). Given their impact on chemical signalling, it is not surprising to learn that DAT, as well as other neurotransmitter transporters are themselves subject to regulation (Manson et al., 2005; Zahniser and Doolen, 2001).

Initially studies were conducted using human derived human neuroblastoma, BE(2)-M17 cells. From the study carried out, these cells showed a steady increase in fluorescence after exposure to the fluorescent false transmitter. However, overall cells showed a weak and inconsistent uptake of the fluorescent neurotransmitter. After failure to improve uptake in these cells, it became necessary to apply the study using an alternative cell line. The phaeochromocytoma (PC12) cell line was then used to carry out subsequent studies. This cell line originates from a rat adrenal chromaffin cells tumour (Fujita et al., 1989; Schimmelpfeng et al., 2004). This cell line is useful for neurobiological studies and when differentiated it induces changes in the cell resulting particularly in increased characteristics of sympathetic neurons

(Das et al., 2004). Results from the undifferentiated PC12 cells showed that the rate of uptake of the fluorescent false transmitter was relatively low, but still was greater than that seen BE(2)-M17 studies. The uptake was however, sufficiently low that any regulation of the transporter might be below detection threshold because of a lack of power to distinguish important effects. Therefore, in order to maximize and attain a reasonable uptake, the PC12 cells were differentiated *in vitro* before used in the experiments.

The process of differentiation can be induced in PC12 cells by various factors, which includes: nerve growth factor (NGF), cytokines, epidermal growth factor and basic fibroblast growth factor (Ohuchi et al., 2002). For the purpose of this study, NGF was used to induce neuronal differentiation of PC12 cells. An important feature of PC12 cells is that they respond to NGF with a dramatic change in phenotype and acquire a number of properties and characteristic of sympathetic neurons (Das et al., 2004). NGF is a peptide required for the survival and development of sympathetic and sensory neurons (Kim et al., 1990). NGF differentiates the cells by acting on the TrkA receptors, leading to the sustained activation of the Raf/MEK/MAPK pathway (Das et al., 2004) and subsequently to neuronal differentiation (Kao et al., 2001). NGF-treated PC12 cells cease proliferation, extend neurites, and become electrically excitable (Ohuchi et al., 2002).

Differentiation of PC12 cells is assessed by quantitative or semi-quantitative morphological methods. These methods include measurement of cell size, the number of cells possessing neurites, and measurement of neurite length (Das et al., 2004).These assessment methods can be difficult in large samples of cells. The reason for differentiating the cell is to ensure cells were exhibiting characteristics of mature neurons which includes neurotransmitter release, membrane excitability and exocytosis (Andres et. al., 2013).

In the present work differentiated cells were seen to develop neurites by the third day of NGF treatment, and the cells were used for experiment on the seventh day. The uptake of the fluorescent false transmitter in differentiated cells was largely increased compared to the undifferentiated ones. Both differentiated and undifferentiated cells displayed a time-dependent uptake of the fluorescent false transmitter, showing monoamine transporter activity. During 30mins washout, cells

were seen to gradually drop fluorescence signal. This result suggests that fluourescent false transmitter was indeed taken up into the cells through one of the monoamine transporter. Given the known transporters on differentiated PC12 cells and NTUA kit used, it was expected that most of the uptake will be through DAT.

In order to investigate and determine if uptake of the fluorescent false transmitter was through DAT, the cells were pre-incubated in GBR 12909 (100nM). GBR is a potent monoamine uptake inhibitor with a high specificity for DAT. GBR is about 2 folds more potent as an inhibitor of dopamine uptake than that of either noradrenaline or serotonin uptake (Melia and Spealman, 1991). In behavioural studies, the effect of GBR has been found to mimic those of psychomotor-stimulant drugs known to inhibit dopamine uptake, including cocaine and bupropion. Like cocaine, it binds to the DAT at the terminal fields of the mesocorticolimbic pathway, where it inhibits the reuptake of dopamine leading to an increase in the extracellular concentration of dopamine (Melnick et al., 2001). Results from this study showed a time-dependent accumulation of fluorescence false transmitter in the differentiated PC12 cells, both in the presence and absence of the inhibitor, GBR 12909. However, when fluorescence uptake is being compared to the control, it shows a 70% reduction in fluorescence signal in the presence of GBR 12909 (Fig. 17). This shows GBR is possibly blocking or inhibiting DA uptake by DAT. The uptake of false transmitter in a preliminary study from our laboratory showed that GBR12909 inhibited the fluorescent false transmitter study but in the presence of NAd blocker desipramine, the uptake was not affected in undifferentiated PC12 cells (Sideway et al., 2011).

NAd transporter is also a member of the NA+/-Cl transporter family (Blakely et al., 1994). Most NAd inhibitors particularly drugs of abuse and tricyclic antidepressant also act on NE, AD and DA transporters, particularly drugs of abuse and tricyclic antidepressants (Parker et al., 2010). In other to investigate the specificity of fluorescent false transmitter, cells were exposed to desipramine (1µM) and uptake of the fluorescent false transmitter in the presence of desipramine was carried out (Fig. 21 and 22). Cells exhibited a significant decrease in NTUA uptake in the presence of desipramine. Desipramine is an antidepressant, which like many works by inhibiting NAd transporter. Selective blockers of the NAd transporter have been hypothesized to also inhibit DA uptake in some parts of the brain, mainly prefrontal cortex.

Evidence from Moron et al. (2002) suggests that DA uptake depend on DAT in nucleus accumbens and caudate areas of the brain, but dependent on NET in prefrontal cortex (Moron et al., 2002). It is therefore possible that the dopamine transporter substrate in NTUA was also transported into the cells through noradrenalin transporter, hence the reduction in fluorescence signals in the presence of desipramine.

In order to understand whether cannabinoids have an effect on DA signalling in the brain, a specific CB_1 receptors agonist, ACEA was used in the study. The effect of cannabinoids on synaptic function consists of inhibition of the release of a variety of neurotransmitters and also the inhibition of electrical activity by a depolarisation. In all experiments carried out (N=6), there was no significant effect in fluorescence uptake in the presence of the CB_1 agonist. This suggests that either that:

- CB_1 receptor activation has no effect on DAT function, or
- CB_1 receptors are not sufficiently expressed in these cells, or
- the agonist was ineffective in these cells.

In order to determine whether endocannabinoid activation might regulate DAT function at rest (indirectly testing hypothesis 3.) the effect of the DAT antagonist AM291 alone was determined. The antagonist AM281 had no significant effect on fluorescent false transmitter uptake. It therefore would be requisite to investigate if PC12 cells express CB_1 receptor seeing as it is the predominant cannabinoid found in the central nervous system and is of importance to this present study.

CB_1 receptors have been reported to be highly present in the central nervous system where it plays a part in synapse density, reward and behaviour (Pertwee, 1997; Takahashi and Castillo, 2006). However the present study is based on the effect of cannabinoid on dopamine transporter in PC12 cells. Molderings et al. (2002) carried out a study aimed to examine CB_1 receptor expression in PC12 cells using PCR and radio ligand binding techniques. Using primers for the rat CB_1 receptor gene, they showed that undifferentiated PC12 cells did not express detectable amounts of CB_1 receptors. In conclusion, undifferentiated PC12 cells were not endowed with CB_1 cannabinoid receptors (Molderings et al., 2002). Absence of CB_1 receptors in PC12 cells was also reported by other studies, (Marsicano et al., 2002). However, it is not clear whether or not differentiated PC12 cells express the CB_1 receptor.

In the present work of CB_1 receptor was investigated using q-PCR method. The q-PCR results indicate that the gene encoding the CB_1 receptor underwent transcription in all samples tested. However, there was a higher transcription rate in the cortex than in the other samples tested. CB_1 receptors have been shown through histology studies to be extensively mapped in rodent brain, with the rat cortex showing a more prominent pattern of CB_1 receptor expression (Tsou et al., 1998).

THC can induce apoptosis in cultured neuronal cells. This apoptotic effect is exerted through THC binding to and activating the CB_1 receptor, which are said to be linked to stress activated protein kinase. However in the presence of THC this process of apoptotic cell death was blocked when the CB_1 antagonist AM251 was exposed to the cells (Downer et al., 2003). Same study was carried out using anandamide (7 µM -10µM). PC12 cells were incubated with different concentrations of ananadamide and massive cell death was recorded with the batch incubated with higher concentration of anadamide. Anandamide exerts most of its effect by activating the CB_1 receptor in the brain (Sarker et al., 2000). The finding that the CB_1 receptor antagonist inhibits apoptosis in neuronal cells induced by THC and anandamide supports the finding from this present study that PC12 cells can expresses the CB_1 receptor. Surprisingly the PC12 cells differentiated to day 8^{th} showed a lower detection of the Cnr1 gene. While this might suggest that the more prolonged differentiation results in a loss of certain function, it is also possible that this is due to variability in the expression amongst cell populations in different experiments. It was also observed that cells with early passage ($p= <11$) had better uptake of fluorescent false transmitter, whereas with increasing passage a noticeable drop in uptake ability rate was observed (although this was not formally quantified).

The study using the fluorescence plate reader was performed in order to establish a more rapid method for testing cellular uptake and regulation. However, these experiments were unable to show, under control conditions, uptake of NTUA in the differentiated PC12 cells. Unsurprisingly, therefore in all conditions of drug incubation with the cells, there was no appreciable effect. This possibly was due to the fact the plate reader used didn't have the optimal excitable wavelength required for NTUA, which is 405nm. Alternatively, the cell density needed to be increased, from the 50,000cells per well plate for the differentiation in this study. However the differentiation process requires cells to be incubated in NGF for 7days. NGF media

changed every day. Cells density may have been lowered due to this process of multiple solution changes: with each solution change some cells can be lost due to detachment.

In conclusion the NTUA is a useful method for detecting neurotransmitter transporter activity in cells using microscopy-based approaches, but the sensitivity is too low for routine use in plate reader experiments with differentiated PC12 cells using the current experimental protocol. The cannabinoid agonist used in this study showed no significant effect on fluorescence intensity in differentiated PC12 cells, despite the presence of the receptor (as assessed with q-PCR). The low expression of the Cnr1 gene detected from q-PCR in this cell type could be the reason. Alternatively, it may be that CB_1 receptor activation has no effect on DAT function.

6.0. Future Work

Against the result of the present study, the following points for the future study can be considered:

- Studies involving cannabinoid should be carried out using cell type other than PC12 cells, that express high levels of the cannabinoid receptors such as the cells from mouse neuroblastoma N1E-115cells and cerebellar cell culture. Study should also be carried out using a second agonist, such as THC, in order to confirm this finding.
- In addition to q-PCR other techniques such as immune precipitation combined with Western blotting should be undertaken to demonstrate the receptors are expressed.

Further refinement of the plate reader approach, including an increase in the plating density and optimisation of the excitation wavelength, would be useful in order to increase the sensitivity of this method, which has the potential to greatly increase the speed with which functional changes in DAT rate can be studied.
For purpose of this study it would be further studies would be required, particularly focusing on the signal transduction pathways linking DAT and CB receptors. Such an interaction would then be explored in a tissue preparation, such as the autonomic innervation of the vasculature (in Prof Janice Marshall's laboratory). Central dopaminergic signaling could also be investigated in rat brain slices containing fields of DAT-expressing terminals.

7.0. APPENDIX

Table 2: Raw data of study carried out on NTUA uptake in BE-(2)-M17 cells.

Table 2A

Time(mins)	NTUA (1:10)	GBR 100nM	S.E NTUA	S.E GBR
0	0	0	0.230161356	0.077963667
10	-0.4216	0.30175	0.252803184	0.11195969
20	0.774	0.4795	0.303845767	0.084846266
30	1.4512	0.672	0.370418075	0.070587219
40	1.7842	0.877	0.422228818	0.093875213
50	2.191	0.95275	0.443204947	0.119651694
60	2.4872	0.9835	0.465769712	0.087438868

***P* = 0.371**

Table 2B

Time(mins)	NTUA	GBR
0	0	0
10	-3.16367	-1.9386
20	3.846833	-0.461
30	4.598097	2.1806
40	5.596333	-0.5452
50	9.450667	-0.5778
60	12.7555	-0.346

***P* = 0.050397**

Table 2C

Time (mins)	NTUA	GBR
0	0	0
10	-13.2193	-11.7155
20	-13.4458	-13.8903
30	-9.05975	-2.967
40	9.3875	1.39025
50	12.45975	5.54475
60	18.919	10.878

***P*=0.713924**

Table 2D

TIME(mins)	NTUA	GBR (100nM)
0	0	0
10	-10.803	-15.703
20	-8.6222	-17.7365
30	-4.4254	-14.114
40	-4.4254	-8.8515
50	3.3876	-3.2805
60	14.625	-4.0475

***P*= 0.0922**

Table 2E

Time(mins)	NTUA	GBR 100nM
0	-0.06717	0
10	1.958167	0.86625
20	4.686	0.842
30	5.5045	1.0025
40	6.866667	1.417
50	7.5285	1.5855
60	9.203	1.653

****P*=0.01585**

Table 3: NTUA uptake in PC12 cells over time.

Table 3A: Change in fluorescence signal in PC12 cells over time. 70-90mins were washout periods.

Time(mins)	Undiffrenciated cells	Differentiated cells	S.E undifferentiated cells	S.E Differentiated cells
0	0	0	5.314534397	8.124044877
10	14.669	56.717	8.481444903	1.613898592
20	38.1205	126.0023333	14.71918349	12.93489967
30	60.1325	187.4393333	19.69513272	23.15497634
40	81.43525	267.1586667	26.72478945	26.25348219
50	106.5865	338.5546667	32.78841239	39.82046711
60	116.931	396.6853333	31.86517845	52.30691478
70	120.04	447.257	29.94988522	56.47672881
80	102.143	495.5353333	22.90208038	67.37933121
90	79.41875	446.0898333	18.08972981	58.40110051

***P*= 0.00514**

Table 3B

Time(minutes)	Undifferentiated cells	Differentiated cells
0	14.67457	-18.6155
10	17.80786	20.07716667
20	23.80429	33.3755
30	27.68914	47.90333333
40	30.05214	54.39366667
50	33.71257	65.723
60	34.067	78.87316667

***P*= 0.296**

Table 3C

Time(mins)	Undifferntiated cells	Differntiated cells
0	0	0
10	26.31683333	53.714
20	41.35416667	121.4925
30	58.2925	188.059
40	73.427	242.228
50	79.16333333	296.87325
60	92.70866667	341.37075

***P*=0.0400**

Table 3D:

Time(mins)	Undiffrenciated cells	Differentiated cells
0	0	0
10	14.669	56.717
20	38.1205	126.0023333
30	60.1325	187.4393333
40	81.43525	267.1586667
50	106.5865	338.5546667
60	116.931	396.6853333

***P*= 0 .0499**

Table 3E

Time(mins)	Undiffrenciated cells	Differentiated cells
0	0	0
10	3.133285714	81.4624
20	9.129714286	202.0868
30	13.01457143	316.9812
40	15.37757143	214.4313
50	19.038	284.364
60	19.39242857	384.56

***P*= 0.0075**

Table 3F

Time(mins)	Undiffrenciated cells	Differentiated cells
0	0	0
10	41.35416667	874.49
20	59.61728571	1325.4362
30	69.64842857	1533.3926
40	83.39314286	1694.8558
50	95.83528571	1795.6158
60	103.4682857	1894.2974

***P= 0 .003**

Table 4:Change in fluorescence signal in PC12 cells over time in presence and absence of GBR

Table 4a: Raw data showing Change in fluorescence signal in PC12 cells over time in presence and absence of GBR (100nM). 70-90mins were washout periods.

Time(mins)	NTUA	NTUA+GBR(100nM)	SE-NTUA	SE-GBR
0	0	0	6.05931824	12.49445
10	96.4308	3.419333333	16.7834994	15.9467
20	112.181	4.530333333	19.254987	18.19094
30	125.4372	25.7175	22.4271069	24.05137
40	165.1378	35.766	25.7327549	26.39442
50	186.6358	47.09766667	27.9564775	30.80649
60	207.634	64.46683333	29.5970412	35.83661
70	195.4214	61.85533333	27.1877483	38.11537
80	189.1864	59.51866667	26.0783508	39.28455
90	178.3462	50.54516667	28.2224972	35.22564

***P*= 0.00028**

Table 4B

Time(mins)	NTUA	NTUA + GBR(100nM)
0	0	0
10	212.65	-16.719
20	260.29	-9.14825
30	321.32	9.581
40	414.81	33.1725
50	512.35	65.3435
60	539.48	96.521

***P*=0.0052**

Table 4C

Time(mins)	NTUA	NTUA + GBR(100nM)
0	0	0
10	132.4215	19.2038
20	269.2463	66.1686
30	411.0475	124.4892
40	533.2325	187.602
50	632.2195	246.8254
60	739.3613	307.9328

***P*=0.0518**

Table 4D

Time (mins)	NTUA	NTUA +GBR(100nM)
0	0	0
10	29.88617	0.85925
20	41.84983	2.19425
30	79.93017	4.34825
40	99.75033	13.0645
50	115.5332	16.58575
60	132.6807	18.4425

***P*=0.0134**

Table 4E

Time(mins)	NTUA	NTUA + GBR(100nM)
0	0	0
10	22.30066667	47.1675
20	22.30066667	31.34525
30	36.21866667	17.2145
40	42.02533333	33.51225
50	40.815	23.653
60	44.42266667	13.091

***P=0.486**

Table 5: uptake of NTUA IN differentiated PC12 cells in the presence and absence of Desipramine (lμM)

Table 5A

Time(mins)	NTUA(1:10)	Desipramine (1μM)	S.E NTUA	S.E Desipramine
0	0	0	5.176066718	9.814145929
10	17.33333333	8.571428571	4.911720676	11.75907282
20	28.33333333	12.78571429	10.25304833	12.08534466
30	39.5	18.71428571	19.85048278	14.76629056
40	52.66666667	24.5	23.23879658	17.93085236
50	72.33333333	32.64285714	19.94993734	18.33009407
60	102.8333333	40.85714286	18.97695971	21.02234383

***P*=0.12**

Table 5B

Time (mins)	NTUA	Desipramine 1μM	SE NTUA	SE Des
0	0	0	13.119145	5.780581
10	40.29	7.85925	16.02011	6.905194
20	52.3306	15.69425	13.987291	9.611358
30	135.903	26.3605	27.823327	6.945371
40	151.2046	35.5645	28.604164	12.55151
50	174.1746	46.58575	30.796434	10.19736
60	241.2186	58.4425	44.394737	8.609224

***P*=0.04**

Table 5C

Time(mins)	NTUA	Desipramine
0	0	0
10	26.55783	18.2425
20	30.06883	25.992
30	38.91867	34.89066667
40	50.765	44.16516667
50	57.81283	59.54583333
60	66.23017	74.183

***P*=0.88**

Table 5D

Time (mins)	NTUA	Desipramine
0	0	0
10	17.29125	6.770666667
20	36.906	12.77933333
30	44.5585	17.08233333
40	54.96575	25.401
50	73.76675	38.71433333
60	92.6955	47.08233333

***P*=0.10**

Table 5E

Time(mins)	NTUA	Desipramine(μM)
0	0	0
10	28.7375	1.9806
20	47.39783	32.0944
30	58.6165	37.8074
40	75.42433	43.5638
50	92.73683	49.0148
60	121.1235	50.3614

***P*= 0.12**

Table 6: NTUA uptake in differentiated PC12 cells in the presence ACEA (100nM)

Table 6A

Time (mins)	NTUA (1:10)	ACEA (100nM)	S.E NTUA	S.E ACEA
0	0	0	18.50786	11.71715
10	16.98867	13.61425	27.06158	11.20988
20	49.29333	38.32775	24.14223	18.61513
30	85.50933	63.31775	26.62188	29.21019
40	114.4513	93.97875	29.06906	43.10169
50	142.4477	115.8753	33.13627	50.4989
60	178.4083	140.3658	40.55381	62.00121

***P*=0.604**

Table 6B

Time (mins)	NTUA	ACEA(100nM)
0	0	0
10	16.353	9.856
20	50.462	24.592
30	89.200	37.649
40	134.081	53.472
50	218.507	73.629
60	239.006	96.333

***P*=0.130**

Table 6C

Time(mins)	NTUA	ACEA(100nM)
0	0	0
10	1.415625	44.41167
20	9.69075	76.13083
30	19.123	118.4398
40	43.7465	160.3723
50	55.1085	190.1902
60	68.97363	227.3113

****P*= 0.029**

Table 6D

Time (mins)	NTUA	ACEA (100nM)
0	0	0
10	10.7573333	179.1698
20	16.9435	128.2211
30	34.1221667	147.1823
40	44.1918333	160.4321
50	55.9386667	178.7635
60	64.113	206.0888

***P*= 0.004**

Table 6E

Time(mins)	NTUA	ACEA (100nM)
0	0	0
10	17.14329	0.6414
20	34.31857	8.8622
30	68.56114	13.6642
40	97.003	14.9426
50	127.131	23.239
60	154.6641	40.4266

***P*= 0.041**

Table 6F

Time	NTUA	ACEA(100nM)
0	0	0
10	30.28129	40.30986
20	62.278	-2.55114
30	85.62971	110.3696
40	100.5724	127.533
50	128.0826	147.0914
60	136.3104	177.614

***P*=0.813**

Table 7: NTUA uptake on PC12 cells in the presence of ACEA in the presence CB1 antagonist AM281(100nM)

Table 7A

TIME (mins)	ACEA(100nM)	AM281(100nM)	S.E ACEA	S.E AM281
0	0	0	5.4556027	10.904109
10	15.97635	19.4689	11.187659	11.177475
20	36.9639	39.98643	15.489146	10.794005
30	62.54781	48.54617	18.901194	10.878564
40	93.09558	74.25403	22.746181	11.648734
50	151.0478	91.0452	26.313502	13.215047
60	180.0846	113.7004	27.525036	13.647139

***P*=0.483**

Table 7B

TIME (mins)	ACEA(100nM)	AM281(100nM)
0	0	0
10	32.39157	13.18329
20	57.35971	27.93971
30	95.79514	49.15571
40	149.2656	64.05686
50	200.944	88.64057
60	255.9107	167.85214

***P*=0.053**

Table 7C

TIME (mins)	ACEA(100nM)	AM281(100nM)
0	0	0
10	18.07	11.2098
20	32.15903	27.8204
30	48.95321	46.71136
40	74.51171	78.65447
50	124.0438	86.982
60	166.0039	100.90376

***P*=0.569**

Table 8: NTUA uptake using plate reader

Raw data of Studies carried out on fluorescence uptake in differentiated PC12 cells pre-incubated with NTUA, GBR, CB1 agonist ACEA and CB1 antagonist AM281.

Time(mins)	NTUA(1:10)	GBR(100nM)	ACEA(100nM)	AM281 + ACEA	AM281(100nM)
0	13374.8	11350.2	12901.2	12881.8	11389.2
5	13039.4	11106.6	12629	12604.2	11139.2
10	12891.2	10982.6	12489.6	12474.2	10990.4
15	12782.2	10893.6	12381.6	12360.8	10887.8
20	12737.4	10826.2	12341.4	12302.6	10830.8
25	12692	10795.6	12274.6	12243.6	10801.2
30	12646	10767.6	12261.4	12212.4	10758.2

Table 9: q-PCR analysis showing the relative quantities

Table 9A: Raw data from q-PCR analysis, showing the relative quantities of the Cnr1 gene in the different samples. (N= 3).

Samples	Relative Quantity (RQ)	RQ2	RQ3	Avg RQ	S.E
1	1	1	1	1	0
2	132.727	120.873	120.145	124.5817	4.078085
C1	1158.044	1439.799	1353.975	1317.273	83.38019
C2	832.427	1098.593	1147.978	1026.333	97.99537
B1	387.395	266.883	394.374	349.5507	41.3829
B2	232.589	589.368	314.784	378.9137	107.8692
Blank	0	0	0	0	0

Table 9B : Raw data from q-PCR analysis, showing the relative expression of Cnr1 normalised to GAPDH. (N=3)

Samples	Rq1	Rq2	Rq3	Av. Rq
1	5.32799E-05	1.64453E-05	2.02045E-05	2.99766E-05
2	0.00337675	0.003290776	0.0022092	0.002958909
C1	0.029377018	0.038701241	0.024665015	0.030914425
C2	0.021160081	0.029177352	0.021101791	0.023813074
B1	0.009926613	0.007132932	0.007233322	0.008097623
B2	0.005979217	0.015664504	0.005712908	0.009118876
Blank	0	0	0	0

Table 9C : Raw data from q-PCR analysis, showing the number of cycles cycle number of the Cnr1 gene in the different samples. (N=3).

Samples	Ct	Ct2	Ct3	Avg Ct
1	39.03133	38.712	38.019	38.58744
2	32.77267	33.195	32.861	32.94289
C1	31.16467	30.886	30.999	31.01656
C2	32.09233	32.097	31.945	32.04478
B1	32.68133	31.255	32.942	32.29278
B2	31.424	32.065	31.041	31.51
Blank	0	0	0	0

8.0. Reference

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