Epidemiological, Pharmacological and Toxicological Investigation of New Psychoactive Substances

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (1) خَلَقَ الْإِنْسَانَ مِنْ عَلَقٍ (2)اقْرَأْ وَرَبُّكَ الْأَكْرَمُ (3) الَّذِي عَلَّمَ بِالْقَلَمِ (4) عَلَّمَ الْإِنْسَانَ مَا لَمْ يَعْلَمْ (5).

(سورة العلق)

In the name of God, the Most Gracious, the Most Merciful

Recite in the name of your Lord who created (1) Created man from a clinging substance (3) Recite, and your Lord is the most Generous (4) Who taught by the pen (5) Taught man that which he knew not.

(Al-'Alaq)

Abstract

New Psychoactive Substances (NPS) are synthesised to produce similar effects to controlled drugs of misuse, with an alteration in chemical structure to circumvent drugs of misuse legislation. One of the most prevalent NPS groups are synthetic cannabinoid receptor agonists (SCRAs). This research was performed to investigate the epidemiology and mechanisms of NPS toxicity.

Analysis of enquiry data from the UK National Poisons Information Service (NPIS) demonstrated that SCRAs were the NPS group most commonly reported in episodes of toxicity. There were increases in reported episodes of NPS toxicity over the last decade, with reductions associated with legal controls for specific NPS, e.g. mephedrone (2010). There has been a recent decline in episodes predating the introduction of the UK Psychoactive Substances Act (2016), which has since continued.

Reports of toxicity resembling serotonin syndrome in SCRA users could be due to structural similarities between indole SCRAs (e.g. JWH-018) and serotonin. This was investigated using ex-vivo rat brain slices. The inhibitory effect on serotonin reuptake observed with fluoxetine was not demonstrated with JWH-018 or CP 55,940 (non-indole SCRA).

Effects of chronic exposure to SCRA were investigated in a human neuronal stem cell model, examining effects on cell viability and gene (RNAseq) and protein expression (western blotting). The SCRAs studied had different toxicity profiles and were more toxic to immature than mature cells, while the cathinone stimulant mephedrone was toxic to mature cells. Chronic low dose exposure to the SCRAs MDMB-CHMICA and 5F-ADB induced changes in gene and protein expression related to protein synthesis, perturbation of mitochondrial function and increased cellular stress.

Toxicity caused by NPS in the UK has recently declined and legislation may have contributed to this. Neurotoxicity, gene and protein expression results suggest that chronic SCRA use could lead to adverse neurological effects.

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Publications and Abstracts

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I. Al-Banaa, A. Balin, S Rushton, S. Thomas. Poisons centre enquiries relating to synthetic cannabinoids receptor agonists (SCRAs) in the UK, 2009-2018. *Toxicology letters,* volume 314S1 (2019).

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Declaration

This thesis is submitted for the degree of Doctor of Philosophy to Newcastle University. The research described within was performed at the Medical Toxicology Centre within the Institute of Institute of Cellular medicine and is my own work unless otherwise stated. The research was carried out under the supervision of Prof Simon Thomas, Dr Sarah Judge and Dr Peter Hanson.

I declare that the work presented in this thesis has not been submitted for any other award and that is it all my own work.

Israa Al-Banaa

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Abbreviations

∆9-THC	Delta-9-tetrahydrocannabinol
5-HT	Serotonin
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
CB	Cannabinoid Receptor
CSEW	Crime Survey for England and Wales
DA	Dopamine
DPM	Disintegration per minute
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FEWS	The Forensic Early Warning System
GABA	Gamma-Aminobutyric Acid
GDS	The Global Drug Survey
GIT	Gastrointestinal tract
MAO	Monoamine Oxidase Enzymes
MT DNA	Mitochondrial DNA
MTF	Monitoring the Future
MT-tRNA	Mitochondrial tRNA
NE	Noradrenaline
NPS	New or New Psychoactive Substances
NPIS	National Poison Information Service
OCT	Organic Cation Transporter
PCA	Principal Component Analysis
PMAT	Plasma Membrane Monoamine Transporter
PSA	Psychoactive Substances Act
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
SCRA	Cannabinoids Receptor Agonist
SERT	Serotonin Transporter
TCDO	Temporary Class Drug Orders
TPH	Tryptophan 5-hydroxylase enzyme
tRNA	Transfer RNA
UGTs	Glucuronosyltransferase
UNDOC	United Nation Office on Drug and Crime
UPR	Unfolded Protein Response
VMAT	Vesicular Monoamine Transporter

Chapter 1 Introduction

The past 10 years have witnessed a substantial increase in the abuse of recreational drugs previously referred to as “legal highs” but now more correctly termed New Psychoactive substances (NPS). These substances are usually synthetic and similar in structure to traditional controlled drugs, but small changes to the molecule are made to evade control of drugs legislation based on exact chemical structure and/or detection by usual drug analysis methods (Johnson et al., 2013). Various terms have been used to refer to NPS, such as ‘research chemicals’, ‘synthetic drugs’, ‘designer drugs’, and ‘herbal highs’ (Winstock and Wilkins, 2011). The term ‘New Psychoactive Substances’ is more appropriate as a scientific term (Corazza et al., 2013), but more recently the term ‘New Psychoactive Substances’ is more often used. (Measham and Newcombe, 2016). NPS are defined by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), as:

“As a new narcotic drug or a new psychotropic drug that has not been scheduled under the 1961 and 1971 United Nations international drug control conventions, and which may pose a threat to public health comparable to the substances listed there in”.

NPS may be provided as synthetic chemicals, plant material or plant material sprayed with synthetic psychoactive drugs (Arunotayanun and Gibbons, 2012). Although previously commonly referred to as “legal highs”, it is important to note that some NPS products may contain controlled substances and not all produce a euphoriant effect (‘high’), with some for example being depressants. The term may have been used by suppliers to suggest to the users that the active substances included are not controlled under Misuse of Drugs Legislation. Users may also believe that the term legal implies that they are safe to use (Vardakou et al., 2011). This may encourage use, especially by young people, and this became common in Europe, Australia and the United States. In response, the European Monitoring Centre for Drug and Drug Addiction (EMCDDA) founded the European Union Early Warning System (EWS) in 1997 and was strengthened in 2005 to monitor and response to problems raised by the NPS (EMCDDA).

A particular challenge for managing the public health impact of NPS use is their availability for purchase via the internet, which facilitates the marketing of NPS as well

as the unrestricted and easy exchange of unedited thoughts and opinions about them (Wax, 2002, Brenda Wasunna 2015). NPS are also commonly sold in specialist shops, often termed ‘head shops’ or by street-level drug dealers who may also sell traditional illegal drugs of misuse. Marketing of NPS products may be enhanced by attractive packaging and their creative and appealing product names which tempt young people to use them and they are sold without any age restrictions (Weaver et al., 2015).

A further public health challenge associated with NPS is that active ingredients of specific branded NPS products may change over time, including after changes in drug legislation. This potentially puts the user in danger of acute toxicity because there may be no information on the safety in humans of exposure to an emerging NPS. In the other hand, the user will be unfamiliar with the active substance and the appropriate dose to use to obtain the desired effects (Davies et al., 2010).

There are two ways that an NPS may be developed. The first classical and generally easier way is by creating a structural analogue of a known psychoactive drug with sufficient changes to the structure so that it is not captured by control of drugs legislation. The second (more challenging) way is by synthesising a drug with similar pharmacological effects to a controlled drug, but with a substantially different structure to avoid detection (Brandt et al., 2014).

There are many types of NPS available; examples are synthetic cannabinoid receptor agonists (SCRAs), phenethylamines, amphetamines, tryptamines, synthetic cathinones, arylcyclohexamines, piperazines, pipradoles, piperidines, aminoindanes, opioid analogues, benzodiazepine analogues and tropane alkaloids (see Section 1.3).

1.1 Risks and effects

Concerns about the public health impact of NPS use arise from the numbers of people using these products and the wide range of chemicals that have been involved. During 2018 alone the EMCDDA reported the identification of 55 new NPS, and by the end of that year more than 730 NPS had been reported to the EMCDDA since their record began (EMCDDA, 2019). Almost all of these have not undergone any clinical testing; while some were originally developed for medicinal use, they did not progress to clinical use, sometimes because of safety issues (Helander et al., 2016). The current efforts to limit the emergence and spread of NPS are limited by a lack of scientific information, the originality of different psychoactive blends and the continuous development of new

substances. The combination of these substances with potentially bioactive material from different plants is a further challenge. Although, chemical analysis performed in December 2008 revealed that the psychoactive effects of some herbal mixtures tested were due to added synthetic cannabinoid receptor agonists rather than the plant material present in the herbal products (Dresen et al., 2010).

Legislative bans on use of one type of emerging drug often results in it being replaced very rapidly by a new uncontrolled drug (Gorun et al., 2010, Uchiyama et al., 2014). As an example, in Germany in 2009 the authorities banned the synthetic cannabinoid JWH-018, which was commonly responsible for the psychotropic effects of 'spice' products at that time. Four weeks after the ban, JWH-018 was no longer identified in spice products, but had been replaced by the newer synthetic cannabinoid JWH-073, which had not yet been regulated (Lindigkeit et al., 2009). This cat and mouse game potentially increases the hazards for users as even less may be known about appropriate dosing and adverse effects of the newer substance, which may have more deleterious consequences than the original drug in relation to drug dependency, toxicity and long term health effects (Baumeister et al., 2015).

A further concern, considering their increasing use, is the lack of pharmacological and toxicological information about NPS. Most of the available data are obtained from retrospective studies and analysis of intoxicated cases reported by the hospitals or poisons centres. Interpretation of these data are made especially difficult because multiple substances may be involved and these are usually not analytically confirmed as part of routine clinical practice. This makes it difficult to link a specific substance to an observed clinical effect (Hohmann et al., 2014). In addition, pharmacological and toxicological data are not available for the metabolites of these newly emerging drugs and these could potentially produce more potent effects than the parent compound (Crews and Petrie, 2015).

The potential serious health consequences of NPS use are also illustrated by the increasing frequency of presentation by NPS users to Emergency Departments (Baumann and Volkow, 2015). Mortality data are also concerning, for example in England and Wales, the number of fatalities ascribed to NPS increased between 2011 and 2018, although there was a transient reduction in 2017, as shown in Figure 1.1 (Office for National Statistics, 2018).

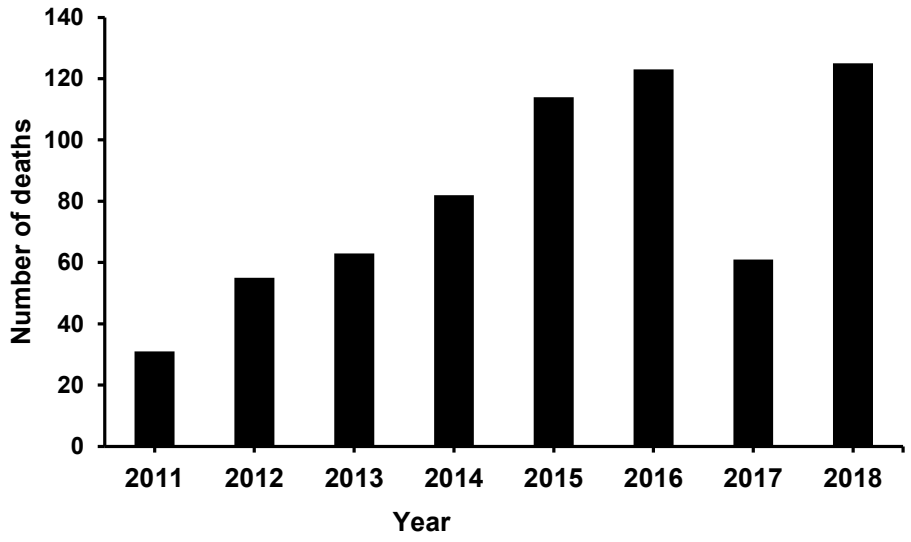


Figure 1.1 Number of deaths related to NPS poisoning in England and Wales (2008-2018).

Furthermore, rapid, efficient and inexpensive methods of detection of new synthetic NPS and their metabolites are not yet feasible. This is due to the complexity of the analytical methods required for each new compound, the lack of analytical reference standards and time required for establishing a standard procedure for each new compound (Favretto et al., 2013, Ibáñez et al., 2014). Synthetic chemists producing NPS may move onto a newer compounds while these analyses are being developed.

In addition, there is a particular lack of information about the longer term consequences of NPS use, which may present a severe challenge to public health (Ayres and Bond, 2012). Risks of social problems such as job loss, financial difficulties, family and relationship problems and criminal behaviour are likely, as is the case for established or 'traditional' drugs of misuse (Hill and Thomas, 2016). Very little information is available on the longer term toxic effects of NPS exposure, which is a particular concern for repeated users (Cooper, 2016). It is possible that NPS have important longer-term effects on the central nervous system; however, the exact mechanisms by which these compounds might exert these effects is not known. NPS affect monoamine neurotransmitters for example dopamine, serotonin and noradrenaline (Iversen et al., 2013). Mephedrone, for example induces serotonergic and dopaminergic neurotoxicity including serotonin and dopamine transporter loss with decreases in tryptophan hydroxylase and tyrosine hydroxylase expression (Martinez-Clemente et al., 2014).

Nagai et al. (2007) reported that several types of designer drugs, including synthetic amphetamines, tryptamine analogues and piperazines strongly act on the CNS and inhibit monoamine uptake and release in rat brain synaptosomes (Nagai et al., 2007). Synthetic cannabinoid receptor agonists may induce neurotoxicity through CB1 mediated cytotoxicity proceeding via apoptosis and this might be responsible for impaired attention and memory loss (Tomiyama and Funada, 2014).

1.2 Prevalence of use of NPS

It is difficult to determine the prevalence of NPS use because of continuous changes in the types, numbers and availability of these compounds. This is further complicated by the difficulty in identifying the chemical composition of the many branded products that have a wide variety of 'street names'. Therefore, most estimates of NPS use arise from general population surveys which have been conducted in many countries using various methods amongst different populations. While population surveys tend to evaluate the reported use of traditional drug of abuse, few surveys include NPS, or the survey may be restricted to specific categories such as synthetic cannabinoids, leaving other NPS not assessed (Palamar and Acosta, 2015). Most of the data on NPS that come from general surveys has its limitations as some groups might be underrepresented. In addition, some surveys focused on NPS use in certain populations at high risk. For instance, psychiatric patients (Stanley et al., 2016), gay people (Measham et al., 2011), prisoners (Ralphs et al., 2017) and dance drug users (Palamar et al., 2016). Data from poisons centres as well as hospital admission and mortality statistics can also help to provide estimation of NPS use and toxicity in the population.

1.2.1 NPS prevalence in the UK

Several countries have recently started to include NPS in their national drug surveys to collect information about the prevalence of NPS use, including in the UK the annual crime survey for England and Wales (CSEW). NPS terminology was first introduced for the 2014/2015 survey. The CSEW has demonstrated that the prevalence of NPS use is low in comparison with some other traditional drugs of abuse, such as cannabis. Cannabis was the most frequently used drug in adults aged 16-24 years in 2018/2019 survey. Prevalence of use over the previous year was 18.6%,16%, 17.1%,15.7%,13.5%, 15.1%, 16.4%,15.7%,16.4%,16.7% and 17.3 for the years 2008 to 2019. For NPS, last year prevalence of use (16-24 years) was 2.8%, 2.6%, 1.2%,

1.2% and 1.4% over the years 2014 to 2019 respectively (Home Office, 2019). In Northern Ireland, the life time prevalence of NPS use was 6% in adults aged 15-36 years and the last year prevalence of use was 6.7% in 2010/11 and 1.6% in 2014/15 for the same age group (The National Advisory Committee on Drugs and Alcohol, 2015). In Scotland, the lifetime prevalence of NPS was 1.8% overall of the respondents in 2017/2018 with the highest prevalence in those aged 25-44 years. In addition, this survey provided the first estimates of the prevalence of SCRA use, which was 2% over the previous year (Scottish Government, 2019).

The Forensic Early Warning System (FEWS) was established in the UK in 2011 to identify NPS present or provided for sale in the UK. The 2016/17 FEWS annual report reported that 57% of seized drug samples contained at least one NPS and 103 different NPS were encountered including 17 NPS new to UK. It was reported that SCRA were the most prevalent NPS, and the total number of new substances identified through FEWS since 2011 is now 67 (FEWS, 2018). The number of seizures of synthetic cannabinoids (spice) in prisons in England and Wales increased dramatically from 15 in 2010 to 86 in 2011, 133 in 2012, 262 in 2013 and 430 in 2014 (UK Government press release, 2015).

Separate studies have also reported NPS prevalence statistics for the UK, including for specific NPS types. An anonymous international survey of 14,966 participants was conducted on-line between November 23rd and December 21st in 2011(Winstock and Barratt, 2013b). The survey was promoted through social media such as Twitter and Facebook and in collaboration with the dance music magazine Mixmag and the Guardian newspaper. More than 50% of the participants were from the UK, 23% from the USA, 5.5% from Canada, 2.8% from Australia and 2% from Ireland. Two-thirds of them were male and the median age of respondents was 26 years. Of the participants, 16.8% reported using synthetic cannabinoid receptor agonists (SCRAs) during their lifetime with 41% of those reporting use of SCRAs in the last year. Of the recent SCRA users, 98% had also used cannabis, while about 50% reported consumption of MDMA and about one third reported intake of mushrooms, cocaine, LSD and/or benzodiazepines during the last year. About 93% of SCRA users preferred cannabis over SCRAs as they perceived the latter to have more undesirable effects. Corazza et al. (2014) reported that 31% of the participants in their on line survey among UK

students had used ‘legal highs’ including mephedrone (41.4%) and spice (10%) and 15.7% did not know what compounds they had consumed (Corazza et al., 2014).

In a study among 533 students aged 15-18 years attending London schools, 20% of the participants reported lifetime use of recreational drugs, with cannabis the most common (18.7 %) and NPS use was reported by only 1.1% of the participant (Penney et al., 2015). In a cross sectional on line survey, of 7700 UK poly drug users’ responding, 326 (4.2%) reported the use of the NPS Methoxetamine (Winstock et al., 2016).

Most of the data on NPS use arising from general surveys has limitations as some groups might be underrepresented. In addition, some surveys focused in NPS use in certain higher risk populations. Therefore, there remains incomplete information on the prevalence and trends in NPS use in the UK.

1.2.2 Global NPS prevalence

The World Drug Report for 2019 has reported that the quantity of NPS seized in 2017 was more than 400 times greater than that recorded in 2001. Over the period of 2007-2017 a total of 66 countries reported seizures of NPS, with this number increasing from 15 countries in 2007 to 45 in 2017 (World Drug Report, 2019). The report also, noted that 36% of the NPS identified in 2009-2018 were stimulants and 30% were SCRA. However, in recent years the emergence of synthetic opioid NPS has increased and has led to serious adverse effects and deaths, particularly in North America.

The Global Drug Survey (GDS) assesses drugs use in a predominately young educated population, across 35 countries with a sample size of 3900 participants. The survey showed that 4.3% of the respondents has used an NPS during the last 12 months and 35.1% of these were younger than 25 years of age (Global Drug Survey, 2019). A US nationally representative survey, it was reported that 1.2% of subjects aged 13-34 years reported use of NPS (Palamar et al., 2015). Tryptamines were the most commonly used NPS followed by psychedelic phenethylamines and SCRA. Other research, however, has suggested that SCRA are the most commonly used type of NPS in the USA. For example, the Monitoring the Future (MTF) survey is designed to monitor substance use in the young US population. This reported that SCRAs were the second most commonly used illicit drug of abuse after cannabis. The prevalence in 12th grade students was 11.4 % in 2011 and 2012, but dropped to 7.9% and 5.8 %in

the following years (Miech et al., 2015). In most recent MTF survey, SCRAs use among 12th grade students had fallen further to 5.5% in 2015, 3.5% in 2016, 3.7% in 2017 and 3.5% in 2018% (Schulenberg et al., 2019). In another study performed between January 2012 and July 2013, 1080 participants in the USA (age range 18-25 years, 53.4% males, from different ethnic origins) were questioned about their use of alcohol and drugs, including SCRAs in the previous 30 days. Of these, 9.3% reported “spice” use and 40% marijuana use on a daily basis over the last 30 days (Caviness et al., 2015).

A recent study reported that the life time prevalence of NPS use in 6 German states ranged from 2.2 to 3.9% making these the most commonly used illegal drugs after cannabis (de Matos et al., 2018). In 2014 the STRIDA project, which monitors the presence and health impacts of NPS use in Sweden, assessed the prevalence in NPS use among drug poisoning cases presenting to hospital emergency departments and intensive care units across the country. Of samples submitted, 83% tested positive for at least one drug and 50% of the cases for more than one drug. More than 50 substances were detected including synthetic cannabinoids, piperazines, substituted phenethylamines, synthetic cathinones, hallucinogenic tryptamines, piperidines, opioid related substances, ketamine and related substances, GABA analogues and phenethylamines. The age range of the cases was 13-63 years (median 20 years) and about 80% were adult males. (Helander et al., 2014) Recently, 14 analytically confirmed intoxications involving number of synthetic opioids were identified, 9 involving fentanyl, 3 involving 4-methoxybutyrfentanyl,1 involving furanylfentanyl and 1 involving 4-methoxybutyrfentanyl and furanylfentanyl together (Helander et al., 2016).

In a study by Martinotti et al. (2015) involving 3011 healthy Italian subjects (44.7% male; 55.3% female) from different Italian cities, aged between 16 and 24 years, 40.3% of the participants reported drug use with cannabis ranked first at 84.1%. NPS use was reported by 4.7%, including mephedrone (3.3%), SCRA (1.2%), saliva divinorum (0.3%), metamphetamine (0.2%) and desomorphine (0, 1%) (Martinotti et al., 2015).

In 2012 a Polish epidemiological study conducted on 10,083 school pupils and 4,428 university students showed that 4.49% of pupils and 1.83% of university students

admitted using ‘designer’ drugs. The prevalence in males (4.74%) was higher than in females (2.77%) (Bilinski et al., 2012).

In Australian cross- sectional surveys among 693 regular ecstasy users, 28% reported use of NPS in the past six months, 20% from the stimulant class (17% mephedrone) and 13% from the ‘other psychedelic’ class (2C-derivatives, DOI, Mescaline, 5-MeO-DMTand DMT) (Bruno et al., 2012).

Collectively, these surveys provide an overview of the prevalence of NPS, but they are not directly comparable as each one has a different definition of NPS and targets different populations.

1.3 Types of New Psychoactive Substances

NPS can be classified according to their chemical structure, pharmacological properties, clinical effects, or a combination of these factors (Liechti, 2015, Hill and Thomas, 2011, Gibbons, 2012). A useful classification is shown in Table 1.1.

Group	Traditional examples	NPS examples
Cannabinoid receptor agonists	Cannabis	Synthetic cannabinoid receptor agonists, JWH-018,HU-210, AM2201, AM1220, RCS4, UR-144, XLR-11, APICA, STS-135,BB-22, LY218324
Opioids	Heroin (diamorphine), Methadone	Acetylfentanyl
Benzodiazepines	Diazepam	Etizolam, diclazepam, phenazepam
Indolealkylamines (tryptamines)	Dimethyltryptamine 4-hydroxy,N,N-dimethyltryptamine (Psilocin),	alphamethyltryptamine (AMT)
Piperazines	Benzylpiperazine	Meta-Chlorophenylpiperazine (mCPP)
Arylcyclohexamines	Ketamine, PCP	Methoxetamine
Amphetamines	Amphetamine, methamphetamine, PMA, PMMA	4-methylamphetamine
Cathinones	Khat	mephedrone, 3-methylmethcathinone, α-PVP, methylone

Benzofurans and difurans	Non	5-(2-aminopropyl)benzofuran (5-APB), bromodragonfly
Aminoindans	Non	2-Aminoindane (2-AI), 5-Iodo-2-aminoindane (5-IAI), 5,6-methylenedioxy-2-aminoindane (MDAI)
D-Series	Non	2,5-dimethoxy-4 bromoamphetamine (DOB), 2,5-Dimethoxy-4- fluoroamphetmine (DOF)
2C-series	Non	4-bromo-2,5-dimethoxyphenethylamine (2C-B), 4-ethyl-2,5-dimethoxy-β-ethyl-phenethylamine (2C-E)
NBOMe compounds	Non	25I-NBOMe
Methylenedioxy amphetamines	3,4methylenedioxymethamphetamine (MDMA) 3,4methylenedioxyamphetamine (MDA)	
Piperidines and pyrrolidines	Non	D2PM,3,4-dichloromethylphenidate
Plant extracts	Non	Kratom, Salvia, ibogaine
Others	cocaine	4,4'-Dimethylaminorex (4, 4-DMAR), dimethocaine, 3-(p-fluorobenzoyloxy)tropane (pFBT)

Table 1.1 Classification of NPS and related traditional recreational drugs. * Overlaps in the structural classification exist such that some chemicals may belong to more than one group.

From all the above categories SCRA has been the NPS group most commonly encountered, therefore these are considered in further details below.

1.4 Synthetic Cannabinoids Receptor Agonists (SCRAs)

The term cannabinoids is used in general for “all naturally or synthetic compounds that can mimic the actions of plant-derived cannabinoids or that have structures that closely resemble those of plant cannabis” (Pertwee, 2005). Cannabinoids act on a cell membrane G-protein coupled receptor family, which is subdivided into CB1 and CB2 receptors. The term SCRAs refers to a “homogenous group of compounds that were originally developed as probes of the endogenous cannabinoid system or as potential therapeutic agents” (Zaurova et al., 2016).

Synthetic cannabinoids were first synthesised in the 1960s to mimic the action of tetrahydrocannabinol (∆9-THC), the active ingredient of *Cannabis sativa* (marijuana, hashish), to increase the therapeutic, anti-inflammatory and analgesic effects of this compound for medicinal benefits. However, it was very difficult to isolate the desired therapeutic properties from the undesirable psychoactive effects of these drugs (Musselman and Hampton, 2014).

The term “spice” became popular in Europe in 2005, as a term describing products containing synthetic cannabinoids as a legal alternative to cannabis (Auwarter et al., 2009). A variety of other names have also been used for cannabinoid containing products such as *Joker, Black Mamba, Kush, Kronic* etc. These products are commonly advertised as incense blends to be burned to scent rooms, meditation potpourris, bath additives or air fresheners and they are usually labelled “not for human consumption”. It has been suggested that the psychotropic effects were derived from the plant material included in the products to allow smoking. The real action, however, results from the addition of a synthetic substance into the botanical mix with agonist action on the CB1 receptor. The ingredients listed on the packaging are often incorrect or inadequate. One gram of “spice” contains 77.5-202 mg of synthetic compound (Auwarter et al., 2009, Simolka et al., 2012).

Spice is usually smoked, using a pipe or by rolling in a cigarette paper. This is popular with users because of the relatively rapid absorption and onset of pharmacological effects. Synthetic cannabinoids are non-polar lipid soluble compounds and are volatilized readily without decomposition under smoking conditions (B. Zawilska, 2011). They can also be ingested as a tea or infusion, or pure powder may be inhaled. Products are also available for inhalation (‘vaping’) using e-cigarettes (Debruyne and

Le Boisselier, 2015). Synthetic cannabinoids vary considerably in chemical structure and, while they all act as agonist at CB1 receptors, some may be not structurally related to classical cannabinoids (Fattore and Fratta, 2011). For this reason, the term 'Synthetic Cannabinoid Receptor Agonist' or SCRA is more precise than 'Synthetic Cannabinoid'.

In 2008, JWH-018 was the first SCRA reported to the EMCDDA. The numbers reported continued to grow and had reached 179 SCRAs by the end of 2017, making this the largest group of NPS monitored (EMCDDA, 2018). The large number of SCRAs, their different chemical structure and the continuous emergence of new compounds every year have made it challenging in terms of identification and monitoring. This problem is further worsened by the lack of available knowledge about their pharmacological and toxicological effects. However, SCRA users may suffer severe poisoning and even death. The numbers of cases of toxicity associated with SCRA use reported to drug monitoring systems and poisons centres has been increasing in Europe, the UK , US and Australia (Tait et al., 2016). Increases have occurred in the UK in spite of legislation introduced in 2009, 2012 and 2016 (see Chapter 2 for further details on legislation). Each new drug may have different pharmacological and toxicological effects and this necessitate the evaluation of the interaction of SCRA with many receptors and systems in the body. The clandestine industry keeps changing the structure of the substances they sell and these synthesized substances had not been evaluated or tested in animals or in humans. The pharmacology of SCRAs must be considered individually for each substance because any modification in the structure may produce in certain instances, important differences in pharmacological action. Recent findings revealed the complex cannabinoid signaling pathways (Demuth and Molleman, 2006) and that SCRAs may target systems other than CB receptors. Therefore, it is important to characterize the pharmacological and biochemical effects of SCRAs not just on CB receptors but also on other pharmacological systems, for example, the monoamine transport system.

1.4.1 Pharmacology of Cannabinoids

Cannabinoid pharmacology has been researched for over 40 years. Cannabinoid agonists are compounds that act on CB receptors. Three main CB ligands have been recognised; endocannabinoids, phytocannabinoids and SCRA (Le Boisselier et al., 2017).

The endocannabinoids are the endogenous agonists for CB receptors. So far, five types of endocannabinoids have been characterised. The endocannabinoids are found in all organs and body fluids (Di Marzo, 2006b).

The recognised psychotropic actions of marijuana is mainly due to ∆9-THC, which is predominantly responsible for the CNS effects in humans including euphoria, alteration in sense of time and hallucinations after high doses (Isbell et al., 1967). Approximately, 60 cannabinoids have been identified in the cannabis plant but, ∆9-THC is the only one with psychotropic effects while the other natural occurring cannabinoids such as cannabinol and cannabidiol lack psychotropic effects but have anticonvulsant (Cunha et al., 1980), antianxiety (Guimaraes et al., 1990), antiemetic, anti-inflammatory and antitumour properties (Mechoulam et al., 2002). Cannabidiol actions may be due to inhibition of anandamide breakdown, antioxidant properties or an interaction with an up till now unidentified cannabinoid receptor, rather than a direct interaction with CB1 or CB2 receptors (Pacher et al., 2006).

SCRAs are potent full CB1 receptor agonists; for example JWH018 is five times more potent than ∆9-THC and this probably explains the stronger psychotropic effects of SCRAs compared with cannabis. Besides SCRA effects on the endocannabinoid system, they might also be able to interact with other targets. For example, JWH-018 binds to 5-HT_{2B} and the $GABA_A$ receptor (Wiley et al., 2016). However, data on SCRA actions at non-CB receptors are limited.

SCRA are highly lipid soluble substances and follow the characteristics of lipid soluble drugs. They are rapidly distributed into fat tissue where they can accumulate resulting in a rapid reduction in blood concentrations after administration (Kneisel et al., 2014). They can pass through the blood-brain barrier and accumulate in brain tissue, leading to a higher concentrations than in the blood (Poklis et al., 2012). SCRA undergo phase I and phase II metabolism. They are first oxidised by cytochrome P450 enzymes, then conjugated with glucuronic acid in the presence of glucuronosyltransferase (UGTs) enzymes to be excreted in the urine (Fantegrossi et al., 2014). These enzymes are found at high concentrations in the liver, but they also present in the human brain. Therefore, the activity of these enzymes may regulate the levels of SCRA in the brain and their activation of CB receptors.

1.4.2 The endocannabinoid system

The endocannabinoid system consists of cannabinoid receptors (CB1 and CB2), endogenous cannabinoids (endocannabinoids), and the enzymes responsible for the synthesis and degradation of the endocannabinoids. It is considered to be a neuromodulator system which has a significant role in CNS development, synaptic plasticity and response to external and internal insults (Lu and Mackie, 2016). It plays a role in many body function such as memory, behaviour, appetite control, etc. (Le Boisselier et al., 2017).

1. Cannabinoid receptors

Up to the present time, two cannabinoid receptors have been recognised, CB1 and CB2 that are encoded by CNR1 and CNR2 genes respectively. However, due to complex pharmacology of the endocannabinoid system there was growing evidence to postulate the presence of further cannabinoid receptors in addition to CB1and CB2 (Begg et al., 2005). Recently, G-protein-coupled receptors (GPCRs) such as GPR18 and GPR55 has been suggested as a potential member of cannabinoid receptors. In addition, transient receptor potential vanilloid type 1 receptor (TRPV1) has been shown to be activated by different cannabinoids (Brown, 2007). In addition, there are many GPCRs that have been suggested as a potential CB receptor such as GPR3, GPR6, and GPR12. However, the absence of selective ligands for these receptors along with their complex signalling pathways is impeding the identification their relationship with the endocannabinoid system (Morales and Reggio, 2017).

CB1 and CB2 receptors belong to the G-protein coupled receptor family that bind to the inhibitory guanine nucleotide binding protein (Galal et al., 2009). Stimulation of cannabinoid receptors leads to inhibition of adenylate cyclase, therefore, inhibiting the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) and activates inwardly rectifying potassium channels and mitogen-activated protein (MAP) kinase (Figure 1.2) (Grotenhermen, 2004). CB1 receptors acts as a neuromodulator through modulation of neurotransmitter release from CB1 expressed in the presynaptic terminals, by inhibition of presynaptic Ca^{++} channels. This may take place by direct interaction with the G protein subunit or indirectly by K^{+} channel opening leading to reduction in neurotransmitter release, including acetylcholine, dopamine, γ-aminobutyric acid (GABA), histamine, serotonin, glutamate, norepinephrine,

prostaglandins and opioid peptides (Grotenhermen, 2004). These findings might explain some of pharmacological effects of cannabinoids.

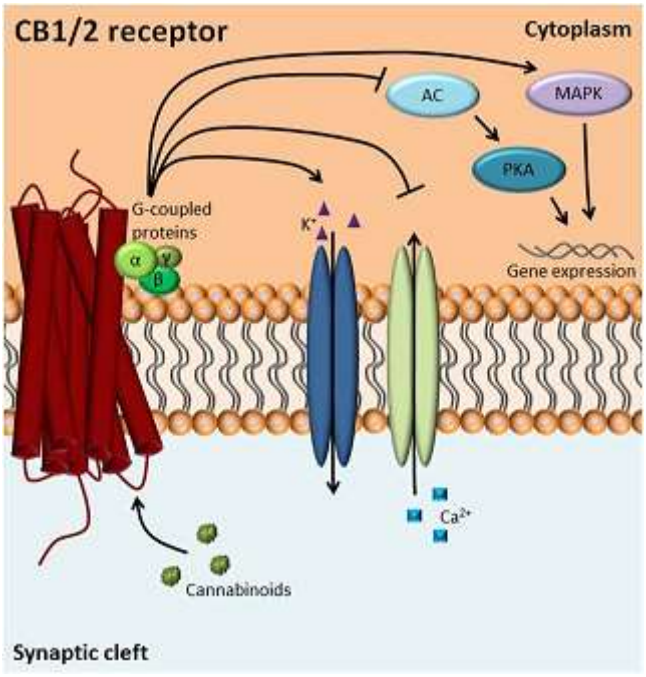


Figure 1.2 Cannabinoids receptors activation. CB receptors activation inhibits Ca^{++} channels and activate K^{+} channels. In addition, CB receptors control gene expression by activation of MAPK and inhibition of adenylate cyclase and cAMP- protein kinase signalling. Figure taken from (Hondebrink et al., 2018).

The CB1 receptor is expressed mainly in the CNS, particularly in the basal ganglia, cerebellum, hippocampus and cortex. CB1 receptors are located in presynaptic neurons, and to a lesser extend in oligodendrocytes and astrocytes. It is also expressed in peripheral neurons and non-neuronal cells, including those in the heart, endocrine glands and gastrointestinal tract (Pertwee, 1997, Di Marzo, 2006a). CB1 receptors are responsible for the psychotropic effects of cannabinoids. (Pertwee, 2005). CB1 receptors are involved in food intake regulation, accumulation of fat and glucose and lipid metabolism (Svíženská et al., 2008). In addition, CB1 is expressed in 78-83% of nociceptive neurons of the dorsal root ganglia indicating a role in pain modulation (Mitrirattanakul et al., 2006).

The CB2 receptor is located mainly in immune tissues and blood cells, but has also been located in the retina and the CNS (Ashton et al., 2006). These receptors are

responsible for the immunomodulatory effects of cannabinoids (Di Marzo et al., 2004) through the induction of apoptosis, inhibition of cell proliferation, inhibition of cytokine and chemokine production, and induction of regulatory T cells (Rieder et al., 2010).

2. Endocannabinoids

This term is used to describe the endogenous ligands for CB1 and CB2 receptors which are capable of binding and activating cannabinoid receptors (Di Marzo et al., 2004). Endocannabinoids are produced within the body and serve as intercellular "lipid messengers" and are considered either neurotransmitters or neuromodulators (Figure 1.3). They have different synthesis pathways and are released from cells upon depolarization and calcium entry and their action is rapidly terminated by enzymatic degradation and reuptake. It is believed that they are synthesized "on-demand" rather than made and stored for later use (Raymon and Walls, 2007, Vardakou et al., 2010). Anandamide (N-arachidonoylethyethanolamine) was the first endocannabinoid isolated from porcine brain in 1992, followed by the discovery of 2-arachidonoylglycerol (2-AG) in 1995. Other endogenous compounds have also been reported, but anandamide and 2-AG (Pacher et al., 2006) remain the best studied.

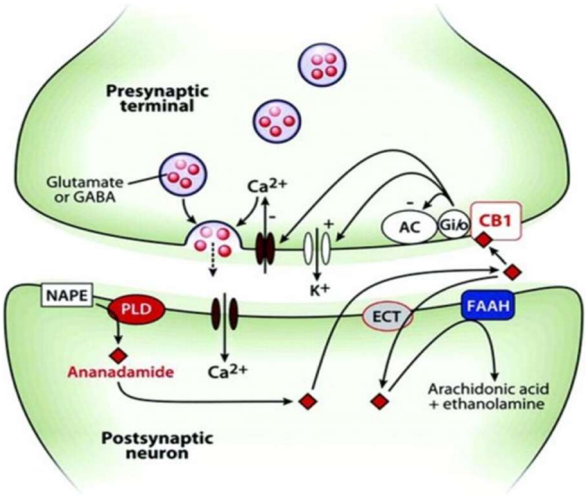


Figure 1.3 The endocannabinoid system as neuromodulator. After neuronal depolarization and calcium entry, the endocannabinoids bind to presynaptic CB1 receptor. CB1 receptor activation result in the inhibition of neurotransmitter release. By doing this, the endocannabinoid system can control different physiological processes such as memory, fear and feeding behaviour. Figure taken from https://nimbinhemp.com/page/medicinal/endocannabinoids.

1.4.3 Clinical toxicity

There is lack of information about the potential adverse effects of recreational use of cannabinoids such as 'spice'. The limited available information is from the medical literature reporting cases of cannabinoid intoxication or from self-reported surveys. Many of the effects of synthetic cannabinoids are similar to those of Δ9-THC especially at high doses of the latter and the duration of action of both ranges from 4-14 hours (Hermanns-Clausen et al., 2013). But some reports demonstrated that the toxic effects of SCRA tend to be more severe and complex than cannabis-related ones (Darke et al., 2020). Anxiety was a prominent feature after smoking spice confirmed to contain JWH-018 and JWH-073 (Schneir et al., 2011). Psychosis and other psychological problems such as panic attacks have also been reported (Müller et al., 2010, Fattore, 2016). Seely et al. (2012) summarised the most common adverse effects of synthetic cannabinoid abuse as affecting the central nervous system, including psychosis, seizures, anxiety, agitation, irritability, memory changes, sedation and confusion. Cardiovascular (e.g. tachycardia, tachyarrhythmia, cardiotoxicity and chest pain) and gastrointestinal (e.g. nausea, vomiting and appetite changes) effects may also occur (Seely et al., 2012b). In a study of patients with analytical confirmation of the presence of MDMB-CHMICA and AB-CHMINACA, depression was the most common CNS clinical symptom (61%); disorientation (45.5%), restlessness/agitation (34.1%), hallucinations (34.1%), generalized seizures (27.3%) and anxiety (18.2%) were also common. Other reported effects included trembling, mydriasis, sluggish pupillary responses, tachycardia, hypertension, and vomiting (Hermanns-Clausen et al., 2018). In addition, most common adverse effects of SCRA reported by undergraduate university students were anxiety, depression, rapid heart rate, paranoia, headache, time distortion, confusion, nausea, vomiting, visual and auditory hallucinations, panic attacks, intensified sensation and racing thoughts (Mathews et al., 2019). Other reported effects associated with the synthetic cannabinoid MDMB-CHMICA use were acidosis, reduced level of consciousness, mydriasis, tachycardia, agitation and tonic-clonic convulsions (Hill et al., 2016). In a review by Kohen and Weinstein (2018), the authors reported that SCRA mimic the effects of Δ9-THC. However, they cause more severe adverse effects including sever psychosis symptoms, agitation, hallucinations hypertension, tachycardia, respiratory difficulties, chest pain, muscle twitches, acute renal failure, anxiety, suicidal ideation, and cognitive impairment (Cohen and Weinstein, 2018). It can be noted that SCRA have been associated with several

neurologic and psychiatric effects and they might cause recurrence of psychosis in vulnerable subjects and trigger psychotic symptoms in in subjects with no previous history (Alipour et al., 2019). Some of these adverse effects are congruent with those associated with serotonin syndrome (e.g., hypertension, agitation, confusion, anxiety and seizures). This can be rather than a direct result of SCRA agonism but could be direct or indirect effect of SCRA on serotonergic system.

SCRAs users may also develop dependence and withdrawal syndromes as also encountered in some cannabis users (Zimmermann et al., 2009). However, clinical effects not generally associated with Δ9-THC have also been reported after SCRA use. These include seizures, hypertension, hypokalemia, agitation and renal failure. These may result from the higher affinity to CB1 receptors of SCRAs compared to Δ9-THC or their full as opposed to partial agonism at these receptors (Pertwee et al., 2010). The possibility also remains that actions may occur via other receptor systems as research on their pharmacology is very limited. Also, unexpected toxicity could arise from unreported co-use with other drugs of misuse.

SCRA-containing products can sometimes cause severe toxicity requiring hospital admission, but most cases are rapidly discharged from the hospital after supportive therapy (Harris and Brown, 2013). To establish patterns of toxicity from SCRAs use, enquiries to the National Poison Information Services in the UK were screened for potential exposure to SCRA-containing products over the period 2007 to 2014. Enquiries were identified involving 510 individuals with probable SCRAs use, with annual incidence rising year on year. Of the patients identified, 80.8% were male and the median age was 21 years; 90% reported using SCRAs alone prior to their acute presentation, while in the remainder SCRAs were used in combination with other substances, mostly ethanol, opioids or benzodiazepines (Waugh et al., 2016). In 433 patients reporting SCRAs use alone, features commonly reported were tachycardia (17%), reduced level of consciousness (16%), agitation or aggression (10%), vomiting (7%), dizziness (6%), confusion (5%), mydriasis (5%) and hallucinations (5%). Chest pain (4%), acidosis (3%), seizures (2%) and elevations in plasma creatinine (2%) were recorded less frequently. A systemic review by Tait et al. (2016), reported that cardiovascular adverse effects, acute kidney injury, generalised tonic- clonic seizures and hyperemesis were the main presentations of SCRA poisoning (Tait et al., 2016).

The potential toxic effects that result from long term use of these compounds or their metabolites are still unknown (Seely et al., 2012a). Previous studies revealed an association between chronic exposure to cannabis and cognitive impairment particularly affecting memory, verbal learning and attention (Broyd et al., 2016). In addition, repeated cannabis exposure is considered as a risk factor (Andreasson et al., 1987) for many psychiatric disorders such as schizophrenia, psychosis (Di Forti et al., 2014) and bipolar disorder (Weinstock et al., 2016). SCRA are often suggested to be more potent than cannabis, it may be anticipated that the long-term effects could be more severe. There is a growing body of literature that reports chronic SCRA exposure inducing similar adverse effects to those of cannabis but more potent and longer lasting (Weinstein et al., 2017, Cohen et al., 2017).

SCRA containing products usually have other additives and adulterants and these may contribute to their effects. In addition, SCRA users might also have used alcohol or other drugs and this further complicates assessment and clinical management. It is important to understand the mechanism of SCRA induced adverse effects with the spectrum of toxicities reported in the literature in order to recognise effective protocols for the management of acute and chronic toxicity.

1.4.4 Classification of SCRAs

SCRAs are classified according to their chemical structure, as follow:

1. **Classical cannabinoids**

These include substances with a similar chemical structure to ∆9-THC (Figure 1.4); examples are nabilone (a licensed anti-emetic) and HU-210. ∆9-THC acts as a partial agonist at CB1 and CB2 receptors, while HU-210 acts as a full agonist and this may explain differences in potency and effects.

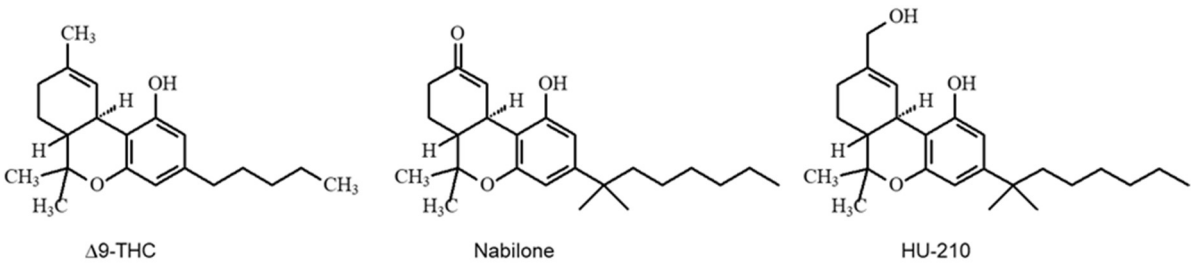
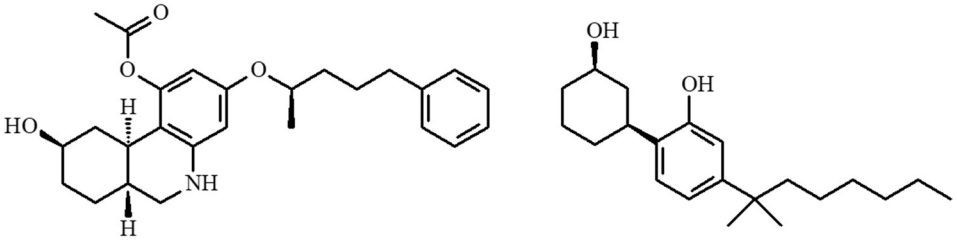


Figure 1.4 Chemical structure of some classical cannabinoids.

2. Non classical Cannabinoids

These compounds were first developed by Pfizer for possible beneficial therapeutic effects. They have a simplified chemical structure consisting of two of the three ring structures of ∆9-THC (Figure 1.5); examples are CP-50,556-1, CP-47,497, and the C8 akyl homolog of CP-47,497, CP-50,556-1, which is also known as levonantradol. Some of these compounds are 30-fold more potent than ∆9-THC on CB1 receptor.



CP 50,558(Levonantradol) CP 47,497

Figure 1.5 Chemical structure of some non-classical cannabinoid.

3. Aminoalkylindoles (AAIs)

This group of SCRAs are subdivided into four groups, the naphthylmethylindoles (e.g. JWH-017), benzoylindoles (e.g. paverlone), phenylacetylindoles (e. g.JWH-250), and naphthoylindoles (e. g. JWH-073). AAIs showed higher relative potency than ∆9-THC on CB1 receptors. In addition, JWH compounds demonstrate various selectivity toward CB1 and CB2 receptors (Figure 1.6).

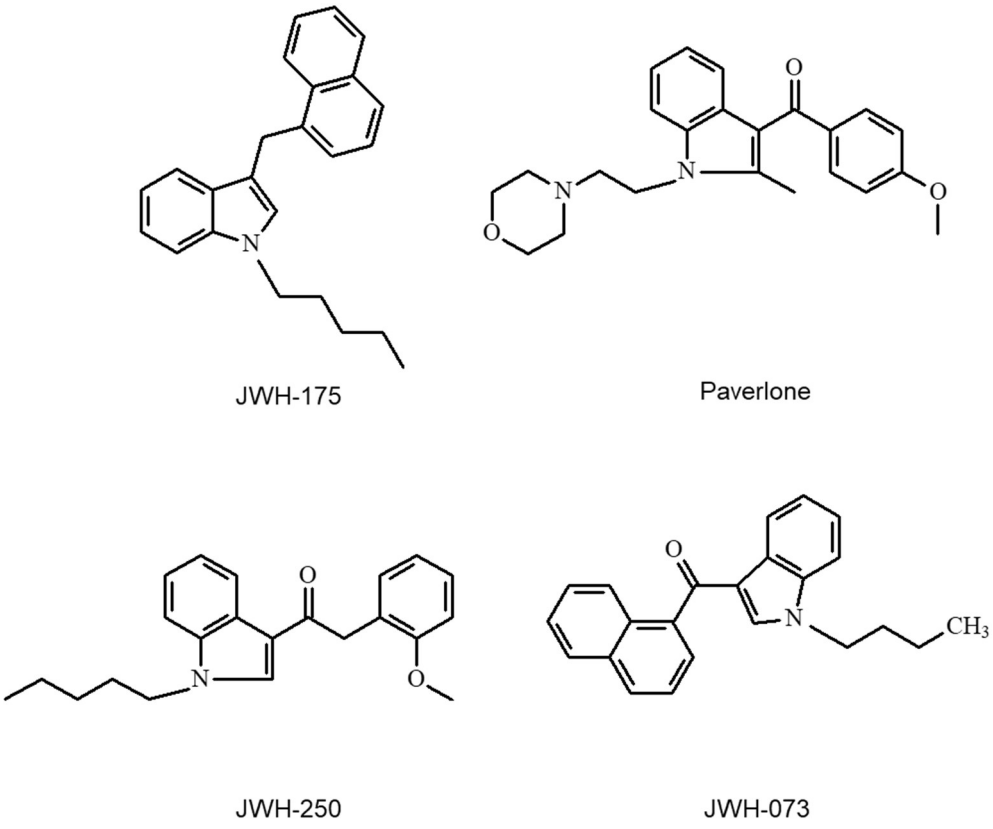


Figure 1.6 Chemical structure of some aminoalkylindoles.

4. Eicosanoids

The eicosanoids are cannabinoids include some endocannabinoids (e.g. anandamide and 2-Arachidonoylglycerol (2-AG), their synthetic derivatives and noladin ether (Figure 1.7). The endocannabinoid anandamide has binding affinity to CB1 Receptor comparable to ∆9-THC and produce pharmacological effect similar to cannabinoid agonists (Lin et al., 1998, Seely et al., 2011, Hohmann et al., 2014).

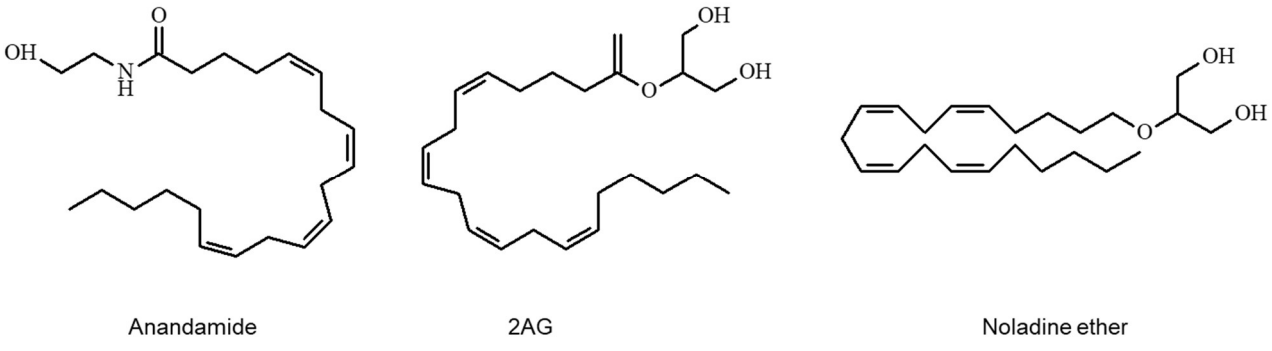


Figure 1.7 Chemical structure of some eicosanoids.

1.5 The Role of Serotonin (5-HT) in Cannabinoid Toxicity

Reports of acute and chronic adverse effects of SCRA are accumulating in the literature (Section 1.4.3) such as tachycardia, agitation, hallucinations, hypertension, vomiting, chest pain, seizures, myoclonia and acute psychosis. These adverse effects could be consistent with possible serotonergic involvement (Louh and Freeman, 2014, Yip and Dart, 2014).

Although, the recognition of possible serotonergic features may suggest serotonergic involvement in SCRA intoxication, a systematic understanding of how the cannabinoid system interacts with serotonergic transmission is still lacking. The involvement of cannabinoids in serotonergic transmission could either be through direct interaction with the serotonergic receptor, increased 5-HT release or uptake inhibition, or indirectly through modulations of these activities via CB1 receptor activation.

Early studies revealed that Δ^9-THC and several of its derivatives were able to inhibit the uptake of ^{3}H-labelled noradrenaline (NE), dopamine (DA), GABA and 5-HT in rat brain (Sofia et al., 1971, Hershkowitz et al., 1977, Banerjee et al., 1975). Acute or perinatal Δ^9-THC exposure produces different effects on 5-HT levels in different brain regions with no change in the densities of 5-HT uptake sites (Molina-Holgado et al., 1993). Subsequent studies that investigated the crosstalk between the 2 systems were mainly motivated by the potential to use the endocannabinoid system as a potential therapeutic target. 5-HT neurons play a role in mediating cannabinoid anti-emetic actions and some types of analgesia by direct inhibition of the 5-HT$_3$ receptor by cannabinoids (Barann et al., 2002). In addition, 5-HT has been involved in cannabinoid-induced hypothermia (Malone and Taylor, 1998), sleep (Mendelson and Basile, 2001) and appetite stimulation (Rowland et al., 2001).

It has been suggested that CB1 receptors are located presynaptically on serotonergic neurons (Nakazi et al., 2000), alongside proteins responsible for re-uptake and monoamine release (Lau and Schloss, 2008). The co-expression of CB1 receptors with different subtypes of 5-HT receptors might further indicate interactions between the two systems at the level of binding sites (Hermann et al., 2002).

Studies examining the effects of chronic cannabinoid exposure demonstrate that this leads to an increase in the 5HT content of the rat frontal cortex (Sagredo et al., 2006),

upregulated 5-HT2A activity and concomitant down-regulated 5-HT_{1A} activity (Hill et al., 2006).

In the central nervous system, an important action of cannabinoids is inhibition of neurotransmitter release (Schlicker and Kathmann, 2001). In addition, cannabinoids modulate the firing of monoaminergic neurons and the release of DA, NE and 5-HT (Fišar, 2012). Moreover, cannabinoid receptors could play an indirect role in the regulation of serotonin transporter (SERT) activity (Kenney et al., 1999) by affecting intracellular levels of cAMP and Ca^{++} which in turn regulate SERT protein (Yammamoto et al., 2013, Ciccone et al., 2008). However, Steffens et al. (2004) showed that the inhibitory effect of the synthetic cannabinoid WIN55212-2 and the endocannabinoid anandamide on DA and 5-HT uptake was mediated by a decrease in Na^+ / K^+-ATPase activity rather than via the CB receptor (Steffens and Feuerstein, 2004). In one study, both in vivo and in vitro effects of ∆9-THC, anandamide and the CB receptor agonist WIN 55,212-2 were investigated on platelet 5-HT uptake. It was concluded that SERT was inhibited acutely by cannabinoids at high concentrations, and the increase in maximal velocity of 5-HT uptake could be due to adaptive changes in the serotonergic system induced by chronic cannabis use (Velenovská and Fišar, 2007). In addition, ∆9-THC affects the organization of the developing diencephalic serotonergic system without affecting the 5-HT uptake site density (Molina-Holgado et al., 1996, Molina-Holgado et al., 1993).Interestingly, it has been reported that repeated exposure to CB_2 receptor agonists can upregulate 5-HT_{2A} receptors in rat neuronal cells that express both 5-HT_{2A} and CB_2 receptors (Franklin and Carrasco, 2013, Franklin et al., 2013b). Modulation of 5-HT_{2A} play an important role in many physiological function such as anxiety (Weisstaub et al., 2006) and the psychoactive effects of the phenylisopropylamine hallucinogens and LSD (Titeler et al., 1988).

Collectively, these studies show complex serotonergic responses to cannabinoids. It is possible that SCRA increase serotonergic activity through different mechanisms, but the structure similarity of indole containing SCRAs with serotonin might allow them to act as competitive inhibitors of 5-HT uptake or activate 5-HT receptors. Further characterisation of SCRA actions on 5-HT systems is needed to advance the understanding of how these drugs affect different brain area.

1.6 Serotonin, 5-HT

5-Hydroxytryptamine (Serotonin, 5-HT) was originally detected as the substance that gives the serum its vasoconstrictive properties (Page, 1954) and was identified chemically as 5 hydroxytryptamine (Rapport, 1949).

The synthesis of serotonin (Hamlin and Fischer, 1951) opened the door for researchers in neuroscience to understand and investigate many of the physiological activities and behavioural disorders in the human body. Interest in 5-HT as a possible CNS transmitter started when Gaddum found that lysergic acid (LSD) acted as a 5-HT antagonist in peripheral tissue and suggested that its central effect might related to 5-HT action (Gaddum and Hameed, 1954). Since then, 5-HT has been shown to act as neurotransmitter in the CNS and as a hormone in the peripheral vascular system. Although over 90% of the total body 5-HT is found outside the CNS, 5-HT plays a vital role in modulation of many behavioural and neuropsychological processes. In the periphery, 5-HT regulates vascular tone, gut motility, primary homeostasis and cell-mediated immune responses (Veenstra-VanderWeele et al., 2000).

1.6.1 5-HT pathways in the CNS

The distribution of 5-HT containing neurons (Figure 1.8) is similar to that of adrenergic neurons, but the serotonergic system is more expansive (Jacobs and Azmitia, 1992). The cell bodies of the neurons are concentrated in the pons and upper medulla, close to the midline and referred as the raphe nuclei. They are clustered in two general groups: a superior group (rostral nuclei) comprised of four groups of serotonergic nuclei, the dorsal raphe, median raphe, caudal linear, as well as the supralemniscal nucleus. The second group is the inferior group (caudal nuclei), that consists of the nucleus raphe obscurus, nucleus raphe pallidus, nucleus raphe magnus, ventral lateral medulla, and the area postrema. The rostral nuclei project into many parts of the cortex and to the hippocampus, basal ganglia, limbic system and hypothalamus, while the caudal nuclei project to the cerebellum, medulla and spinal cord (Azmitia and Whitaker-Azmitia, 1991).

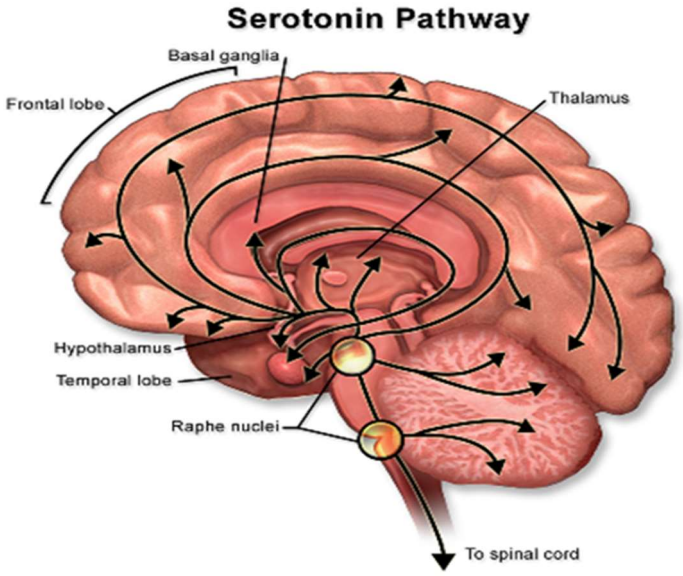


Figure 1.8 The 5-HT pathways in the brain illustrating the serotonergic neurons and the distribution of their axon projections in the brain. Figure taken from Serotonin and motivation: How does serotonin affect motivation? https://en.wikiversity.org/wiki/Motivation_and_emotion/Book/2017/Serotonin_and_motivation.

5-HT is synthesized in the brain from the amino acid L-tryptophan in a two-step enzymatic process (Figure 1.9). The first step is hydroxylation of L-tryptophan by the tryptophan 5-hydroxylase enzyme (TPH) to form 5-hydroxytryptophan; this is the rate limiting step of the synthesis (Fitzpatrick, 1999). Two isoforms have been identified, TPH1, which is mainly localised in the periphery, and TPH2, which is mainly found in neuronal cells and the CNS (Walther et al., 2003). The second step is decarboxylation of 5-hydroxytryptophan by the enzyme hydroxytryptophan decarboxylase to form 5-HT (Fernstrom, 1983). The synthesized 5-HT is then transported from the cytoplasm into storage vesicles via two forms of vesicle monoamine transporter, VMAT1 and VMTA2 (Erickson and Eiden, 1993). VMAT1 occurs in non-neural cells of the periphery, whereas VMAT2 occurs in neurons.

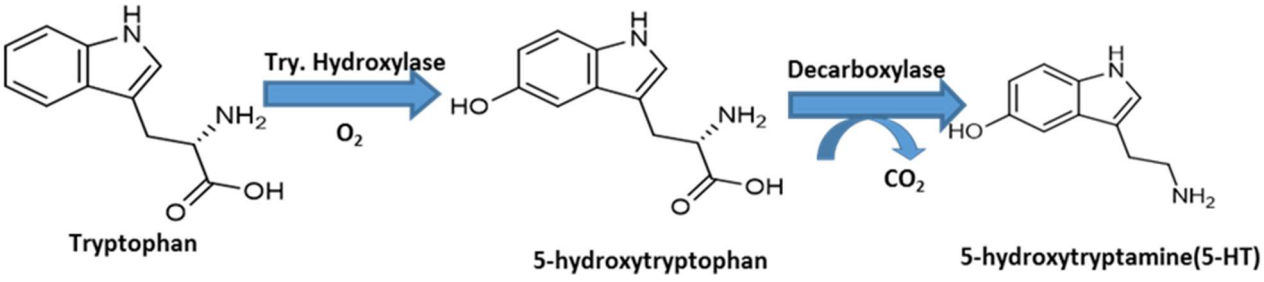


Figure 1.9 Biosynthesis of 5-HT.

The vesicles release 5-HT into the synaptic cleft by exocytosis after activation of calcium channels by an action potential (Elhwuegi, 2004). The intensity and the duration of 5-HT release are controlled by the reuptake of 5-HT into the presynaptic terminal or the glial cells (Figure 1.10). Reuptake into the presynaptic terminal is facilitated by the high affinity serotonin transporter (SERT), which represents the major pathway for presynaptic 5-HT clearance (Torres et al., 2003). In addition, recent studies revealed a second transport system of 5-HT, which consists of the organic cation transporter (OCT) and plasma membrane monoamine transporter (PMAT). According to these findings SERT is termed the uptake 1 transporter and OCT and PMAT the uptake 2 transporter and these play important roles in serotonin uptake (Zhou et al., 2007).

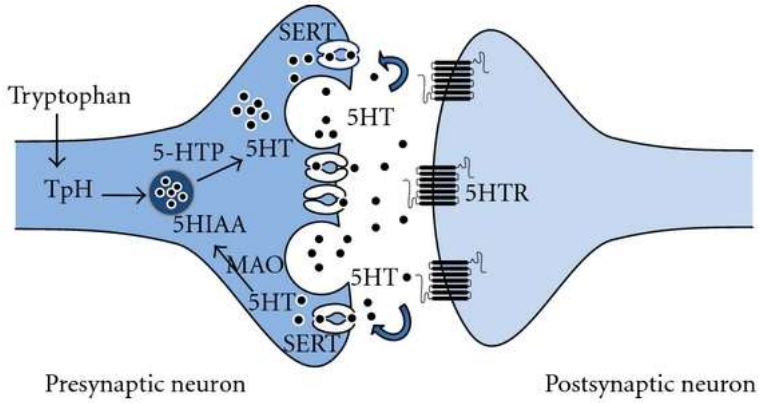


Figure 1.10 Diagram of serotonergic nerve terminal illustrating the synthesis, release, reuptake and metabolism of 5-HT. TpH: tryptophan hydroxylase; 5-HTP: 5-hydroxy-L-tryptophan; 5-HT: serotonin; SERT: serotonin transporter; MAO: monoamine oxidase; 5-HIAA: 5-hydroxyindoleacetic acid; 5-HTR: serotonergic receptor (Buller et al., 2012).

Degradation of 5-HT in serotonergic neurons occurs through oxidative deamination, catalysed by monoamine oxidase enzymes (MAO) (Shih et al., 1999). MAO occurs in two forms in the brain, MAO-A, which has higher affinity for 5-HT and other monoamines, and MAO-B, which has higher affinity for phenylethylamines. Both are located in the outer membrane of mitochondria (Green and Youdim, 1975). 5-HT is catabolised by MAO-A into 5-hydroxyindole acid aldehyde which is further oxidised by aldehyde hydrogenase enzyme into 5-hydroxyindole acetic acid (5-HIAA) which is

excreted in the urine and can be used as indicator of 5-HT production in the body (Virkkunen et al., 1995).

1.6.2 Pharmacology of 5-HT

The actions of 5-HT are numerous and complex and influence a broad range of physiological systems. The diversity in 5-HT action is due to differences in 5-HT receptors and sub receptors. Currently there are 14 known receptors subtypes, divided in 7 classes (5-HT 1-7). These receptors are different in protein structure and the affinity for various agonist or antagonist ligands (Hoyer et al., 1994). All the 5-HT receptors are G-protein-coupled receptors with the exception of 5-HT_3 receptors, which are ligand gated ion channel receptors (Green, 2006). Consequently, efforts are concentrating on the identification of selective ligands more specific for receptor type to enhance drug treatment with fewer adverse effects (Hoyer et al., 2002). The following sections outline briefly the main sites of 5-HT action.

1.6.2.1 Central nervous system

All brain regions express multiple serotonin receptors in a receptor subtype-specific fashion (Lucki, 1998). 5-HT is involved in many vital physiological processes in the brain including sleep (Jouvet, 1999), appetite (Blundell, 1976) and behavioural functions such as aggression, sexual behaviour, pain sensitivity, sensorimotor reactivity and learning (Lucki, 1998), as well as in psychiatric disorders such as schizophrenia, clinical depression and anxiety. Moreover, deficiency in serotonergic neurons in aging may contribute to late-age depression and Alzheimer disease (Meltzer et al., 1998).

1.6.2.2 Gastrointestinal tract (GIT)

5-HT has long been assumed to play a major role in GIT function since its isolation and characterisation as ‘enteramine’ in 1952 (Erspamer and Asero, 1952). More than 90% of body serotonin exists in the gut wall with only 10% located in neurons. 5-HT that is located in the enterchromaffin cells plays essential role in several aspects of gut function including secretion, motility and sensation (Gershon, 1999). The action of 5-HT in the gut is complex. Different subtypes of 5-HT receptor are present throughout the enteric nervous system and the gut muscles. Alterations in 5-HT synthesis or

uptake have been implicated in bowel disorders, including irritable bowel syndrome and ulcerative colitis (Coates et al., 2004).

1.6.2.3 Platelets and blood vessels

Platelets uptake 5-HT synthesised in the gut via SERT and store it in storage granules. When platelet aggregation starts the serotonin is released in to the circulation and causes further enhancement of platelet aggregation (Vanhoutte, 1991). In addition 5-HT promotes vasoconstriction of the surrounding blood vessels and facilitates haemostasis if the endothelium is intact, but if the endothelial is damaged, 5-HT can exert a harmful vasoconstrictive effect (Kaumann and Levy, 2006). Interestingly 5-HT can either cause vasoconstriction or vasodilation, depending on the type of receptors expressed in each vessel and in the surrounding smooth muscle (Ullmer et al., 1995).

1.7 Neurotoxicity of Synthetic Cannabinoids Receptor Agonists

The data on the harms associated with acute and chronic use of SCRAs is limited and still emerging. To date, there are few studies that have investigated the toxicology or metabolism of SCRA. While considerable research has been carried out on cannabis, it cannot be assumed that the risks connected with cannabis use is similar with those associated with SCRA. Since the main targets of cannabinoids; whether they are natural or synthetic, is the CNS, the focus of this thesis is on the toxic effect on the brain.

Recent studies on regular cannabis users have demonstrated that chronic cannabis exposure induces functional and structural changes in the human brain. Weinstein et al. (2016) reviewed the brain-imaging, pharmacological and neurobiological studies of cannabis users. They revealed that regular use of cannabis results in volumetric and structural changes in the grey and white matter in the brain, in particular in the hippocampus and the amygdala. Regular cannabis use resulted in reduced dopamine transporter occupancy and reduced dopamine synthesis but not in reduced striatal D2/D3 receptor occupancy compared with healthy control participants (Weinstein et al., 2016). In a study of SCRA users Nurmedov et al. (2015) reported that grey-matter volume in the left cerebellum and thalamus was reduced in SCRA users in comparison to healthy volunteers (Nurmedov et al., 2015). Furthermore, Zorlu et al. (2016) reported that SCRAs users had a significant reduction in white matter in numerous brain areas including the left temporal lobe, subcortical structures and brainstem (Zorlu et al.,

2016). These studies suggest potential neurotoxic effects of SCRA use and the need for further research. Therefore, the next section highlights some of the *in vivo* and *in-vitro* studies of SCRA cytotoxicity.

1.7.1 In vivo cytotoxicity studies of SCRAs

In vivo animal studies revealed that JWH-018 and JWH-210 reduce locomotor activities and cause neuronal damage through distortion of nucleus membranes in the core shell of the nucleus accumbens (Cha et al., 2015). Moreover, both cannabis and SCRA decreased both short- and long-memory retention in mice (Barbieri et al., 2016, Fehr et al., 1976, Heyser et al., 1993, Basavarajappa and Subbanna, 2014). In addition, JWH-018 and its derivatives cause locomotor suppression, antinociception and hypothermia, as does Δ 9 –THC and other types of SCRA (Compton et al., 1992, Wiley et al., 2012, Vigolo et al., 2015, Ossato et al., 2016). JWH-018 impairs sensorimotor function in mice (Ossato et al., 2015), increases indices of anxiety (Macrì et al., 2013), and increases dopamine release in the nucleus accumbens shell of mice (De Luca et al., 2015). Finally, AM2201-induced seizures which might be related to rapid increases in glutamate release in the hippocampus (Funada and Takebayashi-Ohsawa, 2018). However, withdrawal signs have been reported after withdrawal of repeated exposure with higher potency SCRAs including CP-55,940, WIN-55,212-2, and JWH-073 (Oliva et al., 2003, Aceto et al., 2001, Nacca et al., 2013).

1.7.2 In vitro cytotoxicity studies of SCRAs

There have been few studies investigating the cytotoxicity of SCRAs. Koller et al. (2013) reported JWH-018, JWH-073, JWH-122, JWH-210 and AM-694 induced cell membrane damage at buccal (TR146) and breast (MCF-7) derived cells at concentrations higher than 75μM, but they did not affect mitochondria, protein synthesis, or lysosomal functions at the same concentration (Koller et al., 2013). Couceiro et al. (2016) reported that JWH-018 metabolites were more toxic than the parent compound on neuroblastoma SH-SY5Y and human kidney cell lines and these toxicities were not mediated via the CB1 receptor (Couceiro et al., 2016).

Bologov et al. (2011) evaluated the effects of the cannabinoids CP 55,940, HU-210, WIN 55,212-2 and ∆9-THC on the neuroblastoma cell line N18TG2. They suggested that the tested cannabinoids induced cytotoxicity when the cells were under stress, but failed to induce toxicity under optimal cell conditions. They concluded that the various

physiological responses to cannabinoids, which have different intracellular mechanisms, depend on the state of the cells (Bologov et al., 2011). Almada et al. (2017) reported that the synthetic cannabinoid WIN-55,212 induced cell cycle arrest and reduced cell viability in a human choriocarcinoma cell line through disruption of mitochondrial membrane potential and activation of caspases -9 and -3/-7. This action was mediated through the CB1 receptor (Almada et al., 2017). Likewise, WIN 55,212–2 was reported to induce cell death and induce cytotoxic effects in a renal carcinoma cell line (Khan et al., 2018). Another study reported the concentration dependant cytotoxicity of the synthetic cannabinoids CP-47,497, CP-55,940 and CP-47,497-C8 in a NG 108-15 cell line. The study suggested that cell death was through caspase cascade activation leading to apoptosis and this effect was mediated via the CB1 receptor (Tomiyama and Funada, 2014). Similarly, Koller et al (2014) reported the cytotoxic properties of the synthetic cannabinoid CP-47,497-C8 was through interfering with protein synthesis and causing cell membrane and DNA damage at a concentration of more than 10 μM in buccal derived (TR146) and liver derived (HepG2) cell lines (Koller et al., 2014).The cytotoxic properties of cannabinoids, however, remain contentious. Pereira (2014) reported the cytotoxicity of JWH-250 at 50 μM, whereas JWH-073 was not toxic in a SH-SY5Y cell line (Pereira, 2014). Some studies have reported that SCRAs (Bileck et al., 2016) can cause genotoxic damage to DNA that may have adverse health effects (Koller et al., 2015, Koller et al., 2013, Ferk et al., 2016).

Currently, no definitive mechanism by which SCRAs induce cytotoxicity and cell damage has been established. Tomiyama and Funada (2011) suggested the role of apoptosis through the activation of the caspase-cascades signalling pathway, whereas Couceiro et al. (2016) proposed that cell death was induced through necrosis and this was not mediated by CB1 receptors. Almada et al. (2017) reported that reduction in cell viability in WIN-55,212 exposed cells resulted from the disruption of the mitochondrial membrane potential and activation of caspases -9 and -3/-7. Recently, Oztas et al. (2019) reported that the cytotoxicity of the synthetic cannabinoid AKB48 in SH-SY5Y cells was due to an increase in reactive oxygen species (ROS) production and significant increase in gene expression level of NF-κB and MAPK8. They concluded that lower doses induce apoptotic effects, while both apoptotic and necrotic effects were induced at higher doses (Oztas et al., 2019).

Overall, the information available on the safety and toxicity of SCRA is limited and differs considerably in methodology and SCRA analysed. Most of the research on SCRA-induced cytotoxicity tested individual SCRA on different cell lines that varied in the type of receptors expressed. Furthermore, most of the above studies tested high concentrations of SCRA, far above those observed after human use. In addition, most of the tests were for acute short-term exposure to establish the mechanisms of cell death. Data on the long-term exposure effects of SCRA are scarce and the changes they induce on long term use are still not known.

1.8 Mitochondrial role in cellular stress responses

Stress signalling pathways play an important role in maintaining cellular homeostasis. Cells can react to stress in different ways, from activating survival pathways to initiating cell death, which ultimately eliminates damaged cells. The early response of the cells to a stressful stimulus may provide protection against the insult and allow recovery from it. Nevertheless, if the harmful stimulus is unresolved, then death signalling pathways will be activated (Hotamisligil and Davis, 2016). The cellular response to stress depends on the kind and severity of the insult which in turn can activate different defence mechanisms. Stressors can be physical or chemical in nature and can be intrinsic such as DNA mutation and deletion or calcium overload, or extrinsic such as environmental toxins and chemicals (Eisner et al., 2018).

Mitochondria are responsible for oxidative phosphorylation and energy production in form of ATP. In addition, they are responsible for controlling intracellular Ca^{++} signalling and metabolism, thermogenesis and ROS production and they act as initiators of programmed cell death (apoptosis) (Scheffler, 2001). In view of their vital role in cellular functioning, mitochondria are one of the first responders to different stressors that perturb cellular homeostasis (Manoli et al., 2007). Many stressors target non-mitochondrial cell components, but often pathways converge on the mitochondrion, because of its main function in producing energy and signalling survival and adaptation to stressors (Picard et al., 2018). Other stressors interfere with mitochondrial functions directly, involving energy production, calcium signalling cell death and cell dynamics (Meyer et al., 2018). The mitochondrial response to cellular stress and signalling pathways involved is illustrated in Figure 1.11.

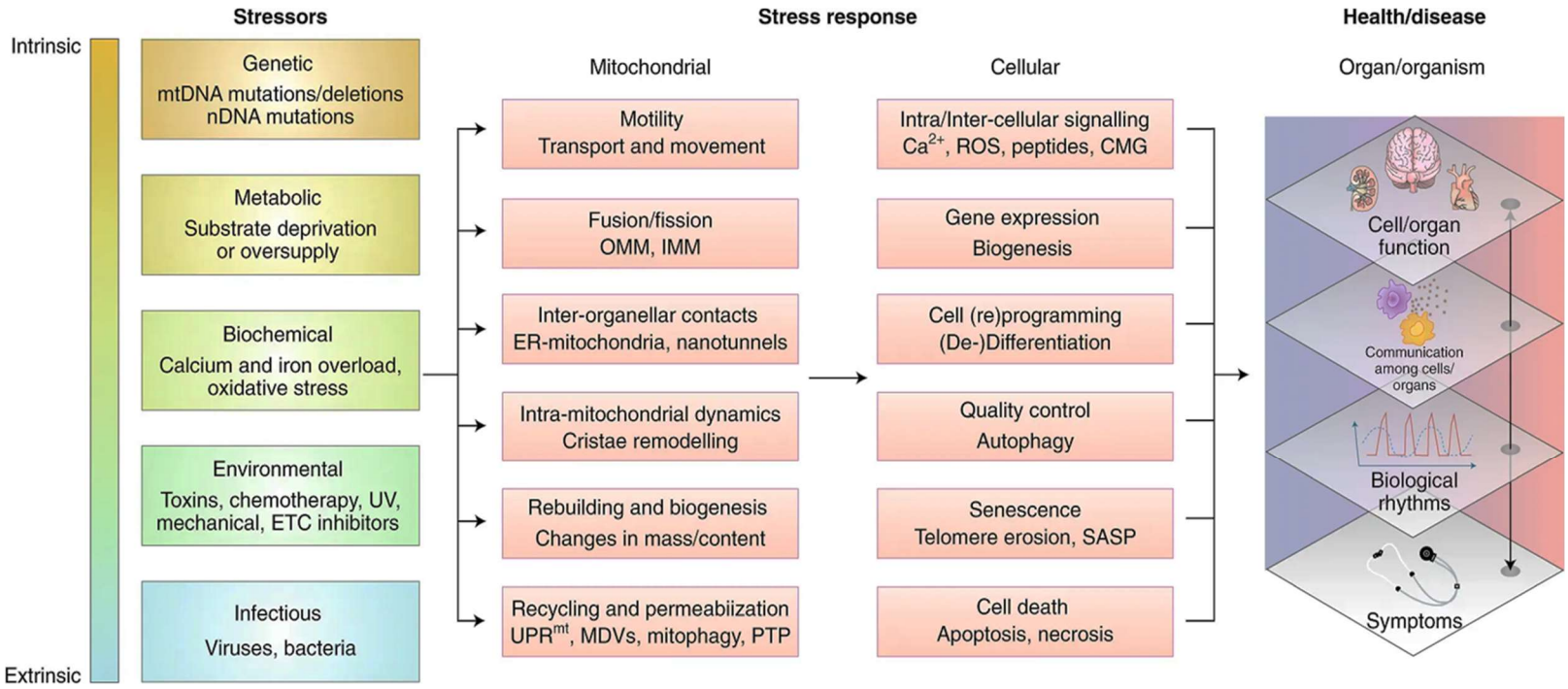


Figure 1.11 Mitochondrial response to different cell stressors. Outlines how mitochondria response to intrinsic and extrinsic stimulus. Different responses may arise and consequently lead to physiological and pathological changes. UPR^{mt}, mitochondrial unfolded protein response; MDVs, mitochondria-derived vesicles; PTP, permeability transition pore; CMG, circulating mitochondrial genome (also ccf-MT-DNA); SASP, senescence-associated secretory profile. Figure taken from (Eisner et al., 2018).

One of the directly affected targets is mitochondrial DNA (MT-DNA, Figure 1.12). This encodes 13 protein subunits, two rRNA and 22 tRNA that are vital for the function of oxidative phosphorylation system, which consists of four respiratory-chain complexes and adenosine triphosphate (ATP) synthase (Gustafsson et al., 2016 1282). Mutations in MT-DNA lead to interference with respiratory chain complexes and ATP synthase, which interferes with oxidative metabolism and disturbs electron transport chain (ETC) function and this impacts on ATP production (Suomalainen and Battersby, 2018).

Mitochondria are sensitive to oxidative damage caused by reactive oxygen species (ROS) (Turrens, 1997). ROS are reactive oxygen intermediates, they usually result from excitation of O_2 to from a superoxide radical, hydroxyl radical or hydrogen peroxide. They are produced as by-products of cellular metabolism, mainly in the mitochondria or in cellular responses to xenobiotic (Ray et al., 2012). Normally, ROS are neutralised by the cellular antioxidant defence system, but when there is an increase in their production they can oxidize and induce destruction of cell components such as lipids, protein and DNA including MT-DNA (Holmstrom and Finkel, 2014). Oxidative damage to protein can lead to modification in cellular protein structure and consequent loss of function and accumulation of the unfolded proteins, which can impair cell function, leading to endoplasmic reticulum (ER) stress and activation unfolded protein response (UPR) (Kupsco and Schlenk, 2015).

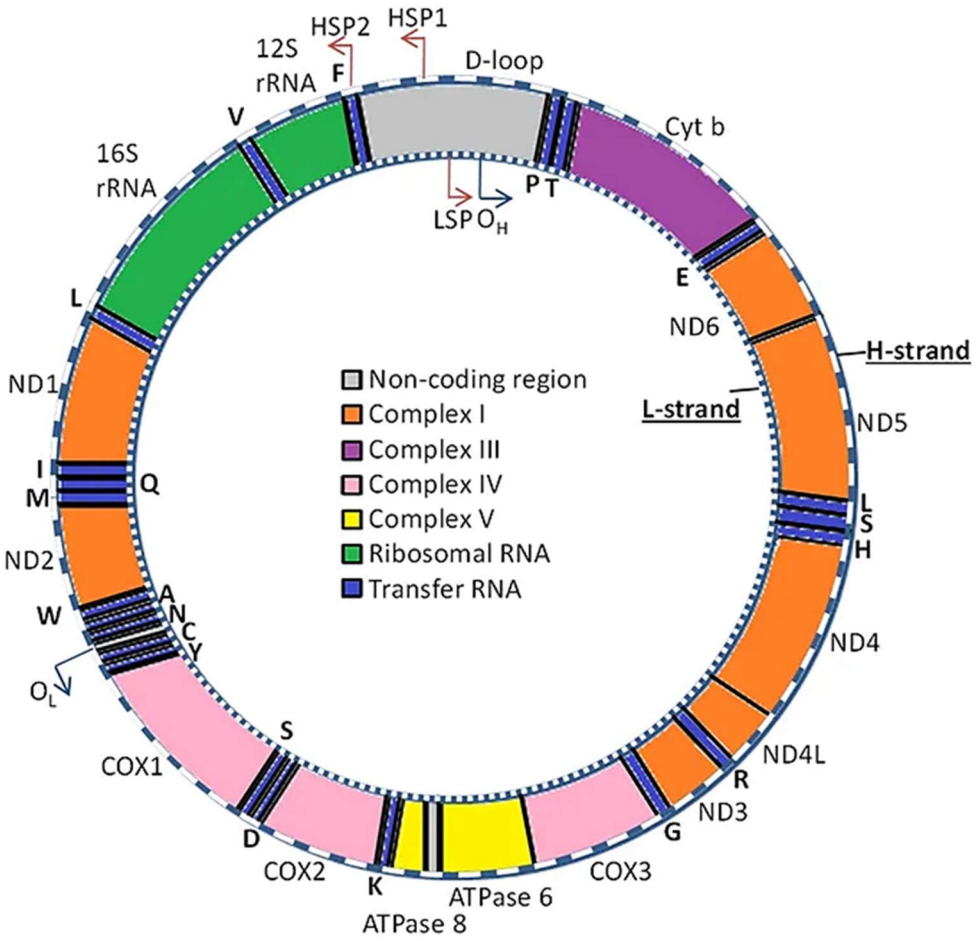


Figure 1.12 Mitochondrial DNA (MT-DNA). The protein-coding genes encode for the complexes required for oxidative phosphorylation (Complex I: orange, complex III: purple, complex IV: pink, complex V: yellow). Figure taken from (van der Wijst et al., 2017).

1.9 Unfolded protein response (UPR)

The UPR is a series of signalling pathways, activated in response to accumulation of unfolded or misfolded proteins (Walter and Ron, 2011). Proteins are synthesised in the endoplasmic reticulum (ER) lumen. The UPR activation aims initially to rectify the errors in protein synthesis. This done by reduction in protein synthesis and translocation into the ER and increasing ER capacity to handle unfolded protein by activation the machinery that is involved in protein folding. If the disruption is prolonged and fails to restore homeostasis, UPR triggers apoptosis and cell death (Ron and Walter, 2007). ER stress is triggered by hypoxia, glucose starvation or oxidative stress.

1.10 Thesis objectives

The research presented in this study aims to evaluate the prevalence of NPS in the UK for the last 10 years and explore some of the pharmacological and toxicological properties of NPS and in particular SCRA. Specific aims were as follows:

1. To identify the numbers of NPS poisoning in the UK by analysing data collected by poisons centre.
2. To evaluate the effect of SCRA on 5-HT uptake in a rat brain slice model.
3. To evaluate the neurotoxicity of some NPS and in particular SCRA using human stem cells and to explore the mechanisms of the toxicity observed.

The following research was therefore conducted:

1. An investigation of the prevalence, trends and demographic characteristics of people experiencing toxicity after use of NPS, as reported in telephone enquiries to the UK National Poison Information Service (NPIS) (Chapter 2).
2. Research testing the hypothesis that SCRA containing an indole structure can affect serotonin uptake without the involvement of CB1 receptors (Chapter 3).
3. Studies of the cytotoxic properties and changes induced by repeated exposure to SCRA, using a human neuronal stem cell model (Chapter4).

Chapter 2 Epidemiology of NPS toxicity

2.1 Introduction

Over the last decade the numbers of NPS involved in misuse and the numbers of people presenting with NPS-associated toxicity have increased at an unprecedented rate; this proliferation poses a substantial challenge to drug policy and carries a significant hazard to public health (World Drug Report, 2017). The UK has one of the highest rates of NPS use in Europe after the Netherlands (Global Drug Survey, 2017). In 2016 58% of NPS were purchased online in the UK and there is evidence of adverse health effects, with NPS users three times more likely to end up seeking emergency medical treatment (Global Drug Survey, 2016).

In spite of the widespread interest in the social and health impacts of NPS use, there is insufficient epidemiological data in the scientific literature and popular media about NPS in comparison to traditional drugs of abuse (Bersani et al., 2014). This is in part because, while routine data collection is in place in many countries for traditional drugs, it is challenging to adapt this to the rapidly evolving use of NPS.

In the UK information is available about diagnosis in patients admitted to hospital or presenting at emergency departments, but this not specifically available for presentations after use of NPS as coding does not involve this level of detail and also commonly coding are not very accurate (Wood et al., 2011). In any case, the NPS involved may not be characterised accurately because the laboratory analysis required is not done as part of routine patient care. Data on causes of death is published and this may include information on deaths related to NPS, but information about specific NPS is not included (Office for National Statistics, 2016) and detailed analytical toxicology is not performed on in all drug related deaths. Other data sources regarding NPS are also available such wastewater or urinal analysis, festival screening, data from seizures of drugs and case reports or case series. In addition, the information on the prevalence of use of NPS could come from small-scale studies among specific population sub-groups, for instance club attenders, military forces or representative population-based surveys among the general population.

Table 2.1 shows the potential data sources regarding NPS that might help to understand the pattern of NPS toxicity with their strengths and limitations (Wood and Dargan, 2012, Hill and Dargan, 2018).

Data Source	Strengths	Limitations
Analysis of drug product		
Drug seizures by law enforcement (Police, Border Force etc)	Provide quantitative data about: • Types and amounts of drug seized • Purity of the marketed drugs • Identifies new drugs appearing in the market	Do not reflect the number of users involved or the frequency of toxicity
Analysis of voluntarily submitted samples, e.g. WEDINOS, FUSE		
Routinely collected health information		
Emergency department attendances or hospital admissions	High-volume national-level data	Coding may not be available for some individual substances, especially NPS Coding may be inaccurate Data may be reported by drug group rather than specific substance No analytical confirmation available to identify individual NPS
Poisons centre data (e.g. NPIS)	Data are collected continuously using consistent methodology. High-volume national-level data about individual substances Time trends in episodes of drug toxicity can be measured indirectly. Characteristics of toxicity can be described Can identify new and unfamiliar exposures	Lack of analytical confirmation. Data depend upon voluntary reporting by health professionals or members of the public (not UK) Rates of reporting may decrease with increasing familiarity about the substances involved
Referrals to drug treatment / addiction services	Data are collected continuously using consistent methodology. High-volume national-level data about individual substances	Lack of analytical confirmation No information available on acute toxicity Coding often not available for NPS

Mortality statistics (e.g. ONS, NRS, NISRA)	National-level data available Analytically confirmed drug prevalence Reflect most extreme forms of toxicity	Inconsistent drug analysis ordered by coroners – may not include emerging drugs of misuse Drugs identified may not be the cause of death Delay to statistics being available Collected in different formats in the various devolved administrations
Population surveys/information		
Population surveys such as CSEW	Reflect the prevalence of reported drug misuse in the population (e.g. aged 16- 59 in the CSEW)	Relies on user reports No analytical confirmation available No information about toxicity provided Information on some NPS not collected
Internet discussion and blogs such as Drugs forum and Erowid	Detailed information dose, routes of administration and desired and unwanted effects.	No analytical confirmation of the NPS used. Unrestricted and unedited thoughts and opinions from enthusiast about NPS effects.
Small-scale studies among specific population sub-groups such as festival goers	Provide a picture of prevalence of use of NPS in that setting	May not reflect other drug user groups No analytical confirmation Used predetermined questions that may limit any additional information
Analytical surveys		
Clinical studies of drug users (e.g. IONA, STRIDA)	Comprehensive analysis of clinical features and outcomes Analytical conformation of substances involved	May require patient consent Not all drug misuse may qualify for entry May not cover whole country
Wastewater and urine analysis	Identifies analytically substances of specific interest	Prevalence of use or toxicity cannot be determined
Others		
Case reports or case series	Detailed clinical data provided	Limited case numbers Some do not include analytical confirmation of the drug(s) involved

Table 2.1 Sources of Data about NPS in UK.

All these approaches have their limitations and therefore the overall assessment of NPS use, trends and effect remains challenging and incomplete.

While use of specific NPS can be suppressed by legal controls based on chemical structure, including the UK Misuse of Drugs Act (1971), this can prompt the emergence of further NPS with chemical structures modified to avoid capture by pre-existing legislation. The aim of prohibition of the use of drugs of abuse is to reduce their use and the associated health and societal impacts. Such legislation often targets specific drugs or drug groups on the basis of their chemical structures and associated health and societal impacts using an evidence-based process which usually takes some time. Modifications of a chemical structure may produce a drug that is no longer legally controlled but retains activity as a potential drug of misuse. Trying to cope with the increased number of NPS, the UK government introduced temporary class drug orders (TCDO) to bring new emerging NPS under control more rapidly. A TCDO bans a drug or chemical for 12 months to allow time for further assessment; after that the drug is either controlled permanently using the Misuse of Drugs Act, if sufficient evidence of harms is available, or extend the TCOD to gather more evidence or it reverts to becoming legal again (Home Office, 2011). However, even using this regulatory approach to classifying drugs, it is often not possible to match the speed of their appearance on the market.

The UK government therefore enacted generic legislation to restrict the production, distribution and sale of any psychoactive substance if the substance is likely to be used for its psychoactive effects. This Psychoactive Substances Act (PSA) was enacted into law in the UK on 26th May 2016. Some substances are exempted, including alcohol, nicotine, caffeine and licensed medicines (Home Office, 2015b).

Research presented in this chapter investigated the prevalence, trends and demographic characteristics of people experiencing toxicity after use of NPS, as reported in telephone enquiries to the UK National Poison Information Service (NPIS) over the last decade. The NPIS provides poisons information and clinical advice via telephone to health care professional across the UK about diagnosis, treatment and management of patients who may have been poisoned, including those exposed to drugs of misuse. Telephone enquiries are more likely when the substances involved are unfamiliar, such as NPS. Data are available on temporal trends and demographics

for specific substances and it is possible to extract data for individual NPS as reported by enquirers.

The NPIS is provided by four individual Units, based in Birmingham, Cardiff, Edinburgh and Newcastle, supported by consultants from two further NHS provider Trusts. These are staffed by Consultant Clinical Toxicologists and Specialists in Poisons Information who work together to provide information and advice by telephone to health professional enquirers on a 24/7 basis (National Poisons Information Service, 2018). In addition, the NPIS provides an on-line poisons information database called TOXBASE®, which contains information about diagnosis and management of approximately 17,000 medicines, chemicals and toxins.

Heath professionals managing people presenting with poisoning of any type, including toxicity relating to drug misuse, are encouraged to use TOXBASE® as their first line information source. Most accesses to TOXBASE® are made in this context, but some are made out of interest or for educational reasons and there may be several accesses about the same patient by different health professionals. Access numbers are therefore an indirect measure of clinical activity and interest relating to specific substances.

Telephone enquiries are made to the service by health professionals when the information on TOXBASE® is inadequate or when there is no specific entry, as may happen for a recently emerging NPS. Enquiries are also more likely to occur for unfamiliar substances or for more severe toxicity. Telephone enquiries made for reasons other than obtaining specific management advice about individual affected cases are uncommon. Most seek advice on the management of episodes of acute toxicity and staff of hospital emergency departments and acute medical wards are high volume users.

NPIS data are collected continuously using consistent methodology and time trends in episodes of drug toxicity can be measured indirectly as numbers of NPIS telephone enquiries and as accesses to TOXBASE®. Unlike emergency department attendances, hospital admissions, or routinely available mortality data, NPIS telephone enquiry and TOXBASE® access data are available by specific substances reported. Although the information available has some limitations, it enables the monitoring of emerging new drugs causing toxicity in UK drug users (Wood et al., 2014, Waugh et

al., 2016). Poisons centre data has also been used to monitor drug misuse trends in other countries (Hondebrink et al., 2015, Wood et al., 2014, Maxwell, 2018).

The patterns of enquiries made to the UK National Poisons Information Service about drugs of misuse since 2009 were extracted and the impact of various legislative changes analysed by comparing data before and after these changes. A more detailed analysis, including time series analysis, was performed to assess specifically the impact of the enactment of the PSA in May 2016. Data was also extracted for five common conventional drugs of misuse to allow study of any secondary effects on enquiries relating to these established drugs of misuse.

2.2 Aims

The aims of this study were to describe demographic characteristics and geographic distribution of NPS users experiencing toxicity and to evaluate time-trends and patterns of NPS exposures reported to the NPIS in UK over a 10-year period. The research also aimed to evaluate the public health impacts of legislative prohibitions of NPS use, and other major policy initiatives.

2.3 Methods

Telephone enquiries received by the NPIS are recorded onto a computer database (the UK Poisons Information Database, UKPID) using a pre-set on-line form, in real time as enquiries are answered. The details documented include a unique call ID, the date and time of enquiry, enquirer postcode, patient's age, sex, substances reported to have been taken including co-ingestants or other co-used substances, route of exposure, mode and symptoms of poisoning, treatment already provided, severity of poisoning and the place where the incident occurred. Clinical outcome is logged when this is known, but these data are incomplete. While follow up is attempted for severe or unusual cases, often the only information available is derived from a single telephone call made around the time the patient presents to health services.

2.3.1 NPIS Telephone enquires

Telephone enquiries to the NPIS were extracted for the period January 2009 to December 2018 and reviewed retrospectively. Enquiries related to recreational abuse and intentional intake of recreational drugs were included in the data set, but those concerning therapeutic errors, body packers or stuffers, accidental poisoning or spiked food or drinks were excluded. The drugs of misuse involved were classified manually

into 8 categories according to their principle clinical effects or chemical structures as follows: benzodiazepines, cannabis, stimulants, opioids, hallucinogens, SCRAs, dissociative drugs and ‘products’. Products consisted of branded drug products (e.g. products sometimes previously termed ‘legal highs’) such as “Black Mamba”, “Exodus” “Annihilation”, etc. While the ‘products’ group is likely to contain drugs from other categories, it is not possible to classify these more accurately as the content of products is not usually known and may vary with time and location (Shanks et al., 2012a).

After classification, NPS were extracted from all the various categories according to the 2018 United Nation Office on Drug and Crime (UNODC) definition: ‘NPS are defined as substances, whether in a pure form or a preparation, which are not controlled by the international drug control conventions but which may pose a public health threat’ (UNDOC, 2018). For this study, all drugs that were not already controlled in January 2009 were considered as NPS. New drug legislation has been introduced over the period of study (Table 2.2) and many drugs considered as NPS for this study have been placed under legal control since 2009. In addition, all branded drug products were considered as containing NPS, although the specific composition of products used in individual cases is often not known, and some may include conventional drugs of misuse or no drugs at all. Unknown substances were classified as “not known”, including unidentified white powders or herbal preparations, which might be NPS or other drug of misuse. They were not classified as NPS. Figure 2.1 shows how the data was sorted and classified. Patient age, sex and severity of exposure were also extracted from the NPIS data and age data grouped into predefined age ranges.

Date	Type	Substances affected
December 2009	MDA	Gamma butyryl lactone, 1,4-butanediol (Class C) Benzylpiperazine and related piperazine compounds (Class C) Further group of anabolic steroids (Class C) 2 non-steroidal agents (Class C) First generation SCRA (Class B) Oripavine (Class C)
April 2010	MDA	Mephedrone and other cathinone derivatives (Class B)
July 2010	MDA	Naphyrone and other naphthylpyrovalerone analogues (Class B)
April 2012	TCDO	Methoxetamine, and its simple derivatives.
June 2012	MDA	2-DPMP and other pipradrol-related compounds; (Class B) Phenazepam and esters or ethers of pipradrol (Class C)
February 2013	MDA	2nd Generation SCRA (Class B) Methoxetamine and other related compounds (Class B) O-desmethyltramadol and tramadol? (Class B)
June 2013	TCDO	25I-NBOMe and related compounds 5- or 6- APB and related substance substances, including 5-IT and 6-IT.
June 2014	MDA	NBOMe compounds (by generic definition, Class A) Benzofuran compounds (by generic definition, Class B) Lisdexamphetamine (Class B)
January 2015	MDA	AH-7921 (Class A) LSD-related compounds (Class A) Tryptamines including AMT and 5-MeO-DALT (Class A)
February 2015	MDA	4,4 DMAR (Class A) MT-45 (Class A)
November 2015	TCDO	Methiopropamine
December 2016	MDA	3rd Generation SCRA
June 2017	MDA	U47,700 (ClassA) 12 methylphenidate-related substances (Class B) 16 benzodiazepines (including etizolam, Class C)
November 2017	MDA	Methiopropamine

Table 2.2 Legislation affecting NPS, 2009-2018. SCRA - synthetic cannabinoid receptor agonist, MDA, Misuse of drugs Act 1971, TCDO Temporary Class Drug Order.

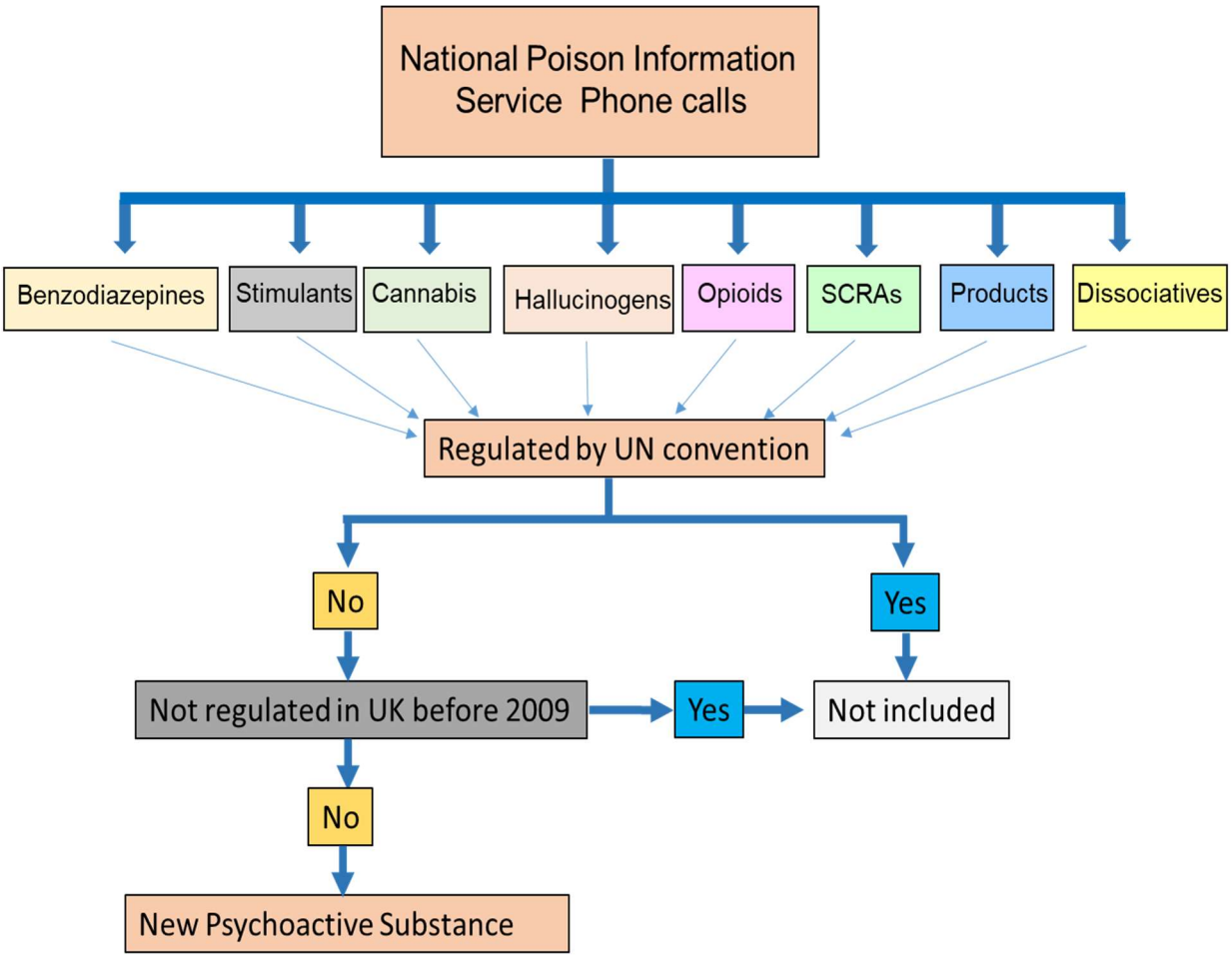


Figure 2.1 The classification of NPIS phone calls and NPS extraction. Each substance reported in the phone calls is recorded into the relevant category. Some enquires reported more than one substance therefore, the number of substances reported are more than the number of phone calls received.

2.3.2 TOXBASE® accesses

TOXBASE® is considered the first line information source for UK healthcare professionals. Its content is updated on at least a 4-year cycle. New substances reported during this time are listed as soon as possible, with priority given to unlisted substances reported in multiple telephone enquiries. Across the whole database, 4,599 product entries were created or updated in the reporting year 2016/2017 (National Poisons Information Service).

TOXBASE® accesses were quantified as user sessions; these are defined as a user logging onto TOXBASE® and viewing the relevant page, including multiple views of the same page, screen refreshes or screen time-outs in the same user session. User sessions from educational establishments (e.g. universities) or NPIS units were

excluded. Because of the very large numbers of products of all types for which TOXBASE® provides information, analysis regarding NPS was restricted to all those drug products likely to contain NPS that had appeared in the top 10 drug products accessed during any of the ten study years, 24 separate products as shown in Table 2.3; as well as all accesses to synthetic cannabinoid receptor agonists and mephedrone. TOXBASE® data cannot be linked back to specific patients and demographic information about users is not available. Finally, 5 conventional drugs of abuse (amphetamine, cocaine, MDMA, heroin and cannabis) were also studied, including telephone enquiry and TOXBASE® access data.

Angel Dust	Herbal highs
Annihilation	Ivory Wave
Bath salts	Jamaican Bubbles
Black Mamba	Liquid Gold
Bonzai	Monkey Dust
Cherry Bomb	Plant Food
Clockwork Orange	Red Raspberry
Diablo XXX Strong as Hell	Sky high
Doves	Spice
Eric 3	Spice Arctic
Exodus	Strawberry
Exodus Damnation	Sweet Leaf Herbal Blend

Table 2.3 Products appearing in the top 10 for TOXBASE® accesses in any single year, 2009-2018.

2.3.3 Statistics

Medians, interquartile ranges (IQR) and mean were used as descriptive statistics and ages of NPS users were compared by the Mann-Whitney U test. To investigate trends

in calls and TOXBASE® sessions for NPS and other drugs of misuse, generalised least squares analysis was used in R, using the nlme package of Pinheiro and Bates (Pinheiro et al., 2018). Monthly NPIS telephone enquiries and TOXBASE® accesses for each NPS and conventional drug of misuse were used as dependent variables in interrupted time series analyses, with the number of months since January 2009 and time since enactment of the PSA used as indices of time. The date the PSA came into law (26th May 2016) was coded as a dummy variable (0= before the point of intervention and 1=after the intervention). Time since PSA enactment was included as a continuous predictor variable. Total calls were log-transformed to normalise the residuals. There was evidence that NPIS telephone enquiries were serially correlated, so models with an autoregressive moving average correlation were used to adjust for the serial dependence. Analyses were first undertaken to investigate trends in calls for NPS and then for the five other drugs of misuse.

2.4 Results

The recreational and intentional intake telephone enquiry numbers to the NPIS involving conventional drugs of misuse and NPS for the period January 2009 to December 2018, classified according to their clinical effects or chemical structures are shown in Table 2.4.

Year	Benzodiazepines	Cannabis	Dissociatives	Hallucinogens	Opioids	Products	SCRA	Not Known	Stimulants
2009	127	96	71	13	245	102	3	45	507
2010	126	116	61	12	211	177	0	82	938
2011	154	94	85	31	223	186	2	65	504
2012	119	91	53	54	212	281	3	127	512
2013	118	108	44	37	219	417	8	150	581
2014	137	102	50	29	176	449	43	163	487
2015	167	113	35	19	228	402	117	124	479
2016	143	106	37	14	184	96	58	84	390
2017	146	105	30	15	202	51	46	71	401
2018	154	126	34	15	193	19	33	65	471
Total	1391	1057	500	239	2093	2180	313	976	5270

Table 2.4 Numbers of drugs of abuse reported to NPIS according to their principle chemical and clinical effect.

During the ten years 2009-2018 there were 3866 telephone enquiries received by the NPIS involving exposure to a NPS, with annual enquiry numbers shown in Figure 2.2.

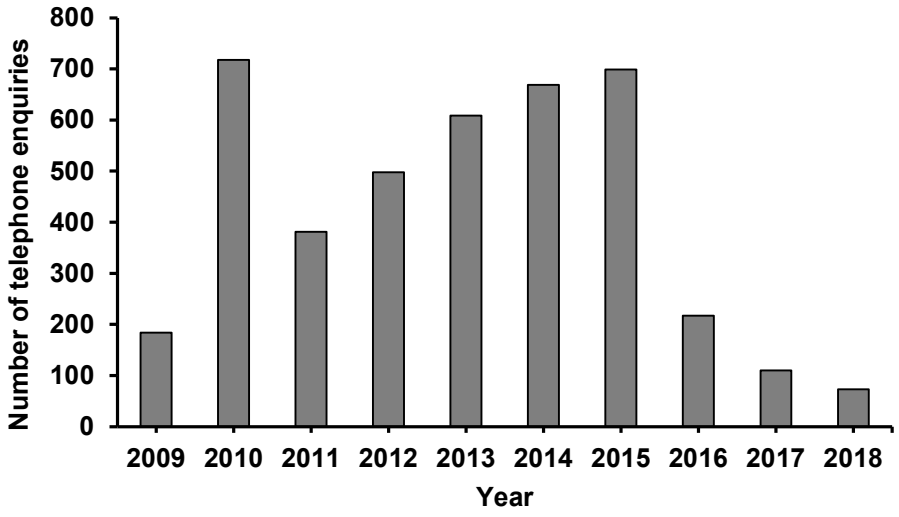


Figure 2.2 Number of Telephone Enquires related to NPS during 2009-2018. For definition of NPS, see section 2.3.1.

Some calls reported more than one NPS and the total number of NPS reported in these 3866 telephone enquiries was 4158. Of all the NPS-related calls received, 2195 (52.8%) involved products (which usually contain one of more SCRA), 1297 (30.2%) stimulants, 315 (7.6%) SCRA, 146 (3.5%) benzodiazepines, 103 (2.5%) hallucinogens, 99 (2.4%) dissociative drugs and 3 (0.1%) novel opioids. The NPS categories reported varied widely between years (Figure 2.3).

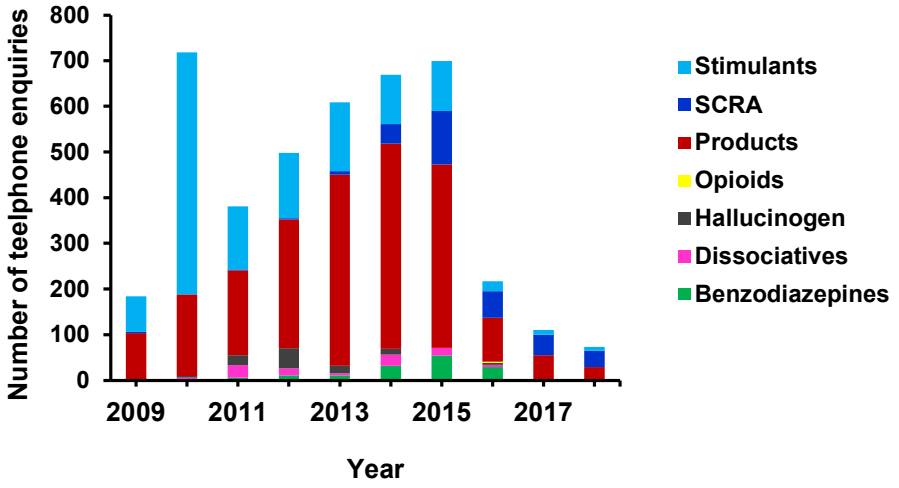


Figure 2.3 Number and categories of NPS reported to NPIS between2009-2010.

2.4.1 Age and gender distribution

The age and sex distribution of users is shown in Figure 2.4. There was a clear predominance of male NPS users (75%) and their median age was 24 years (IQR 19-32). Female users made up 23% of the cohort and had a median age of 20 years (IQR 17-28), which was significantly younger than the male users ($p<0.0001$); in 2% of enquires sex was not recorded. The highest numbers of reported users were in the 16-20 year age group for both males and females. The age distribution was compared across different classes of NPS users. The analysis showed a significant difference between; NPS hallucinogens and NPS benzodiazepines users ($p= 0.017$), and NPS hallucinogen users with NPS dissociative users ($p= 0.017$). There were no significant differences in age among users of other NPS classes ($p> 0.05$). The average age of NPS hallucinogen users was the youngest among all other classes of NPS users (Table 2.5).

Age (Years)	Benzodiazepines n= 111	Dissociative n=81	Hallucinogens n=92	Products n=1913	SCRA n=278	Stimulants n=1172
Median (IQR)	25 (21-30)	24 (20-32)	20 (18- 26)	23 (17-32)	24 (18- 34)	22 (19-29)
Mean ± SD	26.24 ± 7.5	27.3 ± 8.9	23.0 ± 7.9	26.2 ± 10.9	26.7 ± 10.5	25.0 ± 8.7

Table 2.5 Age distribution of different classes of NPS users. Includes only those exposures where the age was known. (Opioids were not included because of very low number).

In addition, the age distribution of cannabis users was compared with that of SCRA users, since SCRA may be used as an alternative to cannabis. The result showed that cannabis users were significantly younger than SCRA users ($p <0.0001$).

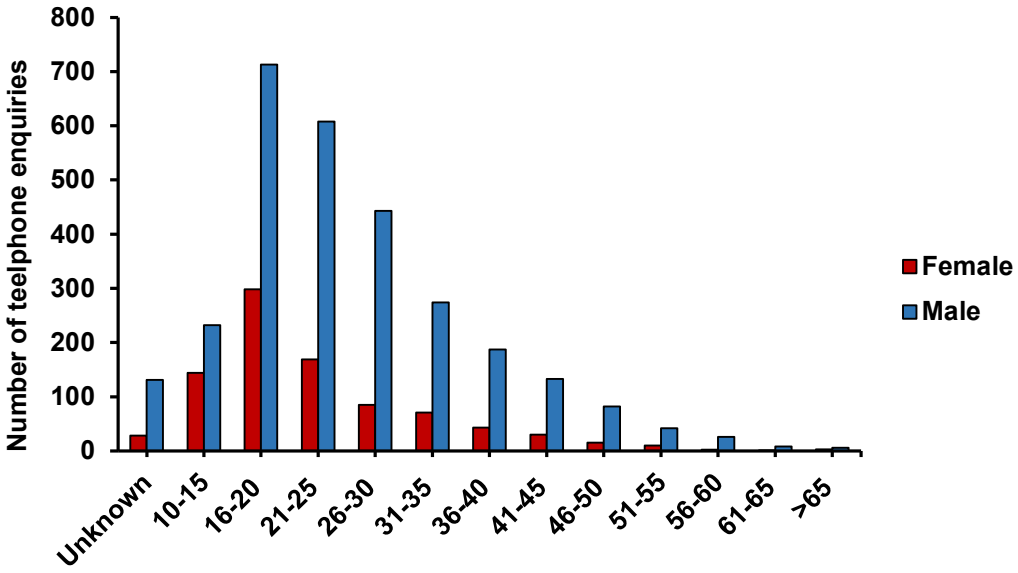


Figure 2.4 Age and gender distribution among NPS users reported to NPIS during 2009-2018.

The age distribution for NPS users was compared with that of the 5 conventional drugs user groups by dividing each group into in 3 age bands (younger than 30 years, 31-55 years and older than 55 years) and median, IQR, mean and the corresponding percentages are in demonstrated in Table 2.6. The use of NPS, MDMA and cannabis was predominantly by younger users; by contrast, 52.8% heroin users were aged between 31 and 55 years. The proportion of users over the age of 55 years was low for all drug groups. Users of NPS were significantly older than users of MDMA ($p<0.0001$) and cannabis ($p<0.0001$), but significantly younger than users of amphetamines ($p<0.0001$), cocaine (($p<0.0001$) and heroin (($p<0.0001$) (Figure 2.5).

Age (Years)	NPS n=3649	Heroin n=668	Amphetamine n=662	MDMA n=1187	Cocaine n=1465	Cannabis n=1010
Median (IQR)	23 (18-31)	32 (26-39)	26 (20-34)	21 (17-29)	27 (22-34)	21 (18-25)
Mean ± SD	25.77 ± 10	32.63 ± 9.3	28.2 ± 10.2	22.36 ± 7.3	28.79 ± 8.9	23.85 ± 10.6
<30 years	82%	46.10%	66.60%	88.50%	62.70%	79.50%
31-55 years	17.50%	52.80%	31.90%	11.10%	36.70%	18.90%
<55 years	0.60%	1%	1.50%	0.30%	0.50%	1.60%

Table 2.6 Age distribution of NPS and conventional drug of users. Includes only those exposures where the age was known. The extent to which different age groups took different drugs was assessed using the Chi-squared test. The Pearson Chi-square was = 677.91, with 10 degrees of freedom and this was significant at p-value < 2.2e-16.

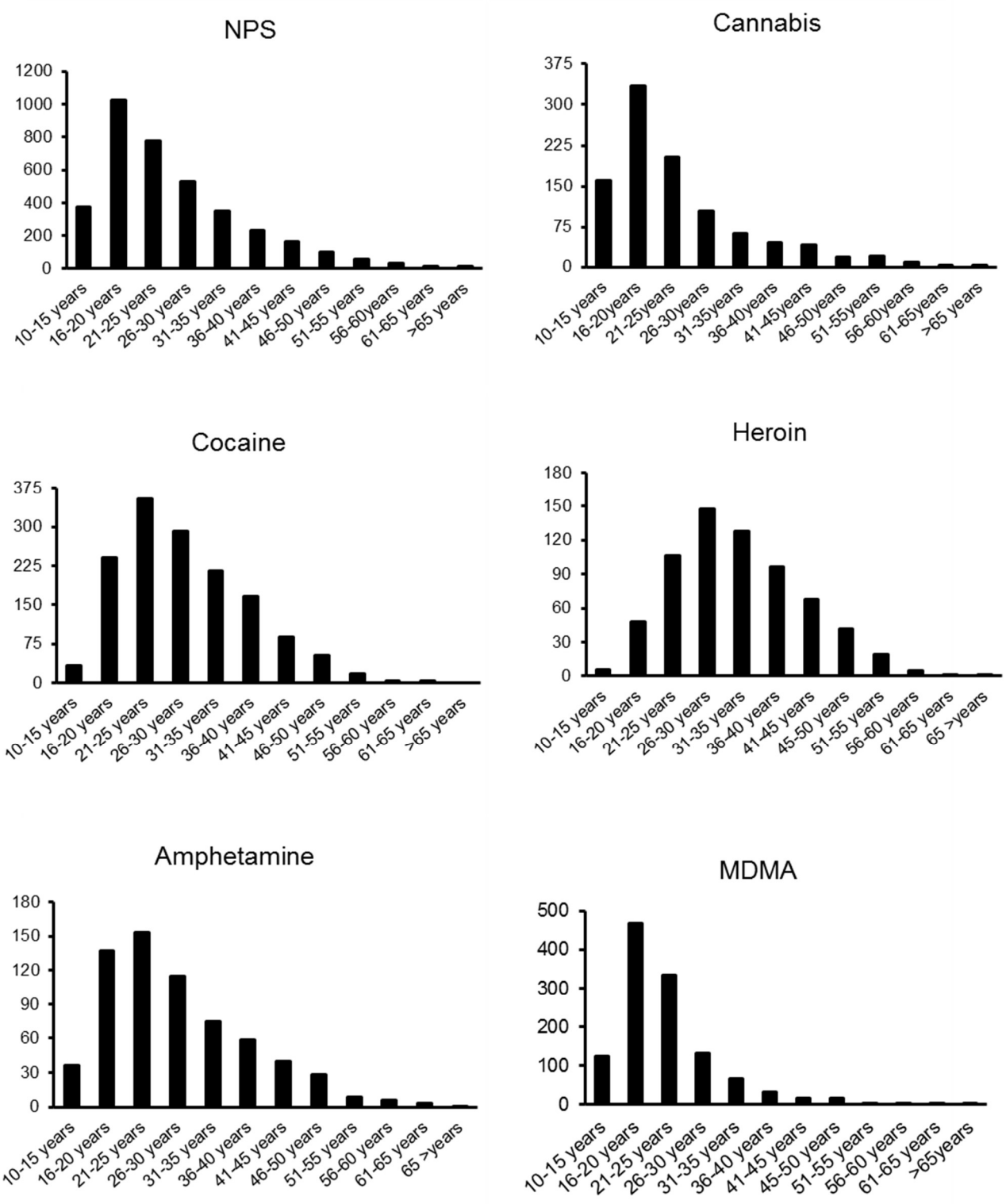


Figure 2.5 Age distribution in NPS and conventional drug users.

2.4.2 Geographic distribution of NPS-related NPIS enquiries

To investigate the distribution of NPS across the UK, the postcodes of enquirers were used. The reported cases were mapped geographically for each year after being corrected for population density (per 100,000 using 2011census data, Figure 2.6). There was very wide variation between areas in rates of NPS telephone enquiries, but without identifiable geographic trends. While increases in NPS use overall are documented, especially between 2012 and 2015, the results did not identify any specific areas from which NPS use originated and disseminated.

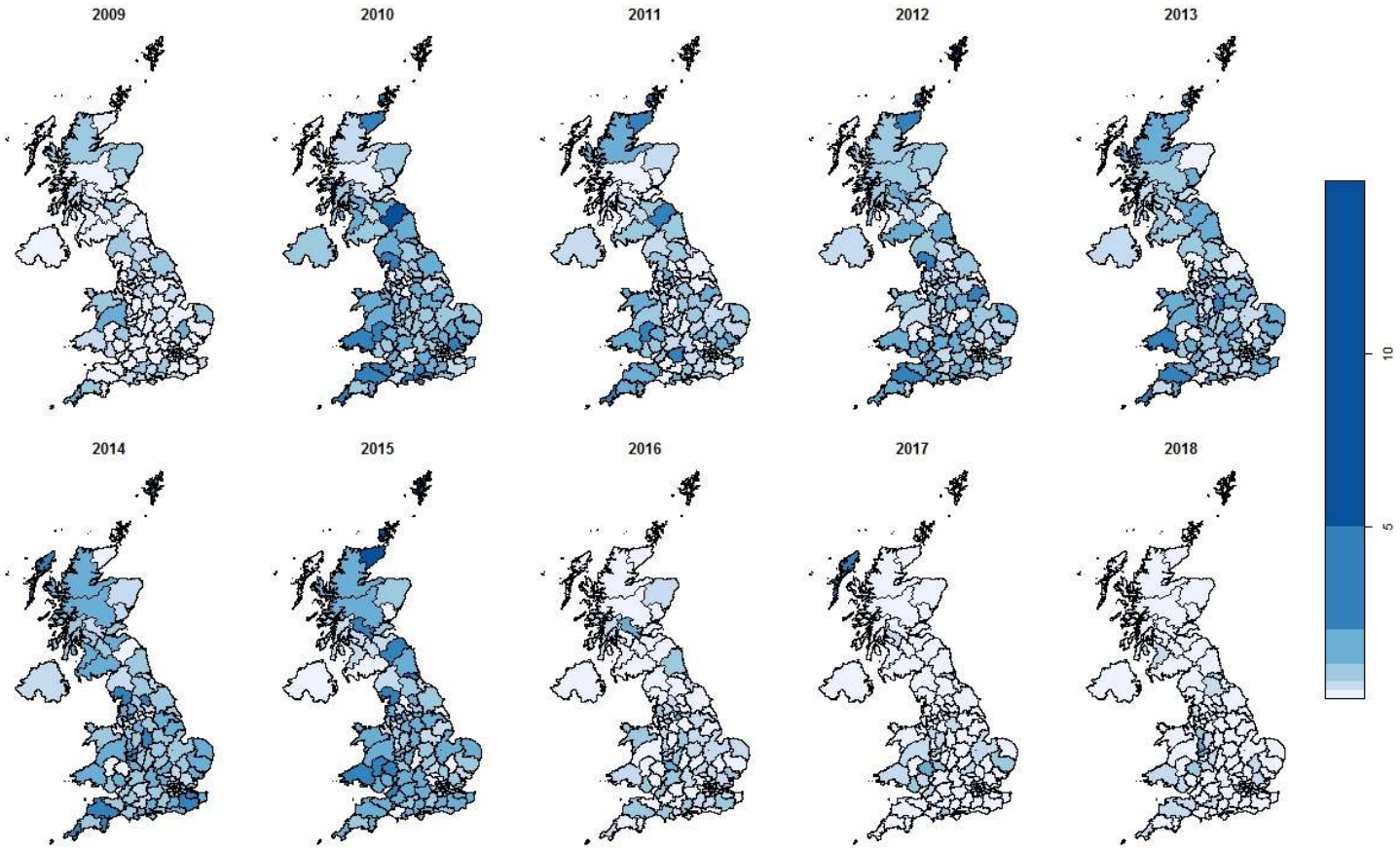


Figure 2.6 Geographical variation of NPS reported to the NPIS in UK during 2009-2018. Showing rates for each area cases with population density per 100,000 population. The darkest shading represents the highest density

2.4.3 Changes in NPS- related NPIS enquiries between 2009-2018

Monthly NPS enquires to the NPIS over a 10-year period are shown in Figure 2.7 A. The largest peak was in 2010 and was largely accounted for by mephedrone enquiries, which made up 44% of the enquiries about NPS reported in 2010 (Figure 2.8 A) and of which 31% occurred in March 2010. After mephedrone was controlled as a class B drug in April 2010 (Home Office circular 10/ 2010), the number of enquiries fell substantially. Another, spike in NPS enquiries was in August 2010 and related to a product called 'Ivory Wave', thought to contain desoxypipradrol (24% of the total enquires in August 2010) (Figure 2.8 B) and 'benzo fury' containing 6-(2-Aminopropyl) Benzofuran (6-APB) (Figure 2.8 C) constituting 25% of the total NPS enquires in August 2010. Calls related to NPS fell in 2011 compared to 2010, but subsequently increased until late 2015, with the continuous emergence of new NPS (Figure 2.8 D, E). Subsequently there has been a decline in NPS enquiries. The increase in incidence of NPS enquiries during 2013-2015 was largely related to branded products (Figure 2.8 F).

Time trends in TOXBASE® accesses for NPS including mephedrone, products and SCRA followed a similar pattern to NPIS telephone enquiries, with a peak in 2010, followed by a rapid reduction and then an increase between 2011 and 2015 and then a reduction (Figure 2.7 B).

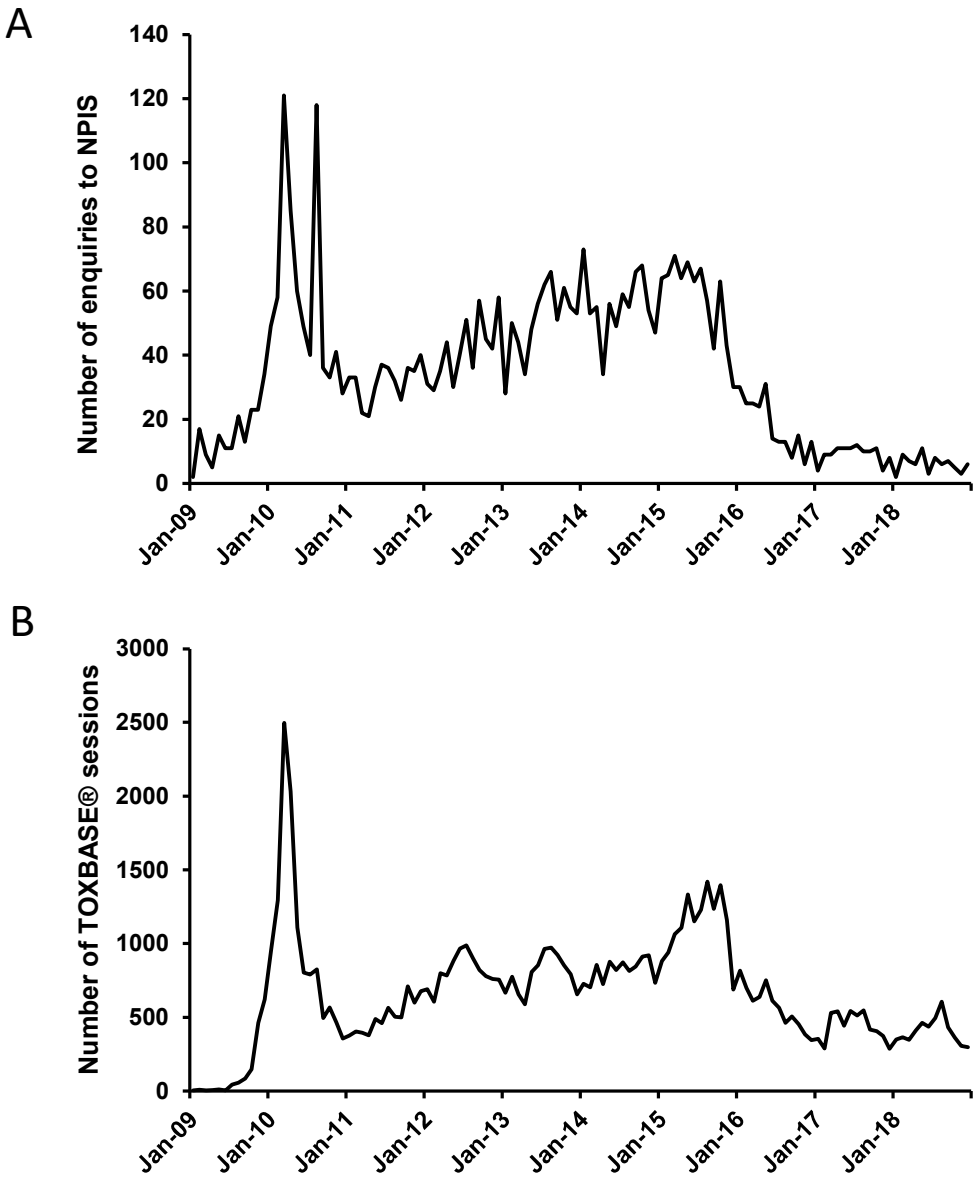


Figure 2.7 Trends in monthly of NPS during 2009-2018. **(A)** Trends in monthly of NPS enquires to NPIS during 2009-2018. **(B)**Time trends in TOXBASE® accesses for top 10 products in any year + mephedrone.

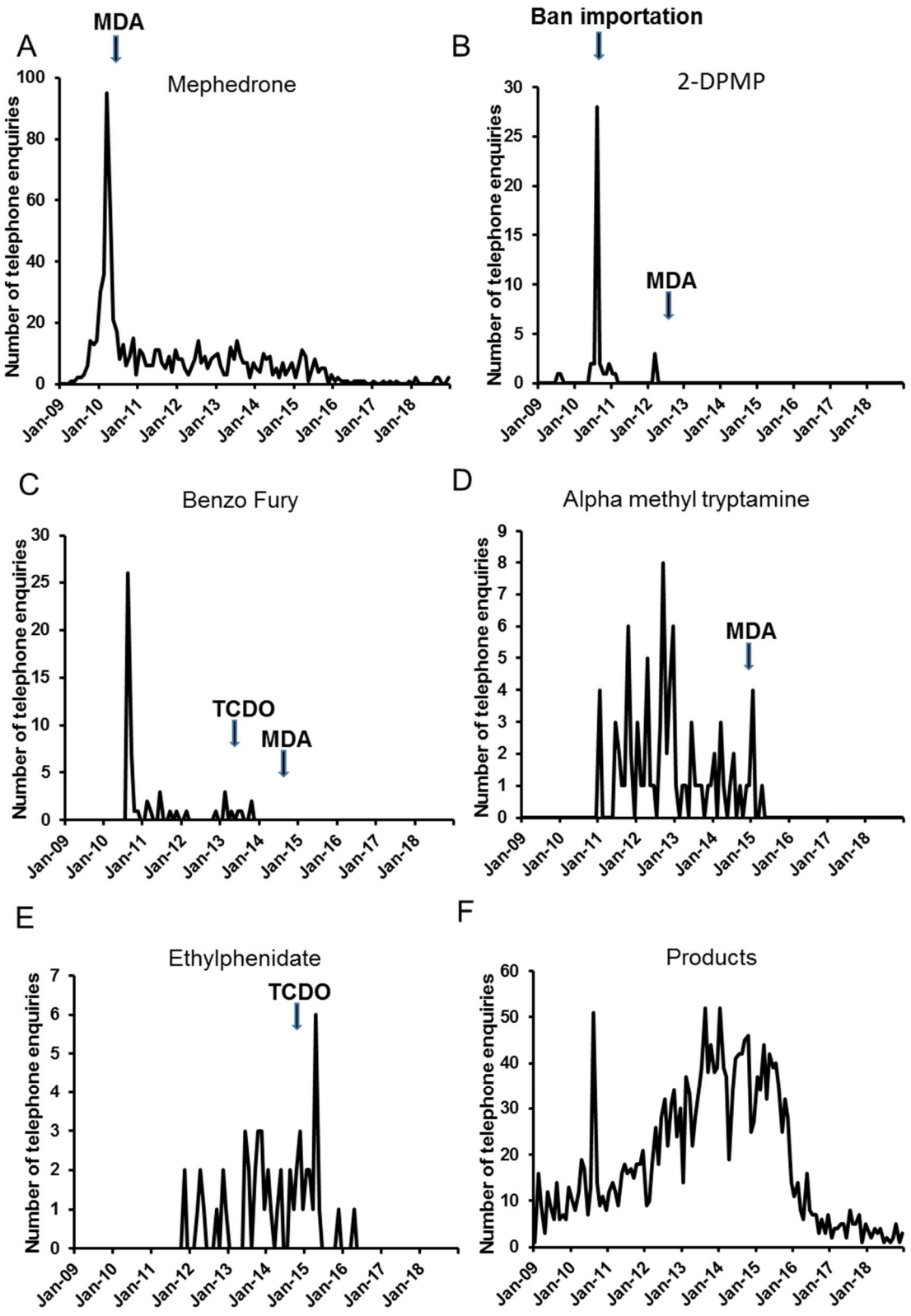


Figure 2.8 Time trend of selected NPS. The arrows refer to the date of the drug regulations. MDA, Misuse of drugs Act 1971, TCDO Temporary Class Drug Order.

2.4.4 Severity of exposure

Poisoning severity is recorded by the scientist receiving the call using the Poisoning Severity Score (PSS) (Persson et al., 1998) which classifies severity as none, minor, moderate or severe. Of the 3866 telephone enquiries involving NPS received during the study period, 598 (15.5%) were graded as PSS none, 2046 (53%) as minor, 869 (22.5%) as moderate and 210 (5.4%) as severe. In 143 (3.7%) the PSS was unknown or there was inadequate data available for accurate classification. The total number of calls reduced over time for all scores, but the proportion of cases with severe poisoning increased over the last 5 years of the study (Table 2.7).

Year	Enquiries	None	Minor	Moderate	Severe
2009	176	24 (14%)	115 (65%)	26 (15%)	4 (2%)
2010	681	77 (11%)	388 (57%)	172 (25%)	23 (3%)
2011	352	42 (12%)	183 (52%)	98 (28%)	16 (5%)
2012	471	66 (14%)	244 (52%)	123 (26%)	19 (4%)
2013	568	107 (19%)	273 (48%)	132 (23%)	31 (5%)
2014	617	104 (17%)	339 (55%)	122 (20%)	24 (4%)
2015	624	102 (16)	335 (54%)	122 (20%)	47 (8%)
2016	195	41 (21%)	81 (42%)	39 (20%)	25 (13%)
2017	107	19 (18%)	54 (50%)	21 (20%)	12 (11%)
2018	75	16 (22%)	34 (47%)	14 (12%)	9 (12%)

Table 2.7 Poison Severity Scores assigned to patients with suspected NPS exposure who were involved in NPIS telephone enquiries.

2.4.5 Legislative impact on NPS

To illustrate the impact of individual legislation on specific NPS, NPIS enquires and TOXBASE® entries were used to examine changes relating to Mephedrone (Stimulant), Spice (SCRA), Black Mamba (branded product sold as SCRA) and the product termed 'Ivory Wave' (which is most likely Desoxypipradrol (2-DPMP).

Figure 2.9 shows that for mephedrone, there were a significant reduction in number of both telephone enquiries and TOXBASE® accesses after specific legislation was introduced. There was also a reduction for 2-DPMP, but that reduction occurred before legislation came into effect. Three legislative changes occurring during the period of study might have affected enquiries about 'Spice' and 'Black Mamba.' which are likely to contain SCRAs. These were control of the so-called 2nd (February, 2013) and 3rd (December, 2016) generation SCRAs and the PSA (May, 2016). There was no convincing relationship between any of these legislative actions and numbers of enquiries for Spice or Black Mamba.

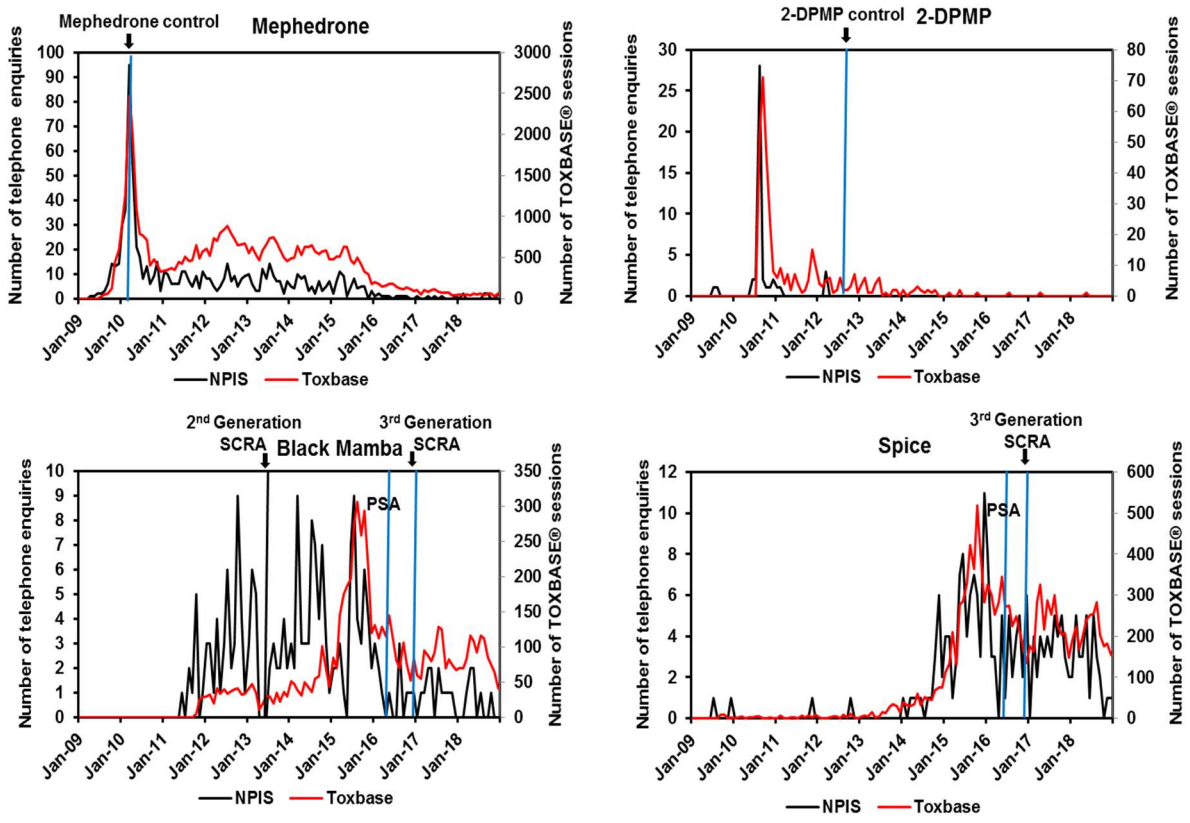


Figure 2.9 Impact of specific legislations on selected NPS in number of enquires reported to NPIS. This includes number of phone enquires and TOXBASE® accesses. The blue lines represent when specific legislations were enacted into law.

To examine the impact PSA on acute health harms related to NPS, NPIS enquires and TOXBASE® entries was analysed using time series analysis. Over the 10 years of the study there were 79,271 TOXBASE® accesses related to the top 10 drug products accessed in any year (24 in total, Table 2.3) and mephedrone. While there were 3,866 telephone enquiries related to NPS received by the NPIS.

	NPIS Telephone enquiries				**TOXBASE® accesses**			
Variable	Estimate	St. Error	T value	P	Estimate	St. Error	t value	P
NPS								
Intercept	3.151	0.114	27.538	<0.001	4.900	0.205	23.843	<0.0001
Months	0.01	0.002	4.743	<0.001	0.029	0.003	7.408	<0.0001
Act	-1.61	0.231	-6.974	<0.001	-1.379	0.414	-3.327	<0.001
Post-Act	-0.04	0.012	-3.418	0.001	-0.035	0.020	-1.712	0.089
Cocaine								
Intercept	2.589	0.071	36.515	<0.001	6.062	0.026	225.91	<0.001
Months	-0.003	0.001	-2.552	0.012	0.008	0.000	15.827	<0.000
Act	0.153	0.143	1.072	0.286	0.050	0.054	0.925	0.357
Post-Act	0.024	0.007	3.372	0.001	-0.001	0.002	-0.679	0.499
Heroin								
Intercept	1.802	0.119	15.173	<0.001	5.548	0.036	153.569	<0.001
Months	-0.002	0.002	-1.011	0.314	0.007	0.001	9.604	<0.001
Act	-0.321	0.24	-1.341	0.183	-0.142	0.073	-1.95	0.054
Post-Act	0.016	0.012	1.373	0.172	-0.006	0.004	-1.535	0.128
Cannabis								
Intercept	2.03	0.076	26.673	<0.001	5.251	0.028	185.185	<0.001
Months	0.001	0.001	0.956	0.341	0.007	0.001	13.275	<0.001
Act	-0.237	0.154	-1.543	0.126	-0.197	0.057	-3.442	0.001
Post-Act	0.015	0.008	1.96	0.052	0.004	0.003	1.547	0.125
Amphetamine								
Intercept	2.183	0.101	21.55	<0.001	5.806	0.04	145.413	<0.001
Months	-0.01	0.002	-4.967	<0.001	0.002	0.001	2.738	0.007
Act	-0.047	0.204	-0.232	0.817	-0.155	0.081	-1.929	0.056
Post-Act	0.009	0.01	0.871	0.385	-0.005	0.004	-1.168	0.245
MDMA								
Intercept	2.188	0.103	21.171	<0.001	5.558	0.041	134.336	<0.001
Months	0	0.002	0.086	0.932	0.01	0.001	13.031	<0.001
Act	0.114	0.208	0.546	0.586	-0.055	0.083	-0.661	0.51
Post-Act	0.005	0.01	0.445	0.657	-0.016	0.004	-3.944	<0.001

Table 2.8 Regression diagnostics relating log transformed number of telephone enquiries. (Left) and TOXBASE® accesses (right) for the time since 2009 ('Months'), the introduction of the PSA in May 2016 ('Act') and the time following it to December 2018 ('Post-Act').

For NPS, there were statistically significant increases in monthly TOXBASE® accesses ($t = 7.408$, $P<0.0001$) and NPIS telephone enquiries ($t = 4.74$, $P<0.001$) between January 2009 and May 2015 for the NPS studied (Table 2.8). There were short term spikes in telephone and TOXBASE® activity in 2010; the first relating to mephedrone and the second to 6-(2-aminopropyl) benzofuran and 'Ivory Wave' (usually desoxypipradrol). There were significant reductions in the number of TOXBASE® accesses ($t = -3.327$, $P<0.001$) and telephone enquiries ($t = -6.974$, $P<0.001$) after the enactment of the PSA in May 2016 compared to the period before, although these reductions began several months prior to that date (Figure 2.10). There have been further significant reductions in telephone enquiries with time after May 2018 ($t = -3.418$, $P < 0.001$) but reductions in TOXBASE® accesses over the same period were not statistically significant ($t= -1.712$, $P= 0.089$).

Regarding other drugs of misuse, Up to the enactment of the PSA, we observed significant increases in monthly TOXBASE® accesses for heroin, cannabis, amphetamines and MDMA, and reductions in telephone enquiries relating to cocaine and (in contrast to the TOXBASE® data) amphetamines (Table 2.8). Comparing the period before and after May 2016, the only significant change observed was an increase in TOXBASE® accesses for cannabis ($t = 13.275$, $p < 0.001$). Since May 2016 there has been an increase in telephone enquiries about cocaine ($t = 3.372$, $p < 0.001$) without significant changes in TOXBASE® accesses. There has also been a reduction in TOXBASE® accesses relating to MDMA ($t = -3.944$, $p < 0.001$) without a significant change in telephone enquiries.

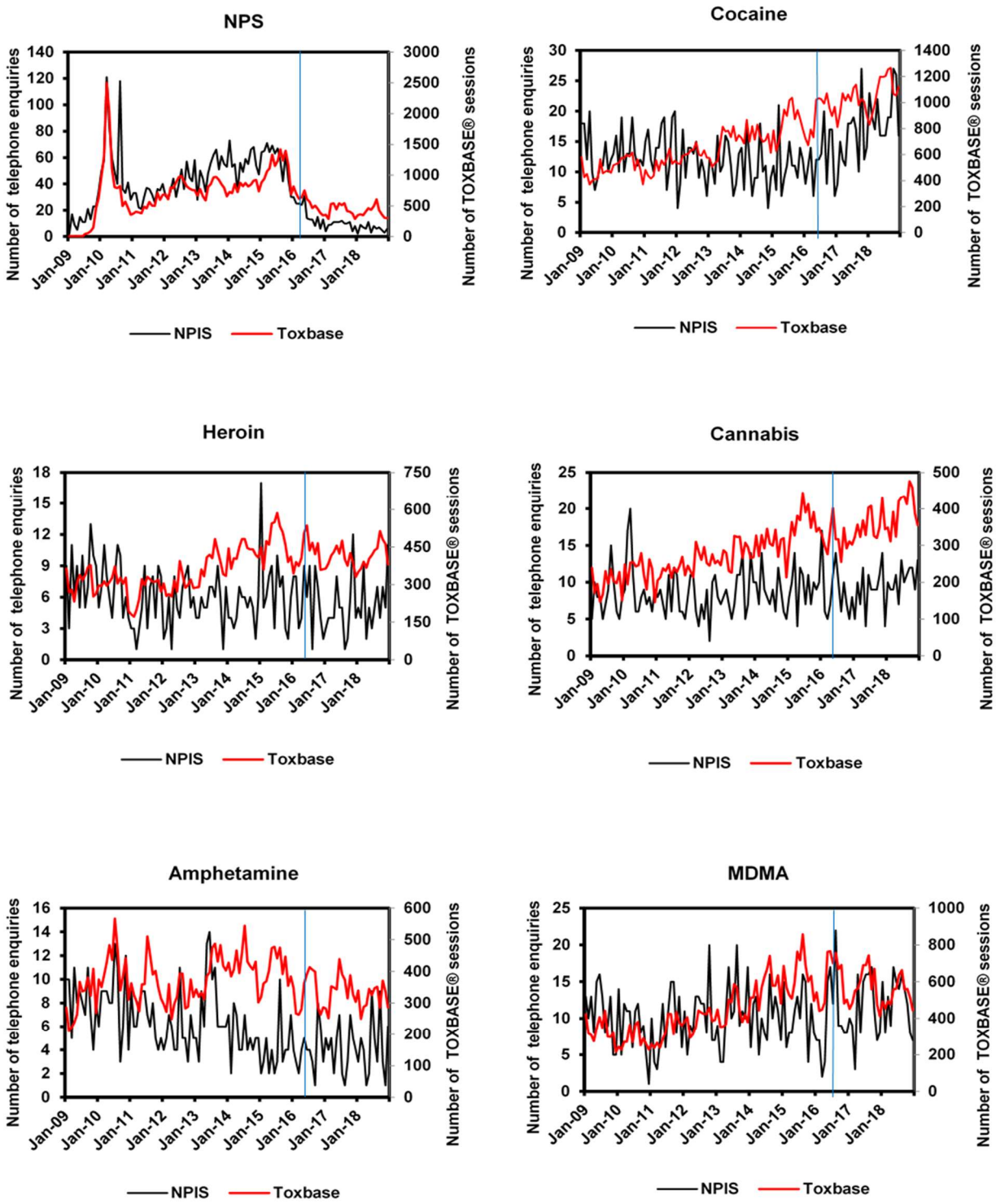


Figure 2.10 Monthly numbers of NPIS telephone enquiries and TOXBASE® accesses relating to NPS and other drugs of misuse. The blue line represents when PSA was enacted as a law.

2.5 Discussion

Over the past decade, the worldwide emergence of NPS has created a new drug phenomenon and represented a new challenge to public health and policy makers. NPS are usually developed to mimic the psychoactive effects of conventional drugs of misuse while circumventing drug control legislation based on chemical structure. These drugs vary widely in their chemical, pharmacological and psychoactive properties. Little is known about their toxicological effects and social impacts, which necessitate risk awareness and careful monitoring.

Studying the epidemiology of drug misuse, especially that involving NPS, is challenging. Although various data sources are available in the UK, all of these have limitations. Some quantitative data are available from drug seizures made by Customs or law enforcement agencies. This provides the types and amounts of drugs seized annually and the purity of drug product being marketed but does not reflect the number of users of the drugs involved. Data are also available on emergency department and hospital admissions which provides useful information about drug morbidity, but these data are unhelpful for NPS because routine coding does not provide the required level of detail for individual drugs. As with poisons centre data, the specific substances involved may not be known because analysis is not done as part of usual clinical practice. Population surveys such as the CSEW reflect the prevalence of drug of misuse in the population aged between16 and 59 in England and Wales (CSEW, 2015), but it does not include the whole population. In addition, the survey only requests answers to questions about specific drugs, which leads to missing information or under reporting of new emerging drugs. Recently, wastewater and urine analysis was adapted as a method to study the pattern of NPS use in term of location and actual substances used (Ort et al., 2018). However, this method cannot provide information on user numbers or any demographic information, (other than a broad geographical indication), and therefore should be regarded as a supplementary technique rather than a substitute (Stephenson and Richardson, 2014). The Welsh Emerging Drugs and Identification of Novel Substances Project (WEDINOS) offers a method for providing analysis and identifying the types of chemical compounds in NPS products at a national level (Drugs, 2016). This can be used as a complementary method in monitoring trends. In addition, The Identification of New Psychoactive Substances (IONA) study was established in 2015 for collecting biological samples from patients presented to

the UK hospital with severe toxicity arisen potentially from NPS poisoning. The study aimed to confirm the analytical findings of NPS and links it with the clinical features in users presented to the hospital (Thomas et al., 2018).

The NPIS data on the harms associated with drug of abuse and can provide a valuable method for monitoring NPS use. This method also has important limitations that are described in detail below.

For this study we used TOXBASE® accesses and NPIS telephone enquiry data as a proxy for clinical activity relating to these new and conventional drugs of misuse. The advantage is that that detailed information is available on the substances reported to be involved, unlike national data sets for emergency department presentations or hospital admissions, which do not provide this level of detail and where coding of the substances involved can be inaccurate (Shah et al., 2011). The disadvantages of NPIS data are that enquiries are not made for all presentations, only those where the health professional believes they need further advice, often after consulting TOXBASE®. Telephone enquiries are therefore more likely for unfamiliar substances or cases with severe toxicity. Also, there may be more than one TOXBASE® access or telephone enquiry for the same case; alternatively, health professionals may look at TOXBASE® when they are not managing a specific patient. In addition, many users are using branded products or drugs purchased online and are not aware of the exact drug they are taking. Therefore, without analytical confirmation there is limited ability to be sure that the drugs reported were in fact the drugs used. Analytical confirmation for NPS is rarely available in normal clinical practice, meaning that exposure data may be inaccurate or incomplete. Finally, NPIS data does not include information about other factors that may be important such as ethnic group or level of education and information on clinical outcome is incomplete.

Branded Products include herbal mixtures sprayed with a synthetic chemical substance (usually a SCRA) or may be branded tablets or powders often containing stimulants. They have made up the largest category in term of number of NPS being reported to the NPIS over the last decade. Although, the exact chemical compositions of these branded products is not known and may change with time, most, especially those that are herbal mixtures designed for smoking (for example ‘Black Mamba’, ‘Cherry Bomb’, etc.), contain synthetic cannabinoids, (Lindigkeit et al., 2009, Uchiyama

et al., 2010, Uchiyama et al., 2009, Langer et al., 2016). Therefore, branded products and SCRA together represent the great majority of SCRA reported to NPIS in telephone enquiries in recent years. These findings are in line with the UK Identification Of New Psychoactive Substances (IONA) study results, which showed that synthetic cannabinoids have been the most common type of NPS identified in patients presenting to Emergency Departments with severe toxicity from NPS exposure since 2015 (Thomas et al., 2018). They are also consistent with the UNDOC statement that "*Globally, synthetic cannabinoids constitute the largest category in terms of the number of NPS reported*" (UNDOC, 2018).

The second largest group reported to the NPIS was the novel stimulants, which showed the highest activity in 2010 with the emergence of mephedrone, 2-DPMP and benzofuran compounds. Subsequently enquiry numbers relating to these have declined (see below). Other categories of NPS were less commonly reported to the NPIS.

2.5.1 Age and gender distribution

It is well documented in terms of the pattern of substance misuse in the general population, that males are more likely to use illegal drugs than females (EMCDDA, 2005) and the data presented here shows the same pattern for NPS. Most NPS users were young and this finding is consistent with other previously published reports on NPS use, where half of the users were younger than 26 years (Barnard et al., 2017). The findings are broadly similar to those reported from other developed countries (Champion et al., 2016, Johnston et al., 2017, Werse and Morgenstern, 2012). The descriptive statistics for age grouping across different classes of NPS showed that NPS hallucinogens users were the youngest among the group. There was a significant difference between hallucinogens and dissociative users, and hallucinogens and benzodiazepine users. This might be due to different motivations for NPS use which varies between substances. Soussan and Kjellgren (2016) reported that common motivation for all NPS classes use was "pleasure and enjoyment". However, for novel hallucinogens and dissociative the main motivations for use were spiritual attainment and exploration (Soussan and Kjellgren, 2016). This might be the reason for the misuses by the younger population as they tend to be more inquisitive. Thus, understanding the reasons younger population prefer different classes of NPS allowing more efficient prevention and subsequently harm reduction.

Cannabis users were younger than SCRA users, this finding was consistence with (Forrester et al., 2012), but other studies reported that there were no significant age differences between SCRA and cannabis users (Mensen et al., 2019, Sznitman et al., 2020). The reason for age differences might be that cannabis is a well-established substance of misuse making it easier to obtain to adolescence and they might perceive cannabis as "harmless". In addition, adults could also be more likely to pursue a 'different' agent compared to young people (Forrester et al., 2012).

In comparison with other traditional drugs of abuse, the age distribution pattern for NPS users was similar to that of cannabis users. This might be because cannabis users might wish to compare the effects of SCRA to cannabis. Younger people may also be influenced by previous legality of NPS which they may interpret as implying safety, in addition to escaping problems associated with illicit cannabis (Winstock and Barratt, 2013a, Champion et al., 2016). Low cost and easy availability may also be relevant. Heroin and cocaine users were significantly older than NPS users and this consistent with the other research; for example the mean age of heroin and cocaine users seeking treatment in Europe is 35 years (EMCDDA, 2019). In general, NPS, amphetamines, MDMA, cocaine and cannabis have shown to be used by younger people. Use of these drugs in younger age groups could interfere their developmental processes and could be associated with many physical, psychological and social harms(Macleod et al., 2004).

2.5.2 Geographic distribution of NPS-related NPIS enquiries

The geographical distribution did not show an identifiable pattern of NPS use in the UK. A limitation was that the post codes analysed were the enquirer rather than the users, as user address is not logged by the NPIS. Enquirer location, however, is still likely to reflect the broad location of drug use as patients are unlikely to travel many miles between drug use and seeking treatment. For the NPIS data, there is a problem with low patient numbers, so the analysis may lack sensitivity in detecting regional differences. As the geographical trends are not easy to monitor, it might be useful to explore the utility of waste water analysis to study the prevalence, patterns and geographical differences in NPS use (van Nuijs et al., 2011, Banta-Green et al., 2016).

2.5.3 Severity of exposure

The PSS measures the severity of poisoning and is a useful tool for comparing poisoning severity between different substances (Persson et al., 1998). It is useful to study severe as well as total enquiry numbers as differences in the way drugs are used might influence these in opposite directions. For example, legal control of a relatively safe drug might result in reduced drug use overall but some diversion to a less safe alternative and a consequent increase in severe cases.

NPS enquiries were usually associated with minor to moderate severity. The annual numbers of reported severe cases decreased each year after 2015, however the proportion of enquires that were severe increased. This could reflect use of more toxic NPS by smaller numbers of people, but an alternative and probably more likely explanation is that clinicians were increasingly confident about managing NPS poisoning and were less likely to make a telephone enquiry unless the patient was severely affected. It is important to point out that in most exposure cases there is poly-drug use often in combination with alcohol and that co-used substances may not be declared by the patient. Therefore, it is may not be known which drug or combination of substances has led to the increased severity.

2.5.4 Changes in NPS-related NPIS enquiries between 2009-2018

The number of NPS enquiries reported to NPIS increased steeply between 2009 and 2010, with a spike in activity in April of that month which was largely accounted for by mephedrone enquiries. This had become popular as a party drug and was perceived by users to be a better “legal” alternative to illicit stimulant drugs such as cocaine and amphetamines (Winstock et al., 2011). Lack of legal control resulted in it being available for sale widely, including in convenience stores and petrol stations as well as via the internet. The Crime Survey for England and Wales 2010/11 reported that mephedrone was the most commonly used NPS at the time in the 16-24 year age group (Home Office Statistical Bulletin, 2011). In March 2010, more than 77 websites were found to sell and deliver mephedrone to users in the EU (EMCDDA). The control of mephedrone as a Class B drug under the Misuse of Drugs Act in April 2010 came after increasing concerns of significant acute intoxication and fatalities (Wood et al., 2010, Wood et al., 2011, Durham, 2011, James et al., 2011, Kamour et al., 2014). This legal control did not eliminate mephedrone use but there was a substantial subsequent reduction in telephone enquiries and TOXBASE® accesses related to the drug.

Separate research also demonstrated a reduction in the number of acute intoxications presenting to one emergency department after legal control (Wood et al., 2013). A gradual recent decline in use has been reported by the Crime Survey for England and Wales (CSEW) with last year use of mephedrone among adults aged 16–59 years being 1.3% in 2010/11, 1% in 2011/12, 0.5% in 2012/13, 2013/14 and 2014/15 then 0.3% in 2015/16, 0.1% in 2016/17 and 2017/18 to 0 in 2018/2019 (Home Office, 2019)

Mephedrone, was soon replaced by other uncontrolled stimulants especially 'Ivory Wave' (mainly 2-DPMP) and 'benzo fury' (6-APB) (Durham, 2011, Kim et al., 2010) and both were responsible for a second spike in NPS enquiries and TOXBASE® accesses in August 2010. Enquiries related to each of these compounds fell even before legal control, which occurred in June 2012 for 2-DPMP (Class B via MDA) and in June 2013 for 6-APB (by Temporary Class Drug Order). This reduction in use before legal control may have been resulted from the prohibition of importation of 'Ivory Wave' in November, 2010 (UK Government press release, 2010) because of perceptions of its serious effects on physical and mental health.

By early 2011, NPS-related enquiries were at a lower level than in 2010, largely because of the reduction in enquiries relating to stimulants including those described above (Figure 2.3). Subsequently there was a gradual increase in NPS enquiries reported to the NPIS, with the greatest contribution from branded products which are most likely to contain one or more SCRAs (Dresen et al., 2010). The main factors influencing the pattern of use of NPS were novel marketing and easy availability and accessibility (Weaver et al., 2015). This increase also occurred in the USA (Wood, 2013, Palamar et al., 2015) and in Europe (EMCDDA 2015).The changes after 2015 are discussed in more detail below.

2.5.5 Impact of legislation on NPS toxicity

This research has demonstrated significant changes with time in the monthly numbers of health professional enquiries to the NPIS via TOXBASE® and by telephone concerning NPS. Compared with the earlier increasing trend, significant reductions in NPS-related TOXBASE® and telephone enquiry numbers occurred after enactment of the PSA in May 2016, although reductions were already evident during late 2015. They have not been offset by increases in enquiries relating to cannabis, MDMA (ecstasy), heroin, or amphetamines. There has been an increase in monthly telephone enquiries

relating to cocaine, but this was not accompanied by significant changes in TOXBASE® accesses.

This is an observational study so changes that occur at the same time as a specific action can not necessarily be interpreted as being caused by that action. Reductions in NPIS activity relating to NPS occurred before the PSA became law. It is possible that publicity associated with the introduction of the PSA to Parliament in May 2015 made potential users more aware of the potential harms of NPS and therefore discouraged use. However, other actions were also taking place at that time, including the use of existing legislation to supress the sales of NPS (Home Office, 2015a). It was estimated that there were at least 250 retail outlets ('head shops') openly selling NPS across the UK in 2013. Many of these had already closed down before the PSA was enacted (Home Office, 2018), in some cases following actions across the UK by police and local trading standards officials (Shapiro and Daly, 2017). In Scotland activity to close down head shops in October 2015 ('Operation Alexander') resulted in this retail trade being severely curtailed before the PSA was introduced (Scottish Government, 2016); this was followed by statistically significant reductions in NPS-related presentations and admissions for drug toxicity in Edinburgh (Pettie et al., 2018). Similar actions were taken in Northern Ireland under the Consumer Protection Regulation; following these all shops in Northern Ireland were reported to have stopped selling NPS before the Act came into operation (Home Office, 2018). Reductions have also been observed in the numbers of English language websites selling NPS and shipping to UK addresses since October 2015, especially those with registered domain locations in the UK. Overall, almost half had closed before enactment of the PSA and by that date there were no remaining active UK-based sites (Wadsworth et al., 2018).

Other potential explanations for the reduction in NPIS activity after 2015 should also be considered. Health professionals may become increasingly familiar with specific NPS and the management of toxicity, and therefore less likely to seek further information. While this may reduce enquiry workload for specific substances over time, it is unlikely to explain the rapid and substantial reductions in NPS-related enquiries during 2015. The possibility that reduced TOXBASE® activity from 2015 resulted from the loss of interest of health professionals in NPS seems very unlikely at time when the passage of the Act through parliament was associated with considerable media attention. This would also not explain the similar reduction in telephone enquiries, as

these are rarely made for educational reasons but rather to seek advice on management of cases of acute toxicity. We cannot exclude the possibility that dealers selling NPS after May 2016 may have mislead users (and the health professionals managing them) into believing they were buying substances other than NPS, thus explaining an apparent reduction in NPS related enquiries, although this seems unlikely.

Other evidence also suggests reductions in NPS use and toxicity since the enactment of the PSA. The Crime Survey for England and Wales (CSEW) has demonstrated a reduction in prevalence of NPS use overall and in males in 2016/17 and 2017/18 compared with 2015/16 (Home Office, 2019). It also demonstrated a reduction in the proportion of NPS bought from shops, with an increasing proportion purchased via the internet or from street dealers (Home Office, 2019). There have also been successive reductions in the numbers of people presenting to drug treatment services with problematic NPS use in 2016/17 and 2017/18 compared with 2015/16 (Public Health England, 2017).

Concerns have been raised that legislation that prevents open sale of NPS will result in users seeking these substances from street dealers or via the internet, where sellers may have less regard for user safety. After introduction of the Criminal Justice (Psychoactive Substances) Act 2010 in Ireland, NPS-related deaths continued to increase, although the largest increase was sometime later, between 2012 and 2013 (Health Research Board, 2014). So far, however, most UK data suggests reductions rather than increases in NPS toxicity. Hospital Episode Statistics show reductions in hospital admissions in England for the category ‘mental and behavioural disorders due to multiple drug use and use of other psychoactive substances’,(NHS Digital) although in one London hospital the numbers of presentations involving an NPS was higher in the year after compared with the year before enactment of the PSA.(Webb et al., 2018) Fewer deaths related to NPS were recorded in England in 2017 compared with 2016,(Office for National Statistics, 2018) although reductions in NPS related deaths were not seen in Scotland (National Records of Scotland, 2017).

Another potential impact is that former NPS users might switch to conventional controlled drugs. The data presented here does not suggest that this has happened for cannabis, MDMA, heroin, or amphetamines, with no significant changes comparing

the periods before and after enactment of the PSA. There were significant reductions in MDMA-related TOXBASE® accesses in the period since the PSA. These reductions were modest, with annual MDMA TOXBASE® accesses 16% lower in 2018 than in 2015. Over the same time MDMA-related telephone enquiries increased by 17%, although this was not statistically significant. The reduction in TOXBASE® accesses is not in line with the increase in prevalence of MDMA use in England and Wales reported between 2016/17 (1.3%) and 2017/18 (1.7%), prevalence having fallen in the previous 2 years (Home Office, 2019) or the increases in MDMA-related mortality reported over the last decade (Office for National Statistics, 2018)This suggests that in isolation, small changes in TOXBASE® accesses, even if statistically significant, do not reliably predict changes in use or toxicity for drugs of misuse.

Increases in cocaine-related telephone enquiries following the PSA might suggest some NPS users switching to cocaine, although this is speculative and there has been no corresponding increase in TOXBASE® accesses. The CSEW detected a recent increase in the prevalence of reported cocaine use, although this occurred a year after the PSA came into force (Home Office, 2019). There has also been a recent increase in the number of young adults seeking treatment for crack cocaine use (Public Health England, 2017). These increases in cocaine-related activity may not have any relation to the PSA and could reflect the increasing availability of higher purity cocaine in the UK and the associated higher risk of adverse health effects (Crawford et al., 2017).

In summary, this research used NPIS data to provide a snapshot of NPS in UK for the last decade. It is possible to use the NPIS data to describe demographic characteristics of users and to examine time trends and the effects of legislation. NPS started to appear in 2009 with most of the reported cases being male and 34% were between16 and 25 years old. There were dramatic but short-lived peaks in 2010 related to some specific novel stimulants. There was a subsequent gradual increase in NPS-related enquiries until 2015, with most of the reported NPS being SCRAs. There have been reductions in NPIS TOXBASE® access and telephone enquiry activity relating to NPS since the introduction of the PSA to Parliament and its enactment. These changes are consistent with other data demonstrating reductions in prevalence of use, hospital admissions, drug treatment presentations and NPS-related deaths. The observed changes, however, started to occur some months before the PSA came into effect and are to some extent offset but a recent increase in telephone enquiries related to

cocaine. It remains important to track adverse health and social consequences of NPS use, alongside those of traditional drugs of misuse and to study the impact of legislative and other measures intended to reduce these.

It would be useful if more details added to NPIS data such as the ethnicity, level of education, etc. Further research is required to elucidate the mechanism of action and toxicological consequences of acute and chronic use of NPS, with those most commonly reported being the priority. This is discussed further in the chapters that follow.

Chapter 3 Effects of indole containing and non-indole containing SCRAs on 5-HT uptake

3.1 Introduction

Products marketed as herbal mixtures, sometimes referred to as “K2” or “Spice” have been found to contain SCRAs such as CP-47,479, CP-47,479-C8, JWH-018, JWH-073, MAM-2201, AKB-48-5F, HU-210 and many others (Lindigkeit et al., 2009, Uchiyama et al., 2010, Assemat et al., 2017). Knowledge of the pharmacological and toxicological profiles of these drugs is very limited and there may be differences between individual SCRAs. The pharmacology of SCRAs is thought to relate to the interaction with cannabinoid receptors (CB), but they may also interact with non-CB receptors or neurotransmitter systems. However, much of the research to date has focused mainly on binding affinities to CB receptors and structure- activity relationships of different SCRAs or their cannabimimetic activity rather than interaction with other receptor systems. Although SCRAs can produce psychotropic effects similar to those of cannabis, there is a higher incidence and severity of adverse effects with SCRAs (Chase et al., 2016, Tait et al., 2016, Wiley et al., 2016). Effects such as tachycardia, agitation, hallucination, hypertension, minor elevation of blood glucose, vomiting, chest pain, seizures, myoclonus and acute psychosis have been documented (Hermanns-Clausen et al., 2013, Brewer and Collins, 2014, Sweeney et al., 2016). Some of these features are similar to those observed in the serotonin syndrome, which is caused by excessive serotoninergic transmission and may be due to an increase in 5-HT release or reductions in its reuptake and /or metabolism (Boyer and Shannon, 2005). Serotonin syndrome is clinically characterised by symptoms such as tachycardia, hypertension, hyperthermia, myoclonus and agitation.

Many SCRAs contain an indole ring within their structure, similar to a moiety found in 5-HT as shown in Figure 3.1. SCRAs containing this indole moiety could potentially bind with 5-HT receptors or act as a 5-HT reuptake inhibitors, as there is evidence that chemicals containing the indole moiety can be potent 5-HT reuptake inhibitors (El-Subbagh et al., 2002, Mignani et al., 1993). SERT regulates the serotonergic activity in the brain by clearing 5-HT following its release into the synaptic cleft. Inhibition of SERT activity therefore results in increased 5-HT concentrations in the synaptic cleft and subsequently increases in duration and intensity of 5-HT action (Blakely et al.,

1994). The increase in 5-HT concertation in the CNS will lead to a spectrum of toxic manifestations ranging from mild to severe effects, depending on the extent that 5-HT is increased (Buckley et al., 2014). This structural similarity has led to speculation that, in addition to cannabinoid receptor agonism, SCRAs might bind to 5-HT receptors or SERT and affect 5-HT neurotransmission (Pascolo-Fabrici and Bonavigo, 2015, Papanti et al., 2014).

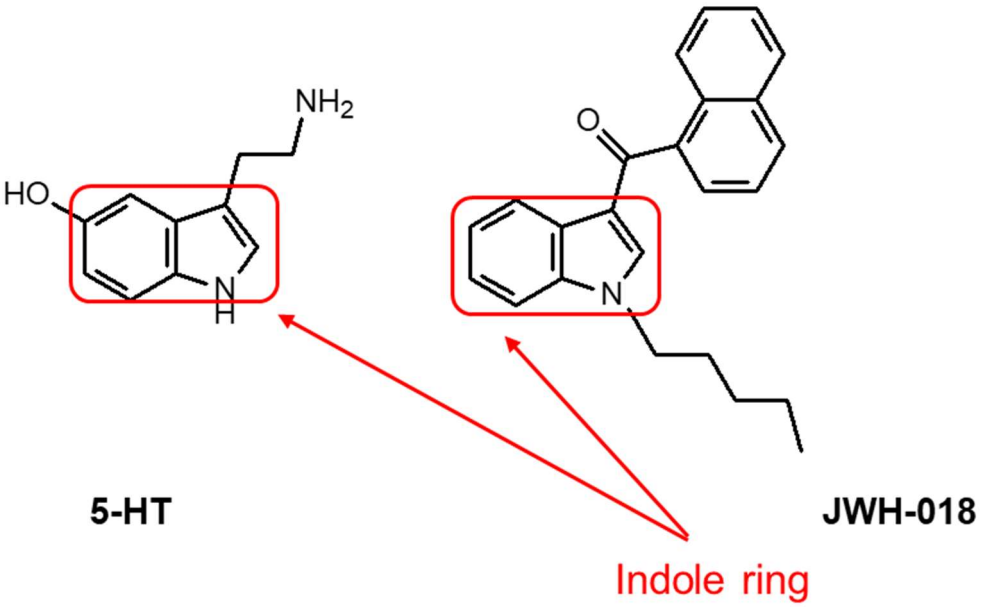
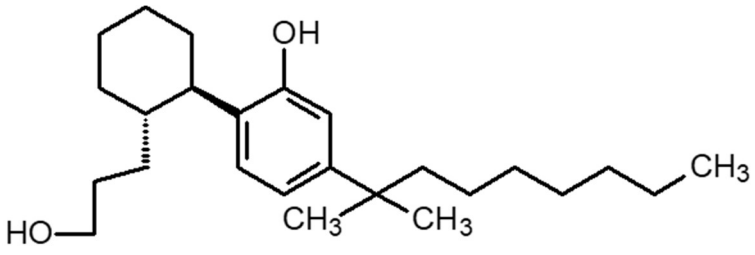


Figure 3.1 Chemical structure of 5-HT and JWH-018 showing common indole moiety found in 5-HT and many SCRAs.

Research presented in this chapter investigated the effects of JWH-018, an indole-containing SCRA on 5-HT transporter (SERT) function. CP 55,940 ,was used as a comparator of a non-indole SCRA (Figure 3.2) to test its effect on 5-HT uptake.



CP55,940

Figure 3.2 Chemical structure of synthetic cannabinoid CP55, 940.

The tested substance, JWH-018 (1-pentyl-3-(1-naphthoyl) indole) is a synthetic cannabinoid receptor agonist belonging to the aminoalkylindole group (Huffman et al., 1994). JWH-018 was one of the earliest SCRAs reported through the European Early Warning System following identification in Germany and Austria in 2008 (EMCDDA 2009). JWH-018 is a potent agonist at CB1 and CB2 receptors with high binding affinities to the CB1 receptor (*Ki*= 9 nM) and CB2 receptor (*K*i= 3 nM) (Aung et al., 2000). CP 55,940 (2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol) is a synthetic cannabinoid developed by Pfizer and subsequently used as a ligand to study cannabinoid receptors (Palmer et al., 2002). It also has high binding affinity for both CB1 and CB2 receptors with binding affinity Ki = 0.5-5 nM and Ki = 0.69-2.8nM respectively (Devane et al., 1988).

3.2 Aims

To investigate the potential effect of a SCRA containing an indole core moiety (JWH-018) on SERT function, utilising ex-vivo rat brain slices and radiolabelled 5-HT. To achieve these two objectives were set.

1. To develop the ex-vivo rat brain slice model to measure 5-HT uptake and validate it using the selective serotonin reuptake inhibitor fluoxetine and the stimulant D-amphetamine.
2. To compare the effect of the SCRAs JWH-018 (indole core) and CP55, 940 (non-indole-containing) on SERT function, by measuring 5-HT uptake in ex-vivo rat brain slices.

3.3 Materials and Method

The 5-HT uptake was measured using radio-labelled 5-HT in rat brain slices (hippocampus) in the presence and absence of SCRAs, JWH-018 and CP 55,940.

3.3.1 Chemicals

Radiolabelled [^{3}H]5-HT (specific radioactivity 91.7 and 82.6 Ci/mmol) was purchased from PerkinElmer (USA). D-amphetamine sulphate, JWH-018 and CP55, 490 were purchased from Tocris (UK). Fluoxetine was purchased from Sequala Research Products Ltd (UK). Pargyline and Rimonabant were purchased Sigma (UK). Perchloric acid was purchased from VWR international (UK). NaCl, Dimethyl sulfoxide (DMSO) and HEPES were purchased from Sigma-Aldrich, (UK). KCl was purchased from Santa Cruz Biotechnology Inc. (Germany), $KH2PO_4$ was purchased from ACROS Organics

(UK). $MgSO_4$ was purchased from Scientific Laboratory Supplies Ltd (UK). Isoflurane was purchased from Abbot Ltd. (UK). Liquid scintillation cocktail (Scintisafe III/code sc/9205/21) was purchased from Fisher Chemicals.

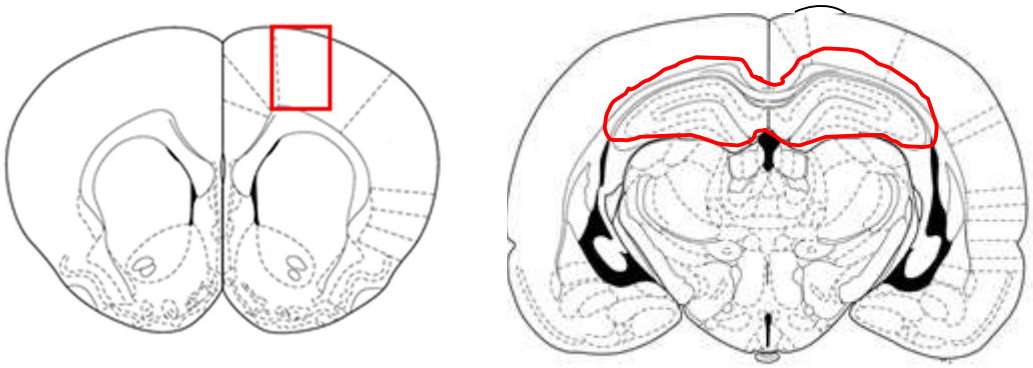
Artificial cerebral spinal fluid (aCSF) composition (mM) was as follows: $MgSO_4$ 2.4;NaCl 124; KH_2PO_4 1.25; KCl 3.25; $NaHCO_3$ 26; $CaCl_2$ 2 ; glucose 10, pH 7.4) was made on the day of the experiment from stock solutions. Sucrose buffer composition (mM) was: sucrose: 200; HEPES 10; MgSO4 7; NaH_2PO_4 1.2; KCl 2.5; $NaHCO_3$ 25; $CaCl_2$ 0.5; d-glucose 10; pH 7.4. This was made in batches and frozen at -20˚C for storage. To allow the frozen sucrose to thaw but remain slushy for the experiment, a 500 ml bottle was transferred to 4˚C the day before the experiment.

Stock solutions of the selective 5-HT reuptake inhibitors fluoxetine (10 mM in water), D-amphetamine sulphate (20 mM in DMSO), JWH-018 (20mM in DMSO), CP 55,940 (20nM in DMSO), the CB1 antagonist rimonabant (10 mM in DMSO) and the MAO inhibitor pargyline (10mM in water) were stored at -4˚C for up to 10 days. Secondary stocks were freshly prepared for each experiment with aCSF. A secondary stock solution of radio-labelled 5-HT was prepared from 91.7 and 82.6 Ci/mmol primary stocks. The final concentration in each test tube was either 7.72 or 8.57 nmol/ml.

3.3.2 Rat brain slices preparation

All experiments were carried out in adherence with the UK Animals (Scientific procedures) Act of 1986. Experiments were conducted on Hooded-Lister male rats (Charles River, Kent, UK). Animals were housed in groups of 3-4 per cage in a temperature-controlled room (21-24 °C) with a 12:12 h light/ dark cycle with ad libitum access to food and water. These rats (n= 47) weighing 200-300g were killed by an overdose of isoflurane, immediately decapitated by small animal guillotine and the brain rapidly removed. The brain was chilled in ice cold sucrose buffer which was oxygenated with 95% O_2 and 5% CO_2. The brain was cut coronally and the cut side of the front half of the brain glued onto a vibratome chuck which was then secured in the vibratome. Coronal brain slices (0.4 mm thick) containing the dorsal hippocampus (n = 34 experiments) or slices containing the frontal cortex (n=19 experiments) were cut using a Vibratome® 1000 (St. Louis, USA) in ice cold sucrose buffer bubbled with 95% O2 and 5% CO2. Slices containing hippocampus (4-5 slices per brain) were taken from bregma 3.3-3.7, and those containing cortex (4-5 slices per brain) were taken from

bregma 2.7-1.2, according to the atlas of Paxinos and Watson (1998) (Figure 3.3). Slices were immediately placed in pre-oxygenated ice-cold sucrose buffer, transported to another laboratory (5 minutes) and then placed in oxygenated sucrose buffer on ice.



Cortex **Hippocampus**

Figure 3.3 Figure depicting brain slices collected for this experiment.
Region of interest are outlined in red. Figures adapted from "the rat brain stereotaxic coordinates" (Paxinos and Watson, 1998).

3.3.3 Uptake assay

After 30-215 min, brain slices were dissected in sucrose buffer and the cortex and the hippocampus were removed. Left and right dorsal hippocampi (n = 8 - 10) or cortical (n = 8 - 10) sections were transferred to oxygenated aCSF (800 µl) in individual test tubes and incubated in a water bath at 34 ˚C for 30 min (Figure 3.4).

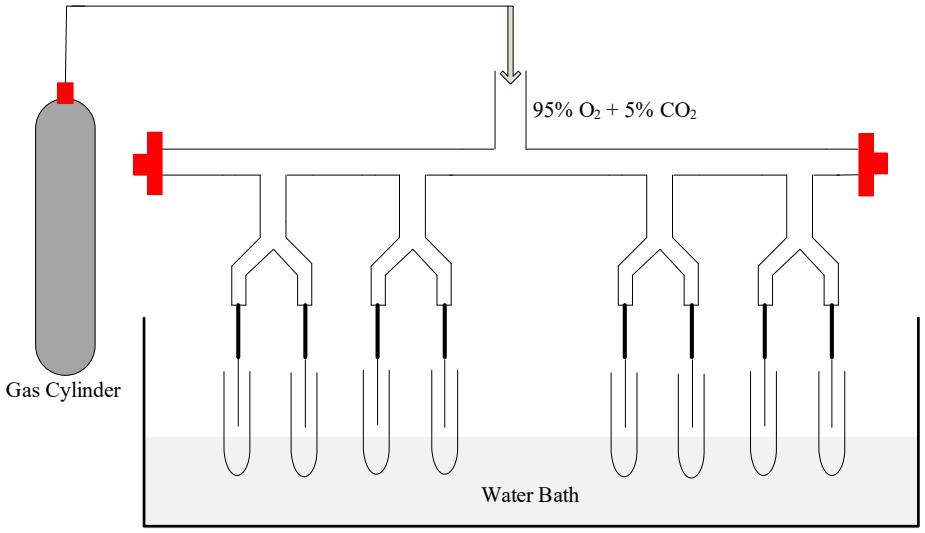


Figure 3.4 Schematic illustration for 5-HT uptake experiment and how to deliver oxygen to the rat brain slices during incubation at 34 ˚C in a water bath.

Secondary stock solutions of 100 µL of the test compounds were added and then 100 µL [^{3}H]5-HT was added to give a final concentration of 7.72 or 8.57 nmol in a total incubation volume of 1 ml.

Table 3.1 shows the final tested compounds concentrations.in each experiment a range of concentrations were tested (2 sections per concentration). This was incubated at 34℃ for another 30 min with 95% O_2 and 5% CO_2. The reaction was stopped by transferring the test tubes into ice and the sections were washed three times using 2 ml chilled aCSF. The sections were weighed and transferred into scintillation vials, and 250 µl of 0.1 N perchloric acid was added to solubilize the tissue (overnight). Subsequently 4-10 ml of scintillation liquid was added to each vial and the radioactivity in the incubation media and in the tissue was recorded using a multi-purpose scintillation counter (Beckman coulter, USA) as disintegrations per minute (DPM). These values were taken as a measure of the amount of 5-HT taken up by the tissue.

Non-specific binding of [^{3}H]5-HT to the brain section tissue may occur by adherence of ^{3}H-5HT to the surface of the section, or by passive diffusion. To assess this, additional sections were incubated with aCSF (900 µl) and [^{3}H]5-HT (100 µL) was carried out at 0° C as a measure for passive diffusion, and at zero time as a measure for the radioactivity adhered to the slices.

Chemical tested	Final concentrations (µM)
Fluoxetine (hippocampus)	0.1, 1, 10 and 100
Fluoxetine (cortex)	0.3, 1, 3,10 and 100
D-amphetamine (hippocampus)	0.01, 0.1, 1, 10, 30 and 100
D-amphetamine (cortex)	0.5, 1, 3, 5, 10 and 100
JWH-018 (hippocampus)	0.001, 0.01,0.1, 1 and 10
CP55-940 (hippocampus)	10
Rimonabant (hippocampus)	1

Table 3.1 Final concentrations of the chemical compounds tested.

3.3.4 Calculations and Statistics

To determine the uptake of [^{3}H]5-HT into sections the presence/absence of SCRA the following calculations were utilised:

Tissue/medium (T/M) ratios were calculated as DPM of tritium in one gram of tissue per DPM of tritium in one millilitre of incubation medium (Shaskan and Snyder, 1970).

The average weight of the hippocampal sections was 5.39 ± 1.7 mg (n=199) and of cortical sections was 5.34 ± 1.6 mg (n=118). The average weight of the sections was used in calculating DPM per gram.

In each experiment, 2 hippocampal or cortical sections were incubated in aCSF with [^{3}H]5-HT at 34 ℃ for 30 minutes in the absence of a test compound. The tissue/medium (T/M) ratio for each of these sections was calculated and the T/M ratios averaged for each experiment. This averaged control value was used as (T/M) for each experiment.

To calculate the passive diffusion, (T/M) ratios for ice control slices were calculated. In addition to the amount of radioactivity adhered to the slices, the (T/M) ratio for slices at 0 time was calculated.

Then the non-specific (T/M) ratios were calculated according the following equation where T/M represents DPM/mg tissue /DPM/ml incubation medium, (T/M) 0 represents T/M value for ice control slices or at 0 time and (T/M) c represents T/M for 34 ℃ controls (absence of test compound).

$$\text{The non-specific uptake} = \frac{(\mathrm{T/M})0}{(\mathrm{T/M})\mathrm{c}} \times 100$$

Then total non-specific uptake for both 0 time and ice controls was subtracted from (T/M) ratio from each experimental slice before further analysis ((T/M) in slice – ((T/M) in slice*non-specific (T/M) ratio) and this represent the specific[^{3}H]5-HT uptake in each slice.

The specific [^{3}H]5-HT uptake was expressed as a percentage of control where T/M= DPM/mg tissue /DPM/ml incubation medium, (T/M)t represents T/M value for brain section with tested concentration and (T/M)c represents T/M for 34 ℃ controls (absence of test compound) :

$$\% \text{ of } [^3\text{H}]\text{5-HT uptake} = \frac{\text{specific (T/M)t}}{\text{specific (T/M)c}} \times 100$$

Statistical analysis was carried out using SPSS (V24; IBM SPSS Statistics USA). Normality of the data distribution was assessed by the Shapiro-Wilko test. Differences between means were tested for significance by one-way analysis of variance (ANOVA) (when data followed normal distribution), or by the Kruskal-Wallis test for data that was not normally distributed. All values were expressed as mean ± standard error.

3.4 Results

3.4.1 Initial assessment of 5-HT uptake assay in hippocampal and cortical sections

Initially, several experiments were conducted to confirm the suitability of rat brain section assay to assess 5-HT uptake.

The competitive effect of increasing concentrations of non-labelled 5-HT (0, 1, 10 and 100 nM) on uptake of labelled [^{3}H]5-HT was tested (10 µM pargyline, a monoamine oxidase inhibitor, was used in the aCSF to maximize uptake). In the absence of non-labelled 5-HT (0 nM; n=6). The average T/M ratio for cortical sections was 9.09 ± 1.5 (n=6) following subtraction of nonspecific uptake (0.048). Specific [^{3}H]5-HT uptake was calculated for 1 (n=6), 10 (n=6), 100 (n=6) nM non-labelled 5-HT. Specific [^{3}H]5-HT uptake was calculated non-labelled 5-HT concentration of 1(n=6), 10 (n=6) and 100 (n=6) nM.

As might be expected, [^{3}H]5-HT uptake was inhibited by increasing concentrations of non-labelled 5-HT; t-tests revealed a significance differences in [^{3}H]5-HT uptake at 10 and 100 nM $t(10)=2.84$, $p < 0.05$ (Figure 3.5).

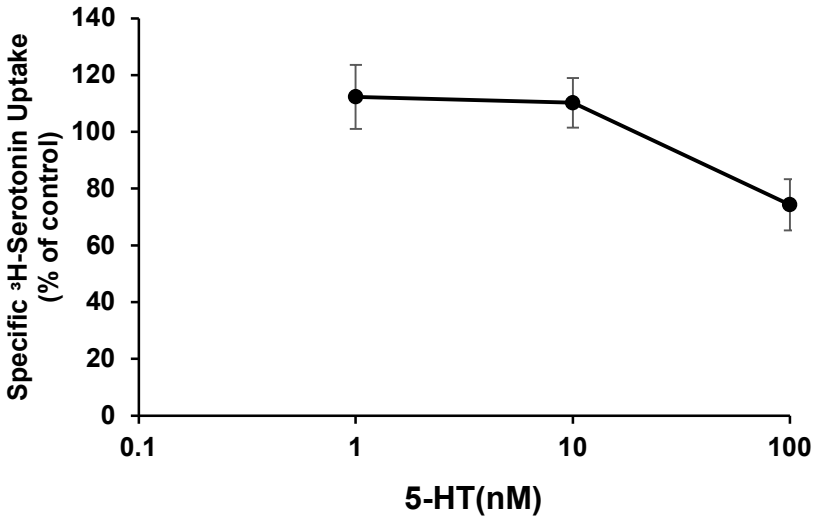


Figure 3.5 Effect of non-labelled 5-HT on [^{3}H]5-HT uptake in rat cortical sections. The uptake is expressed as percentage of [^{3}H]5-HT uptake in control sections. The cortical sections were incubated with 5-HT (0-100 nM) [^{3}H]5-HT for 30 min at 34℃ under O_2/CO_2 atmosphere. Each point represent mean ± SEM of 3 experiments.

To confirm the suitability of using the ex-vivo rat brain sections to assess 5-HT uptake, the accumulation of 5-HT in rat brain slices was assessed in the absence and presence of the selective serotonin reuptake inhibitor fluoxetine.

In the absence of fluoxetine, the average T/M ratio for hippocampal sections incubated for 30 min in aCSF at 34℃ was 8.11 ±1.52 (n=12), the T/M ratio at 0 time was 0.2± 0.03 (n=8) and for the ice control 0.39±0.07 (n=8). Therefore, the T/M ratio at 0 time was 2.48% of the total [^{3}H]5-HT T/M ratio and in 0℃ was 4.9 % of the total [^{3}H]5-HT T/M ratio. The non-specific T/M ratio was subtracted from the total [^{3}H] 5-HT T/M ratio in each experimental slice before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each fluoxetine concentration of 0.1 (n=5). Fluoxetine inhibited specific [^{3}H]5-HT uptake in hippocampal sections in a concentration dependant manner (Kruskal-Wallis H test, $X_{2,3}$ =15.921, p< 0.001; Figure 3.6).

The same experiments were repeated with cortical sections. In the absence of fluoxetine, the average T/M ratio for cortical sections incubated for 30 min in aCSF at 34℃ was 22.6 ± 3.01 (n=6); the T/M ratio at 0 time was 0.16± 0.02 (n=7) and for the ice control 0.8±0.1 (n=7). Therefore, (T/M) ratio at 0 time was 0.69% of the total [^{3}H] 5-

HT T/M ratio and at 0 ℃ was 3.6 % of the total [^{3}H]5-HT T/M ratio. The total non-specific T/M ratio was subtracted from the total [^{3}H] 5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each fluoxetine concentration of 0.3 (n=3), 1 (n=3), 10 (n=6) and 100 (n=3) µM.

Fluoxetine inhibited specific [^{3}H]5-HT uptake in cortical sections (Kruskal-Wallis H test $X_{2,\ 18}$= 11.42, P <0.002, Figure 3.6).

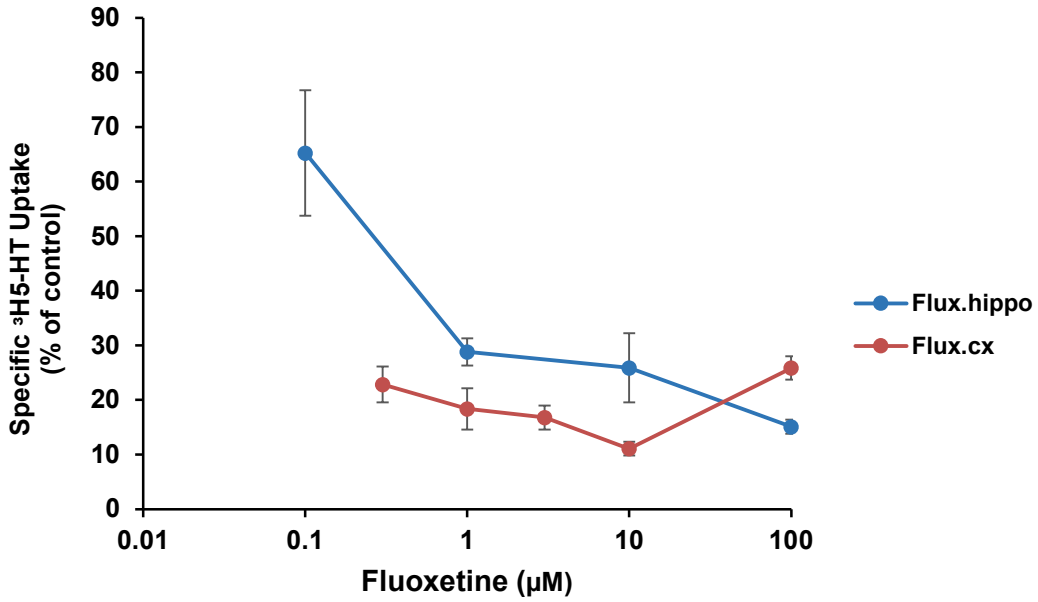


Figure 3.6 Effect of fluoxetine on specific [^{3}H]5-HT uptake in rat brain sections (hippocampus and cortex). The uptake is expressed as the percentage of [^{3}H]5-HT uptake in control slices. The sections were incubated with fluoxetine (0 – 100 µM) and [^{3}H]5-HT for 30 min at 34°C under O_2/CO_2 atmosphere. Each point represents mean ± SEM of (3-5) experiments.

The effect of D-amphetamine on [^{3}H]5-HT uptake was assessed. In the absence of D-amphetamine, the average T/M ratio for hippocampal sections incubated for 30 min in aCSF at 34 ℃ was 9.04± 1.5 (n=9), the T/M ratio at 0 time was 0.2± 0.03 (n=8) and for the ice control 0.39±0.07 (n=8). Therefore, the T/M ratio at 0 time was 0.4 % of the total [^{3}H] 5-HT (T/M) ratio and at 0 ℃ was 4.4 % of the total [^{3}H]5-HT T/M ratio. The non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental slice before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each D-amphetamine concentration of 0.01 (n=4), 0.1 (n=4), 1 (n= 5), 10 (n= 4), 30 (n= 6) and 100 (n=4) µM. D-amphetamine had a variable effect on [^{3}H]5-HT uptake inhibition in hippocampal sections at different concentrations. However, overall [^{3}H]5-HT uptake was significantly inhibited ($X_{2,\ 6}$= 13.805, P <0.03, Figure 3.7).

The average T/M ratio of amphetamine on cortical sections incubated for 30 min in aCSF at 34℃ was 16 ± 2.5 (n=12), the T/M ratio at 0 time was 0.14± 0.02 (n=6) and for the ice control 1 ±0.1 (n=6). Therefore, the T/M ratio at 0 time was 0.85% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 6.08 % of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each D-amphetamine concentration 0.5(n=6), 1(n=8), 3(n=4), 5(n=4), 10 (n=4) and 100 (n=4) µM.

There was no statistically significant effect of amphetamine at the concentrations tested on [^{3}H]5-HT uptake inhibition in cortical sections (Kruskal-Wallis H test $X_{2,\ 5}$ =2.14, p > 0.05; Figure 3.7).

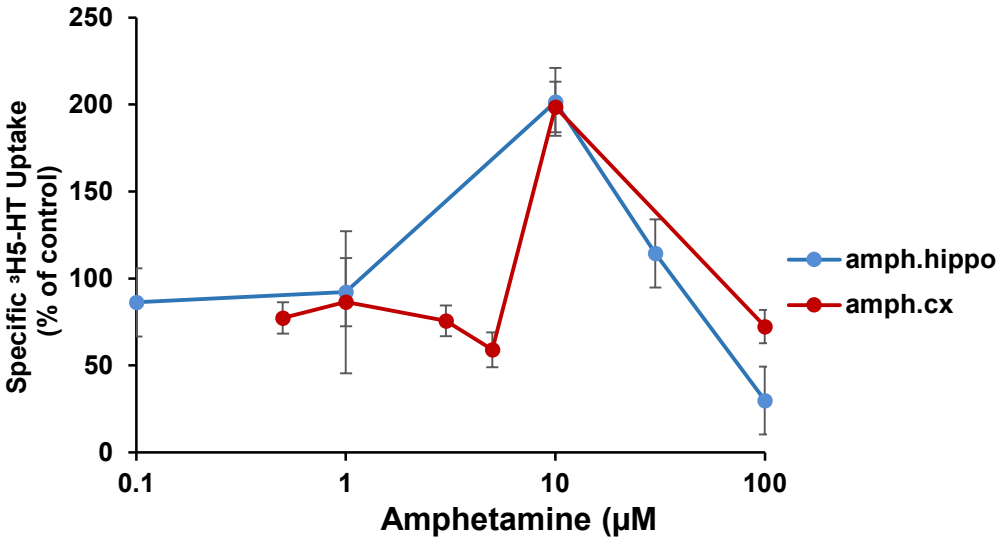


Figure 3.7 Effect of D-amphetamine on specific [^{3}H]5-HT uptake in rat brain sections (hippocampus and cortex). Sections were incubated with D-amphetamine (0 – 100 µM) and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as the percentage of [^{3}H]5-HT uptake in control sections. Each point represents mean ± SEM of 6 experiments.

3.4.2 Ex-vivo brain slice experiment optimization

In the previous experiments, it was evident there was variability between experiments of different days. To reduce the variability between different experiments, the oxygenation of the slices continued during dissection, and they were dissected in aCSF instead of sucrose buffer. In addition, the time between the dissection of slices to the start of incubation reduced from (30-215 min) to (15-30 min). Therefore, the experiments with D-amphetamine were repeated and the results show improvements in uptake and reduced variability.

In the absence of D-amphetamine, the average T/M ratio for cortical sections incubated for 30 min in aCSF at 34℃ was 10 ± 1.03 (n=6), the T/M ratio at 0 time was 0.39± 0.09 (n=3) and for the ice control 1.1 ± 0.05 (n=3). Therefore, the T/M ratio at 0 time was 3.64% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 10.3% of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis. [^{3}H]5-HT

Specific [^{3}H]5-HT uptake was calculated for each of D-amphetamine concentrations 1(n=6), 10 (n=6) and 100 (n=6) µM.

There was a statistically significant effect of amphetamine concentration on [^{3}H]5-HT uptake inhibition in cortical sections (one way ANOVA, $F_{(3, 18)} = 3.8$ $p < 0.05$; Figure 3.8). The optimisation improved 5-HT uptake and reduced 5-HT uptake variability (Two-way ANOVA, $F_{(3, 42)} = 2.66$; $p < 0.05$; Figure 3.8).

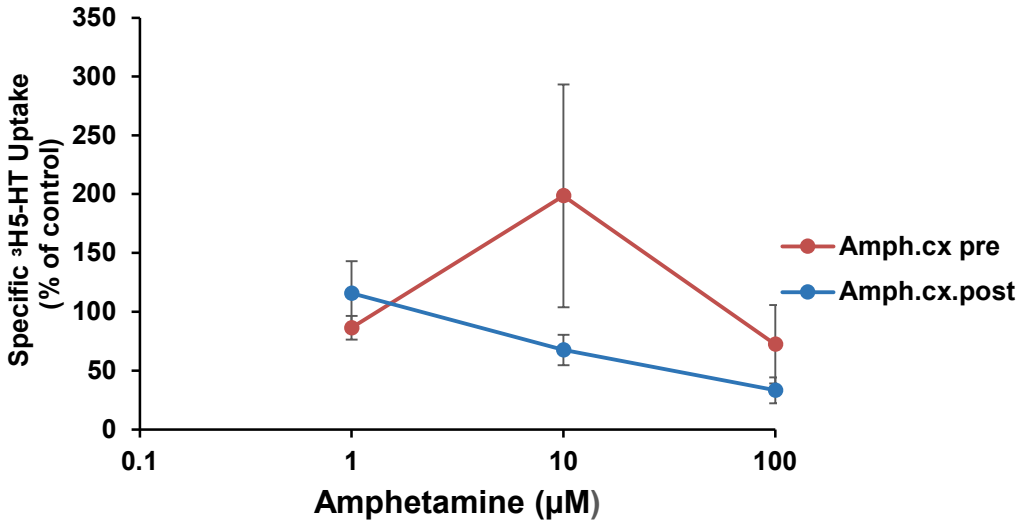


Figure 3.8 The effect of optimisation on percentage of inhibition of [^{3}H]5-HT uptake by amphetamine in rat cortical brain sections. Sections were incubated with D-amphetamine (0 – 100 μM) and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as the percentage of [^{3}H]5-HT uptake in control sections. Amph.cx pre represents the experiments prior to optimisation. Amph.cx post represents the experiments after optimisation. Each point represents mean ± SEM of experiments of 3 experiments.

The uptake inhibition of D-amphetamine in cortical sections was assessed again after the addition of 10 μM of the monoamine oxidase inhibitor pargyline to the incubation aCSF to reduce the metabolism of 5-HT.

In the absence of D-amphetamine, the average T/M ratio for cortical sections incubated for 30 min in aCSF at 34℃ was 19 ± 3.1 (n=6), the T/M ratio at 0 time was 0.2± 0.01 (n=3) and for the ice control 1.32 ± 0.1 (n=3). Therefore, the T/M ratio at 0 time was 1.05% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 6.75% of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each D-amphetamine concentrations of 1(n=6), 10 (n=6) and 100 (n=6) μM.

There was a statistically significant concentration-related effect of amphetamine on [^{3}H]5-HT uptake inhibition in cortical sections in the presence of pargyline (one way ANOVA, $F_{(3,20)} = 29.1$ $p < 0.001$; Figure 3.9). However, a two-way mixed analysis of variance showed no significant main effect of pargyline (Two-way ANOVA, $F_{(3, 38)} = 1.61$; $p > 0.203$; Figure 3.9).

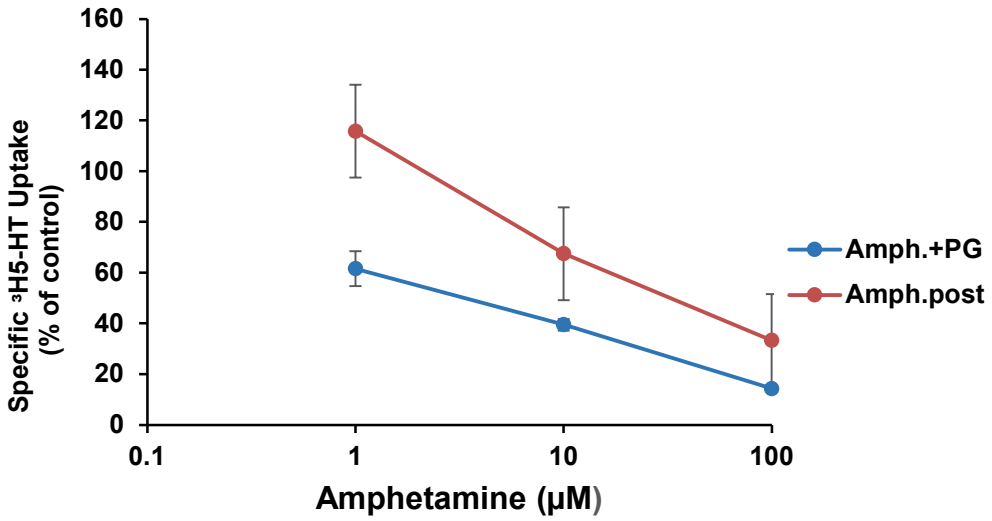


Figure 3.9 The effect of pargyline on [^{3}H]5-HT uptake in the presence of amphetamine in rat cortical brain sections. Sections were incubated with D-amphetamine (0 – 100 µM) and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as a percentage of [^{3}H]5-HT uptake in control sections Amph.post represents the experiments after optimisation. Amph+pg represent the experiments with the addition of 10 µM pargyline. Each point represents mean ± SEM of 3 experiments.

The D-amphetamine and pargyline experiment was repeated in hippocampal sections. In the presence of pargyline.

In the absence of D-amphetamine, the average T/M ratio for hippocampal sections incubated for 30 min in aCSF at 34℃ was 15.27 ± 0.83 (n=6), the T/M ratio at 0 time was 0.2± 0.03 (n=3) and for the ice control 1.03 ± 0.2 (n=3). Therefore, the T/M ratio at 0 time was 1.38% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 6.79% of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each D-amphetamine concentrations of 1(n=6), 10 (n=6) and 100 (n=6) µM.

There was a statistically significant concentration-relate effect of amphetamine on [^{3}H]5-HT uptake inhibition in hippocampal sections in the presence of pargyline (Kruskal-Wallis H test, $X_{2,3}$=19.46; p< 0.001; Figure 3.10).). A two way ANOVA showed a significant main effect of pargyline ($F_{(3, 37)}$ =17.14; p<0.001; Figure 3.10).

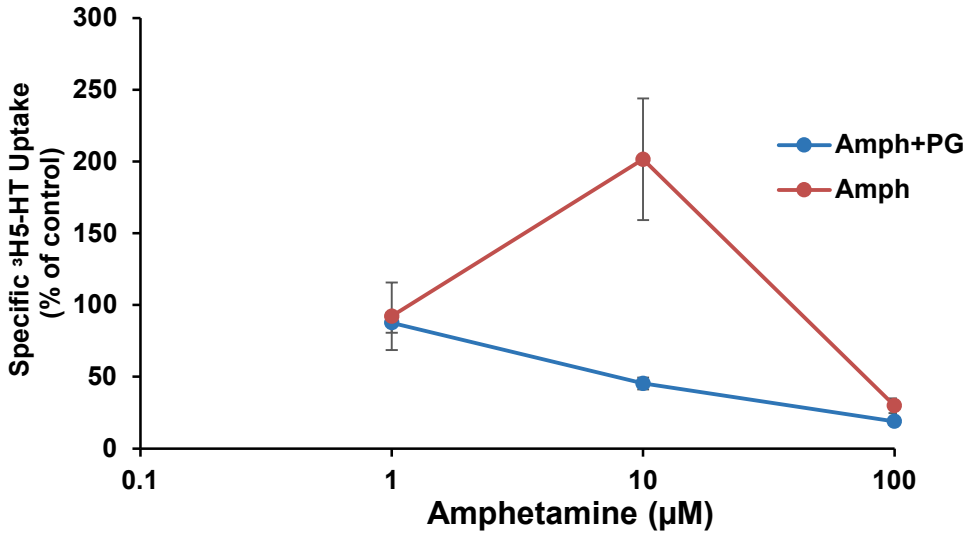


Figure 3.10 The effect of pargyline on [^{3}H]5-HT uptake in the presence of amphetamine in rat hippocampal brain sections. Sections were incubated with D-amphetamine (0 – 100 µM) and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as a percentage of [^{3}H]5-HT uptake in control sections Amph represents the experiments without pargyline. Amph+pg represent the experiments with the addition of 10 µM pargyline to the aCSF. Each point represents mean ± SEM of 3 experiments.

The results after optimization showed there was reduction in [^{3}H]5-HT uptake variability. Therefore, these results suggest that the use of this method can be applied successfully to evaluate the effect of SCRAs on 5-HT uptake.

3.4.3 Effect of JWH-018 [^{3}H]5-HT uptake.

JWH-018 was chosen as an example of an indole derivative SCRAs to assess its ability to inhibit [^{3}H]5-HT uptake on hippocampus.

In the absence of JWH-018, the average T/M ratio for hippocampal sections incubated for 30 min in aCSF at 34℃ was 15.73 ± 1.7 (n=18), the T/M ratio at 0 time was 0.23± 0.02 (n=7) and for the ice control 1.25 ± 0.32 (n=7). Therefore, the T/M ratio at 0 time was 1.44% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 7.9% of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for each JWH-018 concentration of 0 (n = 18), 0.001(n=10), 0.01(n=12), 0.1 (n = 16), 1 (n=7) and 10 (n=6) µM.

There was no statistically significant effect of JWH-018 concentrations on [^{3}H]5-HT uptake inhibition in hippocampal sections (Kruskal-Wallis H test X $_{(2,5)}$ =4.53, p>0.05; Figure 3.11).

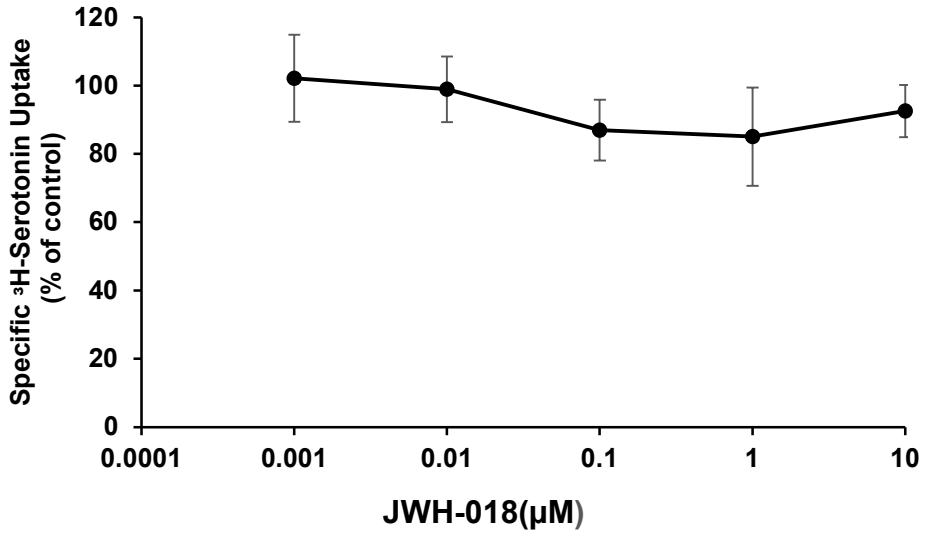


Figure 3.11 Effect of JWH-018 on [^{3}H]5-HT uptake on rat hippocampal sections. Sections were incubated with JWH-018 (0 – 10 μM) and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as a percentage of [^{3}H]5-HT uptake in control sections. Each point represents mean ± SEM of 9 experiments.

To determine if an alternative mechanism of action was counteracting 5-HT uptake, additional experiments were conducted in the presence of CB1 antagonist rimonabant with monoamine oxidase inhibitor pargyline to decrease 5-HT degradation.

In the absence of JWH-018 and rimonabant, the average T/M ratio for hippocampal sections incubated for 30 min in aCSF at 34℃ was 21.47 ± 1.19 (n=12), the T/M ratio at 0 time was 0.27± 0.04 (n=6) and for the ice control 0.98 ± 0.2 (n=6). Therefore, the T/M ratio at 0 time was 1.24% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 4.5% of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for JWH-018 in absence of rimonabant (0.01 μM (n=4), 10 (n=10)), JWH-018 in the presence of 1 μM rimonabant (0.01 (n=4), 10 μM (n=4)) and 1 μM rimonabant (n=8).

The addition of rimonabant did not affect [^{3}H]5-HT uptake in the eight groups of treatment (Kruskal Wallis $X_{(2,7)}$ =2.15, p>0.05; Figure 3.12).

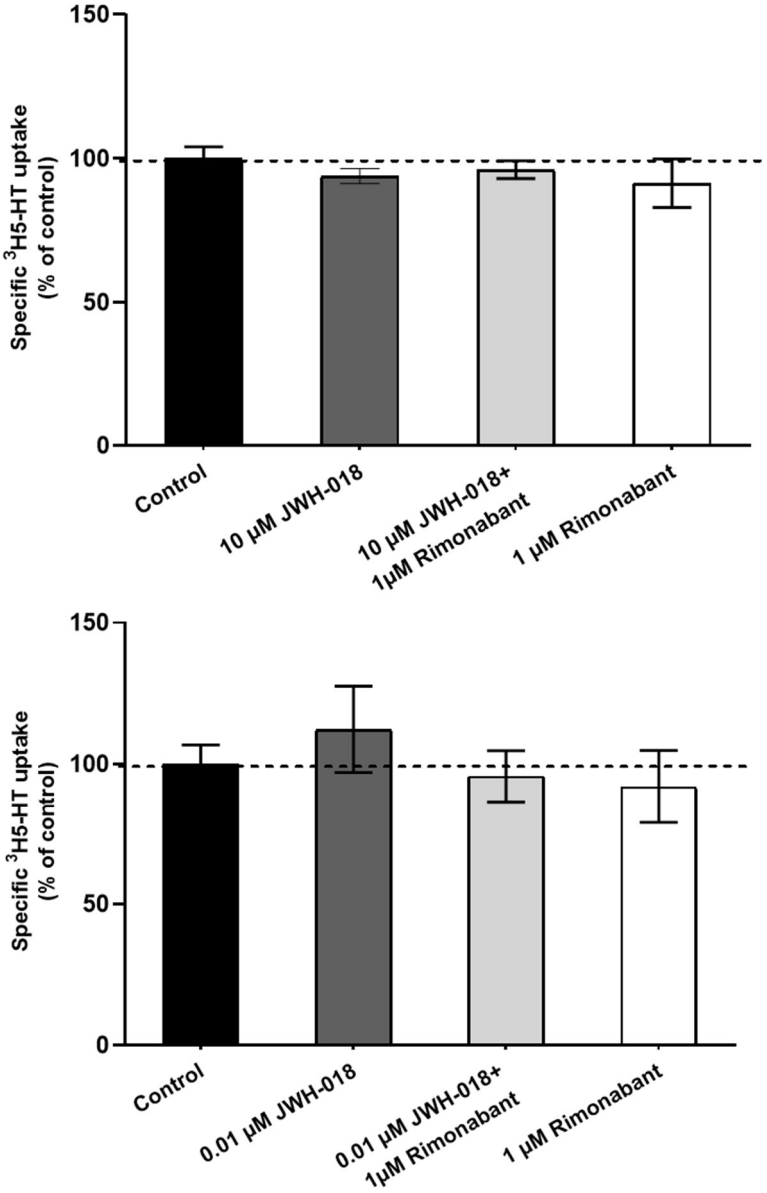


Figure 3.12 The effect of the CB1 receptor antagonist rimonabant on [^{3}H]5-HT uptake in rat hippocampus brain sections. Sections were incubated with JWH-018 (0.01 and10 μM) in the presence of 1 μM rimonabant and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as a percentage of [^{3}H]5-HT uptake in control sections. Each point represents mean ± SEM of 6 experiments.

3.4.4 Effect of CP 55,940 on [^{3}H]5-HT uptake

The effect of the non-indole cannabinoids CP 55,940 on 5-HT uptake was investigated in the presence and absences of 1 μM CB receptor antagonist rimonabant.

In the absence of CP 55,940 and rimonabant, the average T/M ratio for hippocampal sections incubated for 30 min in aCSF at 34℃ was 18.15 ± 2.3 (n=8), the T/M ratio at 0 time was 0.22± 0.05 (n=4) and for the ice control 0.63 ± 0.15 (n=4). Therefore, the T/M ratio at 0 time was 1.19% of the total [^{3}H]5-HT T/M ratio and at 0℃ was 3.4% of the total [^{3}H]5-HT T/M ratio. Total non-specific T/M ratio was subtracted from the total [^{3}H]5-HT T/M ratio in each experimental section before further analysis.

Specific [^{3}H]5-HT uptake was calculated for 10 μM CP 55,940 (n=11) and 10 μM CP 55, 940 in the presence of 1 μM rimonabant (n=10). CP 55,940 has no significant effect on [^{3}H]5-HT uptake in the presence or absence of rimonabant (Kruskal Wallis $X_{(2,1)}$ =0.97, p>0.05; Figure 3.13).

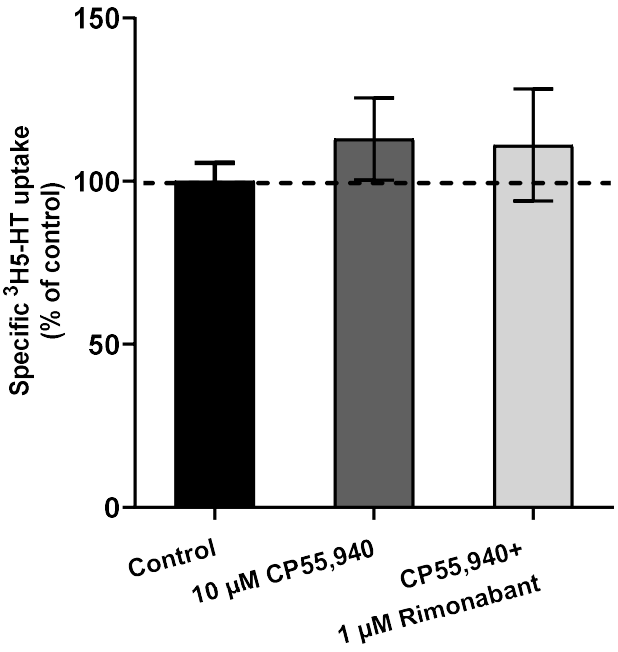


Figure 3.13 The effect of CP 55,940 [^{3}H]5-HT uptake in the presence and absence of the CB1 receptor against rimonabant. Sections were incubated with 10 μM CP55, 940 in the presence and absence of 1 μM rimonabant and [^{3}H]5-HT for 30 min at 4°C under O_2/CO_2 atmosphere. The uptake is expressed as a percentage of [^{3}H]5-HT uptake in control sections. Each point represents the mean ± SEM of 5 experiments.

In summary, these results showed that the SCRAs JWH-018 and CP55, 940 did not inhibit 5-HT uptake, while this experimental model was able to demonstrate that the SSRI Fluoxetine significantly inhibited 5-HT uptake (Figure 3.14).

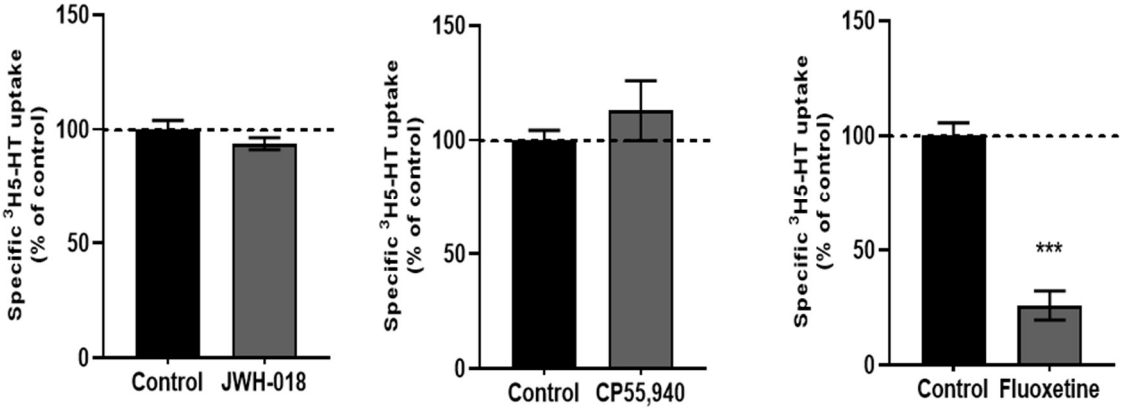


Figure 3.14 Comparison of [^{3}H]5-HT uptake of Fluoxetine, JWH-018 and CP 55,940. The Hippocampal Slices were incubated with ^{3}H-5HT for 30 min at 34℃ in the presence or absence of 10 μM JWH-018, CP 55,940 and fluoxetine. Each point represents the mean± SEM of 3-5 experiments.

3.5 Discussion

The objective of these experiments was to assess the effect of an indole-containing SCRA, represented by JWH-018, and non-indole containing SCRA, represented by CP 55,940, on 5-HT uptake.

3.5.1 Rat brain slice method

Previous published studies examining 5-HT uptake have used either synaptosomes (Whittaker et al., 1964), cultured neurones (Buc-Caron et al., 1990) or rodent brain slices (Shaskan and Snyder, 1970). Synaptosomes, produced from brain tissue homogenate, have proven to be a good model for studying the molecular mechanisms of synaptic transmission. Although, synaptosomes should be fairly uniform during the preparation there may be a loss in functional integrity or low protein yield (Whittaker, 1993, Dunkley et al., 1986). In addition, there are several serotonergic models that have been characterized. For instance, blood platelets have been used as a reliable model to study 5-HT release and uptake, but they lack the ability to synthesis 5-HT (Da Prada et al., 1988). In addition many cell line models have been used such as NG108-

15 hybrid cells (Hilibrand et al., 1987), the medullary thyroid carcinoma (MTC) cell line (Tamir et al., 1989), and the CGP cell line derived from human carcinoids cells (Debons-Guillemin et al., 1982). None of these, however, displays a full serotoninergic phenotype such as active 5-HT uptake, storage, and metabolism (Buc-Caron et al., 1990). On the other hand isolated rat brain slices maintain many "*in vivo"* functions for several hours when maintained in a suitable medium (Collingridge, 1995). Moreover, brain slices provide a highly regulated *ex-vivo* setting while maintaining large parts of the complicated cellular integrity, including cellular barrier and intact circuits. This results in a more comparable *ex-vivo* setting to the *in-vivo* brain in term of pH gradients, active transport systems and cell-cell interaction (Loryan et al., 2013). However, the limitations of this method are the restricted number of slices taken from each brain and the need to keep the slices intact. The rat brain slice method was selected to study the effect of SCRAs on 5-HT uptake as this keeps most of the localised cellular intact. This method also provides a more "realistic" assessment of the effect of SCRA on transmission without excluding the effect of SCRA on other functions.

3.5.2 Development and Validation of the rat brain slice method

To validate the rat brain slice method for 5-HT uptake assessment, non-labelled 5-HT was used (Figure 3.5). These results obtained were consistent with published studies which reported that incubation with increasing concentrations of the non-labelled 5-HT inhibited [^{3}H]5-HT uptake as it increased competition (Blackburn et al., 1967, Hyttel, 1978).To validate the rat brain slice method for 5-HT uptake inhibition, the 5-HT uptake inhibitor fluoxetine was used (Wong et al., 1995). Data obtained showed that fluoxetine inhibited 5-HT uptake in hippocampal rat brain slices in a dose dependent manner (Figure 3.6) which is comparable with a previous studies (Azmitia and Marovitz, 1980) (Table 3.2).

	Fluoxetine concentration	Percentage*
Azmitia and Marovitz study	1	45%
	0.1	57%
Current study	1	41%
	0.1	74%

Table 3.2 [^{3}H]5-HT uptake in hippocampal slices. *percentage of uptake based on 100% of control uptake.

The different incubation times and the 5-HT concentrations used could account for the slight observed differences in uptake inhibition between different studies (Wong et al., 1975, Owens et al., 2001). The effect of amphetamine on 5-HT uptake is more complex than the effect of fluoxetine on uptake. In the current study amphetamine inhibits 5-HT uptake, which is consistent with previous studies (Raiteri et al., 1975, Wong et al., 1973). However, the percentage uptake inhibition varied between different concentrations in a non-concentration dependent manner. This variation in uptake inhibition may be due to different mechanisms of amphetamine increasing 5-HT concentration in the synaptic cleft. Amphetamine has been shown to have monoamine releasing effects (Rothman et al., 2001), i.e. release of 5-HT from serotonergic presynaptic terminals. A drug that influences neurotransmitter release, and at the same time blocks uptake, may promote release of some of the 5-HT previously taken up, making it appear that there is no inhibition.

3.5.3 Ex-vivo brain slice experiment optimization

In the previous experiments, it was noticed that there was substantial variability in 5-HT uptake between the replicates, as demonstrated by large standard deviations. To reduce variability a number of modifications were made to the method prior to SCRA assessment. The slice dissection was conducted in aCSF instead of the sucrose slush; the slices were oxygenated during dissection as transport of 5-HT is enhanced by oxygen (Perez and Andrews, 2005). The slices were dissected before transportation; the time between the dissection and starting the experiment was reduced as longer

incubation times are associated with increased shredding of cells within the slices which might contribute to the variability of data.

Furthermore, the addition of pargyline (MAO inhibitor) significantly increased 5-HT uptake (Table 3.3), because pargyline inhibits the degradation of 5-HT (Ross and Renyi, 1969). In conclusion, the rat brain slice uptake assay was established successfully to be used to test SCRAs and their effect on 5-HT transporter function.

	(T/M) ratio	Number of slices
Cortical sections incubated in aCSF without pargyline	10 ± 1.03	6
Cortical sections incubated in aCSF with 10 μM pargyline	19 ± 3.14	6

Table 3.3 [^{3}H]5-HT uptake in aCSF slices incubated with and without pargyline.

However, there are some limitations related to the rat brain slices method, first a metabolic shaker should be used instead of in house made apparatus (Figure 3.4) to provide more consistence control over the temperature, rate of shaking and oxygen pressure. Secondly, due to the low sensitivity of the method to 5-HT uptake inhibition a more simple and sensitive method such as synaptomsomal preparation could be used. Synaptosomes could be used to optimise the concentrations used of the tested SCRA, before being applied to the rat brain slice method. Thirdly, the underlying reasoning for this part of research was based on the hypothesis that cannabinoids may increase serotonergic activity reported in some clinical cases from SCRA intoxication, however, there is contradictory evidence from a variety of mechanistic studies (Section 1.5). It might have been better to initially start with studying it in animals (murine model) and measuring the level of serotonin directly such as micro dialysis studies.

3.5.4 Effect of JWH-018 and CP55, 940 on [^{3}H]5-HT uptake

The results of this study demonstrate that both JWH-018 and CP 55,940 at the concentrations tested, do not affect [^{3}H]5-HT uptake in rat hippocampus, a brain region receiving serotonergic innervation and possessing 5-HT presynaptic terminals

(Palkovits et al., 1977). These obtained results indicate that that the 5-HT transporter is unaffected by these SCRAs.

Little information is known about the effects of SCRA on serotoninergic systems in the brain. The understanding of the impact of cannabinoids on SERT function could help to understand the interaction between the two systems. Velenovská et al. (2007) investigated the effect of cannabinoids on 5-HT function on platelets from chronic cannabis smokers (Velenovská and Fišar, 2007). They reported that high concentrations of cannabinoids (∆9 THC, anandamide and WIN 55,212-2) can inhibit SERT activity acutely. This inhibition is non- competitive, which indicates that cannabinoids indirectly inhibited SERT activity through the changes on membrane lipids. Although studying 5-HT uptake using platelets models has been shown to be a successful method (Stahl and Meltzer, 1978, Lesch et al., 1993), it does not represent the complexity of the brain where there is an integration and interaction between different receptors, neurotransmitters and /or transporters. In addition, Velenovská noted that high concentrations of tested cannabinoids are required to induce 5-HT uptake inhibition. In contrast, this study tested 5-HT uptake in rat brain hippocampus, where most of the serotonergic features are expressed. In addition, the concentrations range of JWH-018 used in the experiments was chosen to span the expected blood concentrations after inhalation of smoke. Poklis et al. (2012) reported that the blood concentration in mice after 20 min exposure to JWH-018 was 42-160 ng/ml, equivalent to 0.12-0.45 µM (Poklis et al., 2012). Therefore, following the information from Poklis et al. (2012), a concentration range from 0.0001-10µM was used for both JWH-018 and CP55, 940 in this study. Unfortunately, the results of this study suggested that neither JWH-018 nor CP55, 940 affected the uptake of [^{3}H] 5-HT in rat hippocampus. This further suggests that the 5-HT transporter is unaffected.

The initial results show that the indole core structure present in JWH-018 does not act a substrate to compete with 5-HT for SERT binding site. However, to determine this in detail more SCRAs containing the indole core structure would need to be analysed.

3.5.5 The effect of CB1 agonist rimonabant

It has been suggested that CB1 receptors might be located presynaptically on serotonergic neurons (Nakazi et al., 2000). In addition, Lau *et al* (2008) reported the co-expression of CB1 receptors with protein markers for re-uptake and monoamine

release on serotonergic neurons (Lau and Schloss, 2008). Hence, many studies suggested the role of CB1 receptor in modulation of 5-HT release and uptake. Therefore, rimonabant; a potent CB1 receptor antagonist (Rinaldi-Carmona et al., 1994); was used to exclude a direct effect of CB1 receptor activation on 5-HT uptake.

Kenney et al. (1999) suggested that CB1 receptors could be involved in the regulation SERT function (Kenney et al., 1999). They reported that SERT function is decreased in human placenta cells after exposure to the CB1 agonist WIN 55,212-2. It was proposed that the reduction in SERT activity was indirectly through lowering intracellular Ca^{++}, which plays a role in regulation of SERT activity (Turetta et al., 2002). Steffens and Feuerstein(2004), however, reported the inhibitory effect of cannabinoids on 5-HT uptake mediated by a reduction of Na+/K+-ATPase activity rather than CB1 activation (Steffens and Feuerstein, 2004).

Nakazi et al. (2008) had reported that the activation of the CB1 receptor lead to relatively small 5-HT release inhibition (20% inhibition) (Nakazi et al., 2000). Kleijn et al. (2011) stated that the acute stimulation of the CB1 receptor counteracted the effect of selective serotonin reuptake inhibitor citalopram on increasing 5-HT levels in rat prefrontal cortex (Kleijn et al., 2011). The blocking of CB1 by rimonabant moderately stimulates 5-HT release in the rat prefrontal cortex (Tzavara et al., 2003). Taken together, the effect of CB1 activation could inhibit 5-HT release, but this could lead to reduced 5-HT available for uptake and this might mask the inhibition of SERT function. However the results in this study suggested that the blocking of CB1 receptor had no effect on 5-HT uptake as it did not change 5-HT uptake either by itself or in combination with the tested SCRA.

In summary, the results of this study did not explain the occurrence of serotonergic adverse events seen clinically in SCRA users. It was important to test the hypothesis that indole-containing cannabinoids may have a direct effect on SERT function. However, the results suggested that if SCRAs are causing a serotonergic effect this may be via another indirect mechanism. The study tested the parent compound JWH-018, but not metabolites, which are proven to retain activity on CB receptors (Brents et al., 2011). Therefore, we cannot exclude the possibility that these metabolites, which can still contain the indole core, may have an effect on SERT function or other serotonergic receptors that might contribute to a serotoninergic effect.

Finally, this experiment tested JWH-018 and CP55, 940, but, there are diverse preparations or products that have been shown to contain different SCRAs such as AB-FUBINACA, ADB-FUBINACA, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA, ADBICA, and 5F-ADBICA (Banister et al., 2015). These may exert effects directly on the serotonergic or other monoaminergic systems but so far remain uncharacterised. Further investigation into these continually evolving substances should be considered.

Chapter 4 Neurotoxic analysis of NPS

4.1 Introduction

The abuse of NPS, in particular herbal products that contained SCRAs, are an important issue with potential serious consequence for public health. These substances have been perceived as a “safe and legal” alternative to the widely recreationally abused herb “cannabis”, especially in the younger population (UNDOC, 2018). Most SCRAs have the ability to pass blood brain barrier due to their lipophilic properties, and they bind to CB1 receptor with different affinities. Most SCRAs have shown to have binding affinity and agonist activity to CB1 receptor many times higher than ∆9-THC (Banister et al., 2016, Banister et al., 2015, Sachdev et al., 2018). SCRAs are a diverse group of compounds with various chemical structures which bind to cannabinoid receptors with different affinities (Castaneto et al., 2014) resulting in different potencies, metabolism and toxicological profile. Although SCRAs have been shown to have psychotropic effects similar to cannabis (Gunderson et al., 2012), more toxic and serious adverse health effects have been reported with SCRA users. It has been estimated that SCRA users are 30 times more likely to seek hospital admission than cannabis users (Zaurova et al., 2016, van Amsterdam et al., 2015, Winstock et al., 2015).

Over the last 10 years, the number of SCRAs encountered has continually increased, with 179 being notified to the EU early warning system since 2008, (EMCDDA, 2018). The pharmacological effects of this rapidly expanding group of substances is not fully understood, despite there being studies on the structure-activity relationships, binding affinities and pharmacokinetic profile for some SCRAs. Information on the toxicological effects of SCRAs for both short and long-term exposure is still scarce and few studies have been published in the literature regarding the underlying molecular mechanisms of toxicity in human cells (Koller et al., 2014, Tomiyama and Funada, 2011, Ferk et al., 2016, Koller et al., 2013).

The herbal products which are marketed as NPS vary in type and composition of NPS, rendering NPS users unware what they have exactly taken as they consume branded products with different names (Brunt et al., 2017). Zuba et al. (2011) reported that there is a significant difference in the composition of some available branded products

regarding synthetic cannabinoids. He reported that some products contain one SCRA, while other products contain more than two SCRA (Zuba et al., 2011). These findings have been confirmed by other authors, such as Uchiyama et al. (2014), who stated that 60 different combination patterns were detected in 104 products, with 1-7 compounds per product being present. In addition, these compounds belong to different NPS categories for instance SCRA, phenylethylamines and cathinone derivatives (Uchiyama et al., 2014). Alongside this, drugs users' use NPS with other traditional drug of abuse for example MDMA, which instigate to co-administration of multiple drug of abuse that results in adverse effect (Heikman et al., 2016).

Therefore, the evaluation of the toxicological properties of SCRAs, in particular the neurotoxic effects of these widely used substances, is needed.

4.1.1 SCRAs

Although over 179 SCRAs have been identified not all are currently being taken for recreational use therefore for this study two currently in-use SCRAs (taken from the NPIS data), a SCRA analogue and one of the first SCRAs identified in herbal products were examined.

4.1.1.1 JWH-018

JWH-018 (1-pentyl-3-(1-naphthoyl) indole) (Figure 4.1) was chosen as it is one of the first identified SCRAs in herbal products (Auwarter et al., 2009). It is a synthetic cannabinoid receptor agonist from the aminoalkylindoles group. It was developed by the chemist John W Huffman as a potential ∆9-THC alternative for therapeutic uses with less addictive properties (Huffman et al., 1994). It is a potent agonist with high binding affinity for the CB1 (*Ki*= 9 nM) and CB2 receptors (*K*i= 3 nM), which is more potent than ∆9-THC for either receptor (*Ki*=40.4 nM at CB1 and 36.4nM at CB2 receptor) (Aung et al., 2000). Wintermeyer et al. (2010) reported that phase I metabolism of JWH-018 produced at least nine hydroxylated, N-dealkylated, dehydrated and carboxy metabolites (Wintermeyer et al., 2010). Further studying of 5 of the hydroxylated metabolites and the carboxy metabolite revealed that the 5 tested monohydroxylated metabolites, but not the carboxy product, had high activity and affinity at CB1 receptors when tested both *in vivo* and *in vitro* (Brents et al., 2011). Furthermore, these monohydroxylated metabolites also demonstrated nanomolar affinity and acted a potent agonists at CB2 receptors (Rajasekaran et al., 2013). In

contrast the main phase II metabolite, JWH-018-N-(5-hydroxypentyl) β-D-glucuronide (018-gluc) does not have activity on CB1 receptor and antagonises the parent drug activity on the same receptor (Seely et al., 2012a). The human blood concentration of JWH-018 has been shown to decrease rapidly over 3 hours after inhalation through smoking (Teske et al., 2010). JWH-018 was detected in 18 out of 45 post-mortem blood samples analysed in the range of 0.1-199 ng/ml (Shanks et al., 2012b). In a controlled inhalation study conducted by Toennes et al. (2017) concentrations of 2.9 to 9.9 ng/ml were measured in blood (Toennes et al., 2017). Even with low serum concertation, JWH-018 still causes neurocognitive impairment and the euphoric feelings of 'high' (Theunissen et al., 2018).

Animal studies have shown that JWH-018 produces antinociception, catalepsy, hypothermia, hypo-mobility and increased anxiety (Wiebelhaus et al., 2012, Macrì et al., 2013) and induces myoclonus, convulsions and hyperreflexia (Ossato et al., 2015). Moreover, Cha et al. (2015) suggested that JWH-018 and JWH-012 might be induce neurotoxicity through distortion of the nuclear membrane and nuclei in the NA core shell and inducing neuronal cell damages (Cha et al., 2015). In addition, JWH-018 reduced K+ evoked glutamate and GABA release from hippocampal slices and inhibited short and long term memory together with its halogenated analogues in mice (Barbieri et al., 2016). These neurological changes observed in SCRA exposure may reflect the direct or indirect effects of SCRA-induced cytotoxicity and neuronal dysfunction. However, the cytotoxic effect of SCRAs at a cellular level and the mechanisms by which they induce toxicity or molecular changes after acute and chronic exposure in human neuronal cells has not been described previously.

4.1.1.2 MDMB-CHMICA

MDMB-CHMICA (2-[[1-(cyclohexylmethyl) indole-3-carbonyl] 3, 3-dimethylbutanoate, (Figure 4.1) is an indole core SCRA which was detected in Europe and reported to the EMCCDA for the first time in September 2014 (EMCDDA, 2017b). It is a potent CB1 and CB2 receptor agonist with EC50 values of 10 and 71 nM respectively (Banister et al., 2016). MDMB-CHMICA users reported that MDMB-CHMICA is stronger than other SCRAs; this might be due its higher CB1 receptor potency (WHO and Meeting, 2017). MDMB-CHMICA undergoes extensive phase I metabolism producing 31 metabolites that have been identified in human urine samples (Franz et al., 2017), with monohydroxylated metabolites identified as proven biomarkers to detect consumption

(Gaunitz et al., 2018). The adverse effects of MDMB-CHMICA, such as agitation, vomiting, delirium and respiratory depression have been self-reported by users and taken from case reports of acute intoxication that required hospitalization, with some leading to fatalities (Vucinic et al., 2018, Hermanns-Clausen et al., 2018, Abouchedid et al., 2017, Hill et al., 2016, Adamowicz, 2016). However, limited data regarding the cytotoxicity and genotoxicity of MDMB-CHMICA was found in the literature.

4.1.1.3 5F-ADB

5F-ADB, also known as 5F-MDMB-PINACA (methyl-[2-1(fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate], (Figure 4.1) is an indazole core SCRA which was reported to EMCCDA for the first time in 2015 (EMCDDA, 2017a). It is a potent CB1 and CB2 receptor agonist with EC50 values of 0.59 nM and 7.5 nM respectively (Banister et al., 2016). Kusano et al. reported that 5F-ADB undergoes phase I metabolism to produce several metabolites (Kusano et al., 2018); however, there is limited literature available on the pharmacokinetics of 5F-ADB. This SCRA has been reported to be responsible for poisoning and fatalities in several cases/users (Kraemer et al., 2019, Hasegawa et al., 2015, Angerer et al., 2017, Barceló et al., 2017). Like MDMB-CHMICA, no studies were identified in the literature that have examined the cellular toxicity of 5F-ADB.

4.1.1.4 MMB-CHMICA

MMB-CHMICA, also known as AMB-CHMICA (methyl 2-[[1-(cyclohexylmethyl) indole-3-carbonyl]amino]-3-methyl-butanoate, Figure 4.1) is structurally related to MDMB-CHMICA, having an additional methyl group at the butanoate group. It has been shown to be metabolised by hydroxylation and O-demethylation (Mardal et al., 2018). It is a potent SCRA which binds to CB1 and CB2 receptors with EC50 values of 3.5 and 12 nM respectively (Banister et al., 2016).

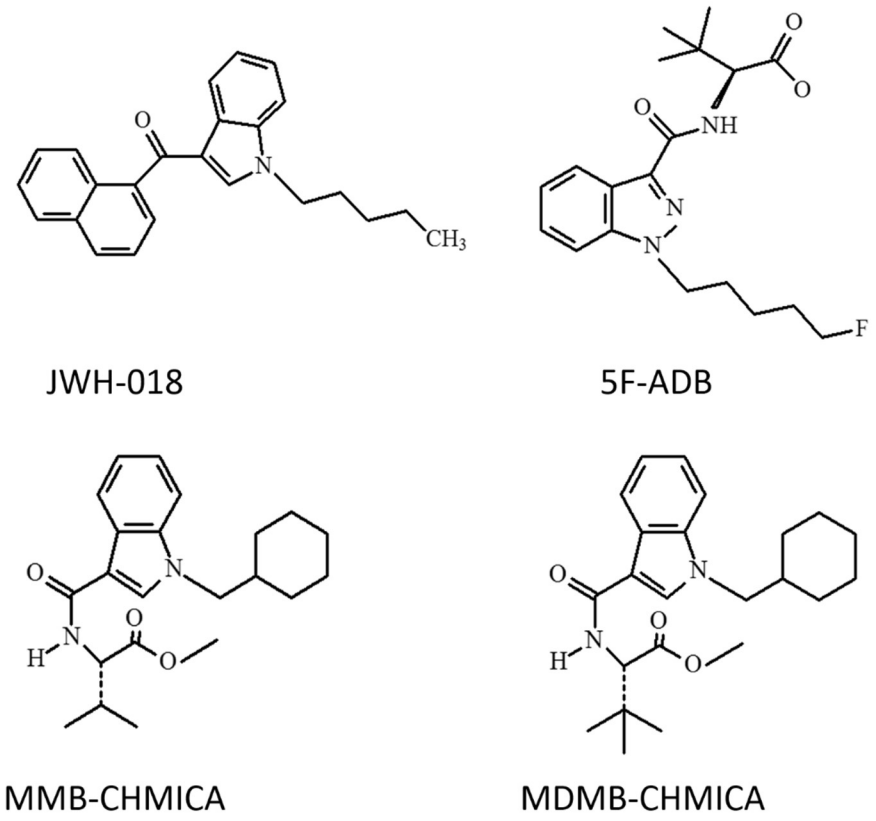


Figure 4.1 Chemical structures of the investigated synthetic cannabinoids.

4.1.2 Stimulants

4.1.2.1 Mephedrone

Mephedrone (4-methylmethcaininoe) is a cathinone stimulant (Figure 4.2). It was first synthesised in 1929, but appeared as recreational drug in the mid-2000s (EMCDDA, 2011). It acts by enhancing monoamine release and blocking monoamine reuptake through the inhibition of monoamine transporters in the brain (Papaseit et al., 2017). It is metabolised by phase I metabolism into six metabolites (Meyer et al., 2010). There is considerable evidence of severe acute intoxication and fatalities relating to mephedrone (Busardò et al., 2015, Schifano et al., 2011). The data in the literature as yet does not clearly describe the mechanism of action and toxicity of mephedrone (Pantano et al., 2017).

4.1.2.2 25I-NBOMe

25I-NBOMe (4-iodo-2,5-dimethoxy-N-(2-methoxybenzyl) phenethylamine) is a stimulant hallucinogen (Figure 4.2). It was synthetized in 2003 as radiolabelled 25I-

NBMOE to study 5-HT receptors, and was first reported to EMCDDA as a recreational drug in 2012 (EMCDDA, 2014). 25I-NBOMe is a potent 5-HT2A receptor agonist with *K*i = 0.44 nM (Braden et al., 2006). It undergoes extensive phase I and phase II metabolism (Leth-Petersen et al., 2016). Severe intoxication cases and fatalities have been reported with 25I-NBOMe (Hill et al., 2013, Walterscheid et al., 2014, Poklis et al., 2014). Like other NPS, limited data areavailable about the pharmacology and toxicology of 25I-NBOMe in humans.

4.1.3 Classical drugs of misuse

4.1.3.1 Delta-9-tetrahydrocannabinol (∆9-THC)

∆9-THC is the psychoactive ingredient of the plant *Cannabis sativa* and it is responsible for the psychoactive effects of cannabis (Gaoni and Mechoulam, 1964). It acts as a partial agonist on CB1 (Ki=40.4 nM) and at CB2 receptor (Ki 36.4nM) (Aung et al., 2000). In addition, it acts on other receptors such as the opioid and benzodiazepine receptors and interacts with protein and nucleic acid metabolism and the prostaglandin pathway (Burstein et al., 1982, Cichewicz, 2004, Grotenhermen, 2003). Unlike SCRA, ∆9-THC is well studied. Briefly, it is highly lipid soluble and rapidly absorbed after inhalation, reaching peak concentrations in blood after 5-7 minutes (Ohlsson et al., 1980). Once absorbed, ∆9-THC is metabolised by oxidation by CYP2C9 and CYP3A4 to produce hydroxylated active metabolite, which undergoes oxidation to add several carboxylic groups and produce inactive 11-nor-9-carboxy-Δ9-THC metabolite. Then this inactive metabolite undergoes conjugation with glucuronic acid, which is regarded as the main metabolite (Bornheim et al., 1992, Yamamoto et al., 1987). In mice ∆9-THC, produce antinociception, hypothermia, catalepsy, impairment of memory and learning and aggressive behaviour (Wiley and Martin, 2002).

4.1.3.2 MDMA

MDMA (3, 4-methylenedioxy-methamphetamine), commonly known as ‘ecstasy’ (Figure 4.2) is a stimulant and entactogenic recreational drug chemically related to amphetamine. It was first synthesised in 1912 and used for psychotherapy, then subsequently misused as recreational drug (Benzenhöfer and Passie, 2010). It acts by directly increasing the release of noradrenaline and dopamine as well as blocking the serotonin transporter (Kalant, 2001). Most MDMA users are poly-drug users, combining MDMA use with substances such as alcohol, cannabis, cocaine, heroin or

LSD (Wish et al., 2006). MDMA is metabolised in the liver mainly by CYP2D6 to several metabolites, some of which retain pharmacological activity (Kalant, 2001) MDMA can cause acute and chronic toxicity, and this is not limited to neurotoxicity (Meyer, 2013, Kalant, 2001).

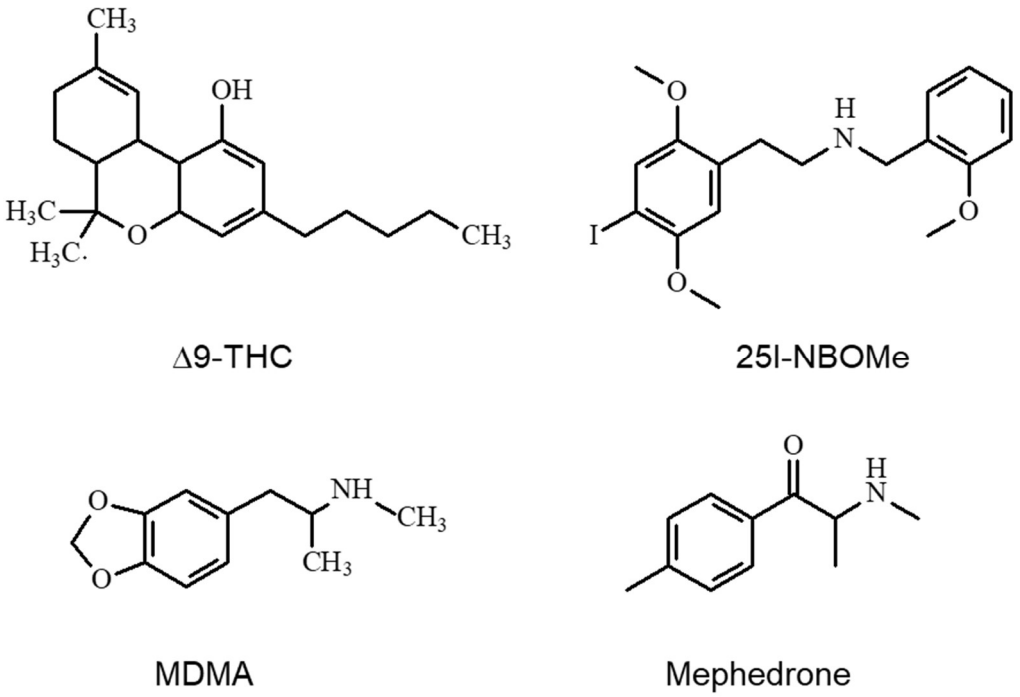


Figure 4.2 Chemical structure of novel stimulants (25I-NBOMe and mephedrone) and classical drug of abuse (∆9-THC and MDMA).

4.1.4 Use of cell cultures in toxicity testing

Toxicology offers many in vivo and in vitro test techniques. While animal experimentation has played a critical role in toxicology, a progression towards an alternative in vitro toxicology using cell-based assays is needed. Cell culture provides a method of studying animal or human cells in a controlled environment. This can include cell biology such as metabolic studies and the study of the effect of drugs and toxic substances on physiological pathways, for example protein synthesis and interactions between cells.

Cell culture has the advantage of allowing the control of physicochemical environment. Experiments are easily performed at relatively low cost and are appropriate for toxicity testing of a variety of chemicals, allowing multiple replicates (Philippeos et al., 2012). Advances in stem cell toxicology testing provided a propitious platform for predictive

studies. Stem cells have the advantage of the ability to extensively proliferate without transformation or differentiate into different cell types making them suitable for functional toxicity and developmental tests (Liu et al., 2017).

In this study, a human neuronal progenitor stem cell model (hNPSC) was used. This has been used successfully as a model to investigate the cytotoxicity of other compounds (Nisar et al., 2015). The use of human neuronal stem cell model provides a further advantage of avoiding the need to extrapolate results across species (Breier et al., 2010, Scott et al., 2013).

4.1.5 Cell viability assay

There are several cytotoxicity assays widely used in pharmacology and toxicology to evaluate the impact of drugs and chemicals in the cells, such as MTT, XTT, and the Alamar blue reduction assay. In this study the Alamar blue reduction assay was employed to evaluate cell viability (Page et al., 1993). This reagent is not toxic to the cell, and does not require cell death to obtain measurements, allowing the continuous monitoring of the same cell cultures over time. In addition this method is more sensitive than MTT (Hamid et al., 2004, O'Brien et al., 2000, Ahmed et al., 1994, Rampersad, 2012).

The Alamar blue reduction assay is a sensitive, simple, non-toxic test that can be applied to high throughput toxicity testing. It measures metabolic properties of the cell through monitoring the oxidation-reduction of the active ingredient Resazurin. Resazurin is water soluble, stable in aqueous media and cell membrane permeable (Rampersad, 2012). Resazurin is a blue low fluorescent dye that is reduced by nicotinamide adenine dinucleotide NADH into the pink highly fluorescent dye called resorufin (Figure 4.3) (Candeias et al., 1998).

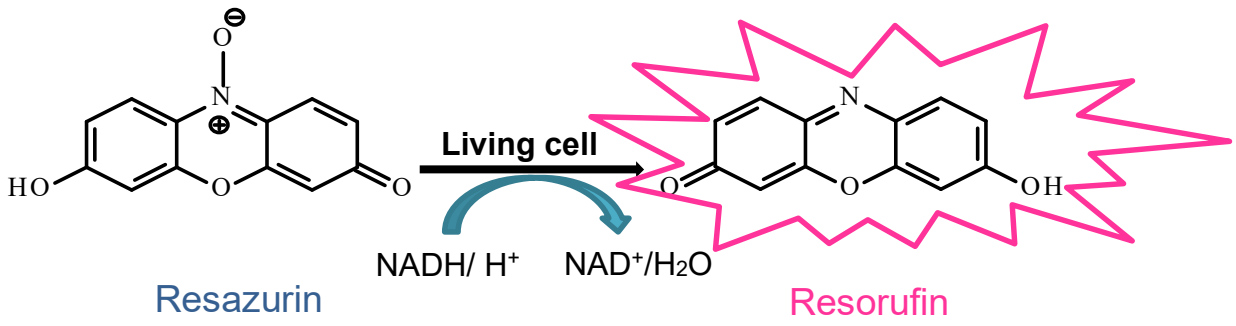


Figure 4.3 Alamar blue reduction assay principle.

4.1.6 RNA sequencing

The information for protein translation is stored in genes as DNA which is transcribed into RNA and ultimately translated into proteins. The transcription of a subset of genes into corresponding RNA molecules denotes the cell's identity and controls its biological activities. These RNA molecules are crucial for explaining and understanding the genome function in development and changes induced during disease (Kukurba and Montgomery, 2015). The assessment of protein expression is important to understand any modifications at cellular level in response to internal or external stimuli. However, it is difficult and time consuming to investigate all the changes that may occur in protein expression. Therefore, studying protein–encoding messenger RNA expression (transcriptome) might be informative as a proxy for envisaging protein expression (Guo et al., 2008). Understanding mRNA expression helps to associate the information on genome with its functional protein expression.

RNA sequencing has been employed as an unbiased genomic approach for identification and quantification studies of messenger RNA molecules in a biological samples and is a valuable tool for investigating cellular responses (Haque et al., 2017). RNA sequencing techniques facilitate the profiling of the whole set of the transcribed genes or all types of RNA (transcriptomes), in addition to quantifying the modifications in expression levels of each transcript in response to various conditions (Wang et al., 2009, Whitley et al., 2016) RNA sequencing was employed in this study to detect any changes in gene expression after treatment with SCRAs and to compare the effect of SCRA usage with ∆9-THC.

4.1.7 Pathway analysis

To understand the RNA sequencing data generated Ingenuity Pathway Analysis (IPA) software can be employed. IPA enables scientists to analyse data generated from microarrays, RNA-sequencing, proteomics and PCR. IPA's data analysis facilitates identification of the significance of data or target(s) of interest in relation to bigger biological systems. IPA enables scientists to investigate more information about proteins, genes and how different drugs and chemical induce these changes (Jiménez-Marín et al., 2009, Zeng et al., 2016). It helps to build 'interactive models of experimental systems'. Importantly, canonical pathways are a set of pre-built pathways curated from numerous sources including the literature. Canonical pathway analysis of

IPA explains how pathways are affected significantly, taking into account the number of molecules shared between a user identifiable set and a pathway.

4.1.8 Western blotting

Proteins are complex molecules that play an important role in regulation of cell function, they are responsible for most of the cell physiological function, including cell shape, structure, catalysing metabolic reaction, transport, etc. Therefore, any changes in cellular protein levels can have an impact on the health and function of cells and the brain itself.

To study potential changes in protein expression in the neuronal cells exposed to SCRA western blotting was performed. Western blotting is used to detect and recognise proteins of interest from a protein lysate/homogenate extracted from cells or tissue. It can also be used semi-quantitatively to determine the level of protein expression change in comparison to a control (health) sample/donor, (Mahmood and Yang, 2012). The principle of western blotting relies upon the separation of protein according to their molecular weight by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). All of the separated proteins are then transferred onto a nitrocellulose membrane producing and the protein of interest is then detected by immuno-reaction with a specific primary antibody targeted to the protein of interest. This primary antibody is then subsequently detected by a secondary labelled antibody. The protein of interest is detected as band at the correct molecular weight by either chemiluminescent or near infrared imaging. The band intensity is related to the level of protein present within a specific linear range (Ma and Shieh, 2006) and is compared between samples to determine a ratio for protein expression change in conjugation with using a house keeping gene (Figure 4.4).

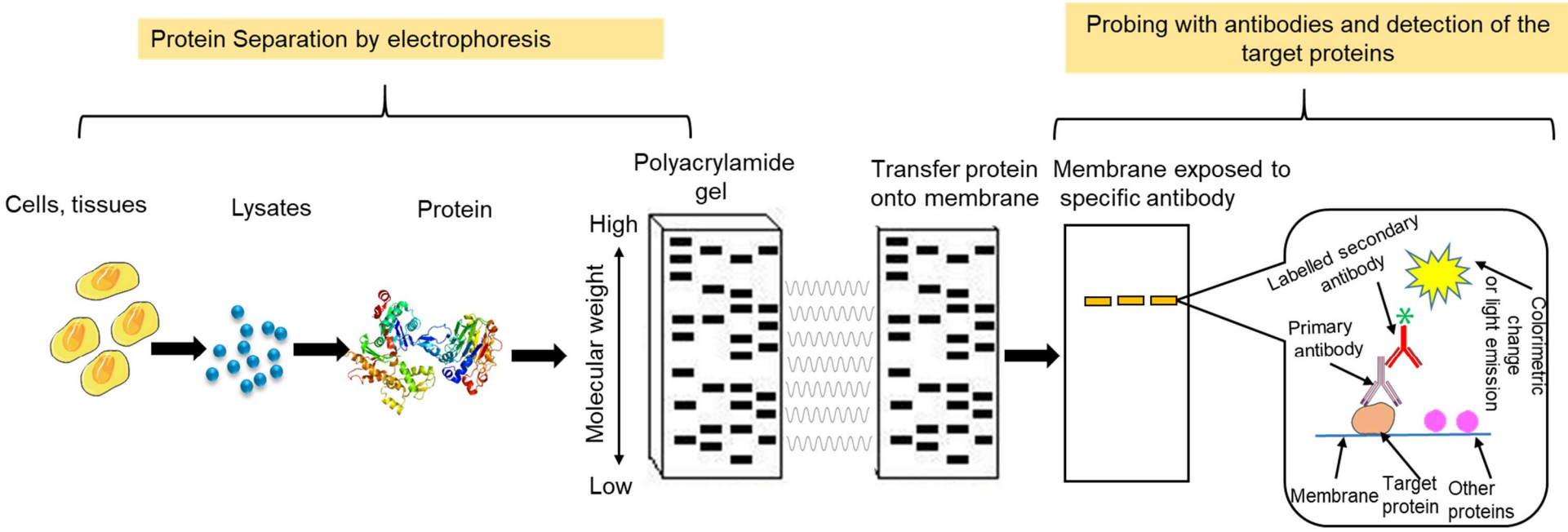


Figure 4.4 Principle of protein separation by western blotting.(Adapted from MBL life science® website).

4.2 Aims

The present study aimed to examine the acute and chronic neurotoxicity of selected SCRAs and comparing them to ∆9-THC, MDMA and selected synthetic stimulants using human neuronal stem cell models.

4.3 Materials and Methods

4.3.1 Cell culture

Human neuronal precursor stem cell (hNPSC) line N1997 forebrain (generated in house), were cultured in growth medium consisting of Advanced DMEM/F12 (Life Technologies) supplemented with 5µg/ml Amphotericin B (Gibco), 1% N-2 (Life Technologies), 2% B27 (Life Technologies), 1% Insulin-Transferrin-Selenium supplement (Life Technologies), 1% penicillin-streptomycin-L-glutamine (Sigma), 1% MEM non-essential amino acids (Gibco), 1.5% D-glucose (Sigma), 2% sodium bicarbonate (Sigma), 3.5 µg/ml ascorbic acid (Sigma), 5µg/ml heparin (Sigma), 10ng/ml leukaemia inhibitor factor (Santa Cruz), 20 ng/ml basic fibroblast growth factor (R&D Systems) and 20 ng/ml epidermal growth factor (R&D Systems). Cells were cultured in T75 flasks coated with 1% Geltrex™ (Life Technologies) in a humidified incubator at 37℃ with 5% CO_2. Growth medium was replenished at 2-3 day intervals by replacing 60% of the existing medium with fresh medium.

The cells were grown to 80-90% confluency before passaging, approximately every 5-7 days. For cell passaging, the medium was removed, and cells were incubated with 5ml (for T75 flask) of Trypsin/EDTA to detach cells for 3-5 minutes. The trypsin was diluted by adding growth medium to the cell suspension. Cells were collected in 15 ml tubes and centrifuged at 250g for 5 minutes. The supernatant was discarded, and cells were re-suspended in fresh growth medium. Then cells were counted using a haemocytometer and seeded into T75 cm^2 flasks (for maintenance) or multi-well plates (subsequent experiments).

To differentiate the hNPSC into mature neuronal cells, the hNPSC were seeded into plates coated with 1% Geltrex™ (Life Technologies) prepared in DMEM (Life Technologies) and grown for 3 days in growth medium. The growth medium was replaced with differentiating medium containing Advanced DMEM/F12 supplemented with 10% heat inactivated foetal bovine serum (FBS) (Sigma), 1% N2 (Life Technologies), 2% B27 (Life Technologies), 1% penicillin-streptomycin-L-glutamine

(Sigma), 5 µg/ml Amphotericin B (Gibco), 1% MEM non-essential amino acids (Gibco), 2% sodium bicarbonate (Sigma), 1.5% D-glucose (Sigma), 3.5 µg/ml ascorbic acid (Sigma), 1% Insulin-Transferrin-Selenium Supplement (Life Technologies) and 5µg/ml heparin (Sigma). Cells were differentiated for 14 days to allow maturation into neuronal cells and the differentiating medium was replenished every other day. The cells allowed to differentiated for 14 days because the alternative splicing of microtubule-associated genes such as EML4 (echinoderm microtubule associated protein like 4), MAP2 (microtubule-associated protein 2), KIF2A (microtubule-depolymerizing kinesin) appeared after 6-10 days of differentiation. These genes are the key indicator in stem cell differentiation (Madgwick et al., 2015).

4.3.2 Cell viability assays

Human neuronal progenitor stem cells (hNPSC) were seeded into 48 well plates, at a density of 50,000 cells/well, coated with 1% Geltrex in DMEM and cultured for 3 days with growth medium. Cells were either tested directly as immature progenitor stem cells or differentiated into mature neuronal cells by replacing the growth medium with differentiating medium. Cells were differentiated for 14 days to allow maturation into neuronal cells with the medium being replaced every other day prior to treatment.

The cytotoxicity of NPS was evaluated using the Alamar Blue reduction assay by repeatedly exposing the hNPSC and mature neuronal cells with JWH-018 (0-10 µM, Tocris), 5F-ADB (0-10 µM, Chiron AS Trondheim), MMB-CHMICA (0-10 µM, Chiron AS Trondheim), MDMB-CHMICA (0-10 µM, Chiron AS Trondheim), Mephedrone (0-10µM, Sigma) and 25I-NBOMe (0-10 µM, LGC Standards). The tested substances have a limited solubility in aqueous solutions (see logP values, Table 4.1), therefore they were solubilised by DMSO and then diluted in 1:1000 in cell culture media.

Chemical substance	LogP value
MDMB-CHMICA	5.31
MMB-CHMICA	4.96
5F-ADB	3.26
JWH-018	6.86
25I-NBOME	4.30
Mephedrone	1.86
MDMA	1.81
∆9-THC	7.68

Table 4.1 LogP values of the tested chemical compounds.

Cell viability was tested after 24 hours, 7 and 14 days exposure by the addition of Alamar Blue reagent. Resazurin sodium salt (Sigma) was dissolved in Dulbecco's Phosphate Buffered Saline (dPBS; Sigma Aldrich) at a concentration of 1 mg/ml. Then the stock solution was diluted to 100 µg/ml with either growth or differentiation media to give 10% Alamar blue reagent. The growth/differentiation media was removed and 350 µl of 10 % Alamar blue in the relevant media was transferred into each well of the 48 well plates. The plates were incubated for 1 hour (for mature cells) and 1.5-4 hours (for immature cells) before duplicates of 100 µl Alamar blue reagent from each well were transferred into a 96 well plate. Alamar Blue reduction was measured by fluorescence at an excitation wavelength of 530 nm, and the emission at 590 nm. Plates were returned to the incubator after the remaining Alamar Blue solution was removed from the wells of the 48 well plate and the cells exposed again to the same concentration of NPS in fresh media. The cells were continually exposed for 14 days and the Alamar Blue reduction assay repeated after 24 hours, 7 days and 14 days exposure. Control cultures were treated with an equivalent amount of vehicle (DMSO).

Table 4.2 showed the concentrations of chemical used and a typical plate layout.

	1	2	3	4	5	6	7	8
A	10 µM	10 µM	10 µM	10 µM	5 µM	5 µM	5 µM	5 µM
B	2 µM	2 µM	2 µM	2 µM	1 µM	1 µM	1 µM	1 µM
C	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.1 µM	0.1 µM	0.1 µM	0.1 µM
D	0.05 µM	0.05 µM	0.05 µM	0.05 µM	0.01µM	0.01 µM	0.01 µM	0.01 µM
E	0.005µM	0.005µM	0.005µM	0.005µM	DMSO	DMSO	DMSO	DMSO
F	Media	Media	Media	Media	H_2O_2	H_2O_2	H_2O_2	H_2O_2

Table 4.2 Layout of the 48 well and the treatment used.

To examine the effect of multiple NPS exposure and the effect of combinations of NPS with other drugs of abuse (poly drug exposure), Immature and mature cell were exposed to combinations of the SCRAs MDMB-CHMICA which is denoted as (A) and 5F-ADB which is denoted as (B) in addition to the conventional drug of abuse MDMA (ecstasy) which is denoted as (C) at concentrations of 1,3,10 and 20 nM each. The combination of exposure included MDMB-CHMICA & 5F-ADB (A+B), MDMB-CHMICA & MDMA (A+C), 5F-ADB & MDMA (B+C) in three concentrations (1, 3 and 10 nM) and MDMB-CHMICA & 5F-ADB & MDMA (A+B+C) in 1,3,10 and 20 nM.

Table 4.3 shows the concentrations used in a typical plate layout. The cells were exposed for 14 days and the cell viability assay analysed after 24 hrs, 7 days and 14 days.

	1	2	3	4	5	6	7	8
A	A+B (3nM)	A+C (10nM)	A+C (1nM)	B+C (3nM)	A+B+C (20nM)	A+B+C (3nM)	DMSO	H_2O_2
B	A+B (3nM)	A+C (10nM)	A+C (1nM)	B+C (3nM)	A+B+C (20nM)	A+B+C (3nM)	DMSO	H_2O_2
C	A+B (3nM)	A+C (10nM)	A+C (1nM)	B+C (3nM)	A+B+C (20nM)	A+B+C (3nM)	DMSO	H_2O_2
D	A+B (10nM)	A+B (1nM)	A+C (3nM)	B+C (10nM)	B+C (1nM)	A+B+C (10nM)	A+B+C (1nM)	Media
E	A+B (10nM)	A+B (1nM)	A+C (3nM)	B+C (10nM)	B+C (1nM)	A+B+C (10nM)	A+B+C (1nM)	Media
F	A+B (10nM)	A+B (1nM)	A+C (3nM)	B+C (10nM)	B+C (1nM)	A+B+C (10nM)	A+B+C (1nM)	Media

Table 4.3 Lay out of the 48 well and the combination treatment used. A = MDMB-CHMICA, B =5F-ADB and C =MDMA (ecstasy).

4.3.3 RNA sequencing to determine gene expression changes due to NPS exposure

hNPSC were seeded into six well plates coated with 1 % Geltrex in DMEM and grown for 3 days in growth medium at a density of 300,000 cells/well. The medium was replaced with differentiating medium and the cells were differentiated for 14 days to allow maturation into neuronal cells, with the medium being replaced every other day. The mature cells were exposed separately to 10 nM of MDMB-CHMICA, 5F-ADB, ∆9-THC and DMSO control (n=4 per treatment). The cells were exposed to this treatment for 14 days and the medium along with the corresponding drug being replenished every other day.

4.3.3.1 RNA extraction and purification

The culture medium was aspirated and 1 ml Trizol (Life Technologies) was added to each well of the 6-well plates. Cells were scraped and transferred to micro-centrifuge tubes, after which 50 µl of 4-Bromoanisole (Sigma-Aldrich) was added and the tubes were mixed for 15 seconds by vigorously shaking by hand and incubated at room

temperature for 2-3 minutes. The tubes were then centrifuged at 12,000 g for 15 minutes at 4°C. The upper aqueous layer that contains the RNA was transferred to a clean RNase-free tube. Equal volumes of 70% ethanol (prepared from 200 proof molecular biology grade ethanol, Sigma Aldrich) were added to obtain a final ethanol concentration of 35%. The tube was vortexed to mix well and then inverted to disperse any visible precipitate. RNA was further purified using the Pure Link® RNA Mini Kit, Invitrogen. The solution was transferred into spin cartridges (with collecting tubes) in 700 µl volumes and was centrifuged at 12,000 g for 15 seconds at room temperature. The flow-through was then discarded and the remaining solution filtered prior to the addition of 700 µl washing buffer I (Pure Link® RNA Mini Kit, Invitrogen) and centrifuged at 12000 x g for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was inserted into a new collection tube. 500 µl of washing buffer II was added and the tube centrifuged at 12,000 g for 15 seconds at room temperature. The flow through was discarded and the washing with buffer II was repeated. The tube then centrifuged at 12000 x g for 1 minute, then the spin cartridge was inserted into a recovery tube and 50µl of RNase free water was added to the centre of the spin cartridge. The tube incubated for 1 minute at room temperature and centrifuged for 2 minutes at 12000 x g at room temperature. The purified RNA was collected in the collection tube.

The initial RNA concentrations were determined by measuring absorbance at 260 nm using a Nano Drop spectrophotometer (2000 Thermo Scientific) using Nuclease-free water as a blank. Purity of the samples was measured as a ratio of A260/A280 (Absorbance at 260nm and 280nm) ratio between 1.8 and 2.0 generally suggesting a pure nucleic acid sample whereas lower ratios suggests protein contamination (Desjardins and Conklin, 2010).

4.3.3.2 RNA sequencing

The extracted RNA samples were sent to the Genomics Core Facility (Newcastle University, UK). All the samples were assessed for quality and quantity using an Agilent Tape station 4200 with RNA Screen Tapes and were sequenced using an Illumina NextSeq 500 platform. The sequencing parameters were 76bp single read and dual 8bp index reads. The samples were prepared using the TruSeq stranded mRNA library preparation kit (Illumina).

4.3.3.3 Bioinformatics information

Bioinformatics analysis to ascertain gene expression changes between exposed and non-exposed cells was conducted at Bioinformatics Support Unit (Newcastle University).

4.3.4 Determination of Proteomic changes that occur due to NPS exposure

hNPSC were seeded into six well plates coated with 1 % Geltrex in DMEM and grown for 3 days in growth medium at density 300,000 cells/well. After which the growth medium was replaced with differentiating medium. Cells were differentiated for 14 days to allow maturation into neuronal cells with the medium being replaced every other day.

The mature neuronal (differentiated) cells were exposed separately to 10 nM of MDMB-CHMICA, 5F-ADB, MMB-CHMICA, ∆9-THC and MDMA (ecstasy), in addition to a combination of NPS and ecstasy (3 nM 5F-ADB combined with 3 nM MDMB-CHMICA and 3nM MDMA). Control cultures were treated with the equivalent amount of vehicle (DMSO). The cells were exposed to this treatment for 14 days, the medium with the correspondent drug being replaced every other day. All treatments were carried out in quadruplicate and the whole experiment repeated three times.

4.3.4.1 Protein lysate preparation of cells

The culture medium was aspirated, and cells were washed twice with dPBS. 250 µl of native lysis buffer (1% Tris-buffered saline (TBS), 0.27 M Sucrose, 1 % Triton X-100, and protease inhibitor (Roche)) was added. The cells were then scrapped and left on ice for 30 mins. Finally, the cells were scraped again, and the cell lysate was collected and stored at -20°C.

4.3.4.2 Determination of protein concentration by the Bradford assay

Bovine serum albumin (BSA, sigma) was used to plot a standard curve of known protein concentration in the range of 0 to 1mg/ml prepared in diluted native lysing buffer. The samples were diluted 1:20 with MilliQ water prior analysis. 5 µl of each diluted sample and standard were pipetted onto 96-well plates in triplicate. 250 µl of Bradford reagent (Sigma) was added to each well and left to incubate for 15 mins at room temperature. Absorbance was measured at 595 nm using a plate reader (TECAN, Infinite 200 Pro reader, infinite M NANO+). Protein concentration was calculated using the standard curve produced from the absorbance of the BSA

concentration samples and the linear regression equation was used to quantify the protein of the unknown samples.

4.3.5 Western blotting

Protein samples were prepared at 1 µg/µl with 4X LDS NuPAGE LDS sample buffer (Invitrogen, UK) and 10X NuPAGE sample reducing agent (Invitrogen, UK). Samples were sonicated for 8-10 seconds, and the proteins denatured by heating for 10 minutes at 70℃.

10 µg (10µl) of protein was loaded into each well of NuPAGE 4-12% Bis-Tris Gel (Invitrogen) and 5µl See Blue molecular weight marker (Invitrogen) was added to the gel. The gel was electrophoresed in NUPAGE MOPS SDS running buffer containing NuPage Antioxidant (Invitrogen) at 120 V for 20 minutes and then at 160 V for one hour. The proteins were then electrotransferred onto nitrocellulose membrane using iBlot Gel transfer stacks (Invitrogen) at 20 V for 8 minutes in an iBlot device (Invitrogen). Proteins on the membrane were then stained with Ponceau S solution (Sigma) to check if the transfer was successful. Then the membrane was appropriately cut and labelled before they were washed for 10 minutes three times with Tris buffered saline with 0.2% Tween 20 (TBS-T).

The membranes were blocked with odyssey blocking buffer (TBS) (Li-COR) for 1 hour at room temperature to block non-specific antibody binding. Membranes were then incubated with specific primary antibodies (Table 4.4) diluted in odyssey blocking buffer containing 0.2% Tween 20 overnight at 4˚C. The following day excess antibody was removed by washing the membranes 3 times with TBS-T for 5 minutes each. Membranes were then incubated with the respective secondary antibody (Table 4.4) in blocking buffer for 60 minutes at room temperature followed by several washes with TBS-T (3 times 5 minutes each). Membranes were incubated with GAPDH antibody conjugated to Alexa Flour® 680 at room temperature for 1 hour. The membranes were then washed with TBS-T twice for 5 minutes each, followed by 3 washes with TBS. All incubations and washings were done with continuous shaking. The membranes were imaged with an Odyssey near- infrared imaging system (LI-COR odyssey FC).

GAPDH was used to normalise the results and the ratio of mean protein of interest/GAPDH were calculated for all samples. The primary and secondary antibodies used in the study are listed in Table 4.4.

Antibodies	Species	Dilutions	Source	Catalogue number
AHR	Rabbit Polyclonal	1:1000	Gene Tex	GTX 129013
BIP (HSPA5)	Rabbit Polyclonal	1:500	Abnova	PAB2462
Beta III -tubullin	Mouse Monoclonal	1:500,000	R&D Biotechnology	MAB-1195
CB1	Rabbit Polyclonal	1:1000	Gene Tex	GTX 100219
COX Va	Mouse Monoclonal	1:500	Mitoscience	MS409
CYP2E1	Rabbit polyclonal	1:500	Abcam	ab 28146
CYP3A4	Rabbit polyclonal	1:500	Cypex	PAP011
CYPOR (G-5)	Mouse Monoclonal	1:200	Santa Cruz	sc-25263
Cytochrome C (A8)	Mouse Monoclonal	1:1000	Santa Cruz	sc-13156
DARPP-32	Rabbit Polyclonal	1:1000	GeneTex	GTX61337
GFAP (F-2)	Mouse Monoclonal	1:1000	Santa Cruz	sc-166481
GSTP1 (FI-210)	Rabbit Polyclonal	1:2000	Santa Cruz	sc-134469
MTCO1	Mouse Monoclonal	1:250	Abcam	ab14705
NDUFA9	Mouse Monoclonal	1:500	Mitoscience	MS111
NDUFS1 (E-20)	Goat Polyclonal	1:500	Santa Cruz	sc-50132
PDI (C-2)	Mouse Monoclonal	1:1000	Santa Cruz	Sc-74551
Phospho Erk 1/2	Rabbit Polyclonal	1:1000	Cell Signalling	9101 S
SR-2A (A-4)	Mouse Monoclonal	1:1000	Santa Cruz	sc-166775
UGT1A1	Rabbit polyclonal	1:1000	Abcam	ab194697
UGT1A6	Rabbit polyclonal	1:1000	Abcam	ab97646
VDAC1	Mouse Monoclonal	1:500	Abcam	ab14734
GAPDH Antibody (G-9) Alexa Fluor® 680	Mouse Monoclonal	1:500	Santa Cruz	sc-365062 AF680
IRDye® anti-mouse secondary antibody	Goat Polyclonal	1:15000	LI-COR	926-32210
IRDye® anti-rabbit secondary antibody	Goat Polyclonal	1:15000	LI-COR	926-32211
IRDye® anti-goat secondary antibody	Donkey Polyclonal	1:15000	LI-COR	926-32214

Table 4.4 Specifications and source of primary and secondary antibodies.

4.3.6 Preliminary NPS effect on metabolism in the brain analysis

The RNA sequencing data showed changes in some metabolizing enzymes; therefore, the following method was done to test the effect of SCRA on drug metabolism. Human neuronal progenitor stem cells (hNPSC) were seeded into six well plates coated with 1 % Geltrex in DMEM and grown for 3 days in growth medium at density 300,000 cells/well. The medium was replaced with differentiating medium and the cells were differentiated for 14 days to allow maturation into neuronal cells, with the medium being replaced every other day. The mature cells were exposed separately to 10 nM of MDMB-CHMICA, 5F-ADB, ∆9-THC and DMSO control (n=6 per treatment). The cells were exposed to this treatment for 14 days and the medium along with the corresponding drug being replenished every other day.

4.3.6.1 Paracetamol metabolism exposure

The culture media was aspirated from all 6 wells of each treatment group, to 3 wells from each group 1 µg/ml of paracetamol diluted in differentiation media was added for 48 hours. The remaining 3 wells per group had the differentiation medium replaced (without NPS treatment) for 48 hours to allow the cells to recover from SCRA exposure. After 48 hours the paracetamol treated cells were lysed and collected together with the corresponding media and kept at -20 C°. The remaining 3 wells, which were allowed to recover from NPS treatment, were then treated with 1 µg/ml of paracetamol in differentiation media for 48hrs, after which the cells were lysed and collected along with the media.

4.3.6.2 Cell and media collection/preparation

The culture medium was aspirated and transferred into 15 ml tube and 150 µl transferred to vials for the mass spectrometer. 250 µl of sterile culture water was added to the wells of the plate, then then cells were scrapped and the cell lysate was collected. The cell lysate was sonicated for 8 seconds and centrifuged for 15 minutes at 20,000 rpm. 150 µl of the supernatant was transferred to mass spectrometer vials for analysis.

4.3.6.3 Liquid chromatography–mass spectrometry (LC-MS)

Cell lysate and the corresponding medium were sent to the Northern Institute for Cancer Research Chromatography lab (NICR), Newcastle University for analysis.

4.3.7 Statistical analysis

The statistical evaluation of the results of experiments was performed with the SPSS software system (V24; IBM SPSS Statistics USA). Results were reported as means ± standard deviations (SD).

Normality of the data distribution was assessed by the Shapiro-Wilko test. The results of the viability assays were analysed by one-way ANOVA followed by the Dunnett's multiple comparison test (when data followed normal distribution) or by the Kruskal-Wallis test. All other analyses were performed with the Student's-test; p-values ≤ 0.05 were considered as statistically significant.

4.4 Results

4.4.1 Cytotoxicity assay of New psychoactive substances

The cytotoxicity of new psychoactive substances on human neural precursor stem cells was evaluated using the Alamar blue assay.

4.4.1.1 Synthetic cannabinoids

To determine the toxicity of SCRAs, hNPSC cells were treated with nine different concentrations of SCRAs ranging from 0.05 to 10μM then assessed for toxicity after 24 hours, 7 and 14 days of exposure. The results showed a statistically significant reduction in cell viability in immature stem cells over a concentration range 5-10 μM as shown in Figure 4.5, with each drug exhibiting different toxicities.

MDMB-CHMICA significantly reduced cell viability by 14% at Day 7 (10 μM, p=0.017) and up to 20% after 14 days of exposure (10 μM, p=0.005, Figure 4.5 A). MMB-CHMICA, the structural analogue of MDMB-CHMICA, was shown to be toxic to cells after 7 days, inducing 13% cell death (5 μM, p=0.024). Further cell death was observed with both 5 μM (23%, p=0.046) and 10 μM (17%, p=0.004) after 14 days of exposure (Figure 4.5 B). 5F-ADB significantly reduced cell viability by 22% at after 14 days exposure (10μM, p=0.038, Figure 4.5 C).In contrast, the data from the Alamar Blue reduction assay on mature cells treated with synthetic cannabinoids (JWH-018, 5F-ADB, MMB-CHMICA and MDMB-CHMICA) showed no significant effect (p>0.05) with less than 10% cell death shown after 2 weeks exposure for all the SCRAs analysed (Figure 4.6). JWH-018 was the one of the first SCRA to be abused in the spice products and was included as a comparator to currently abused SCRA.

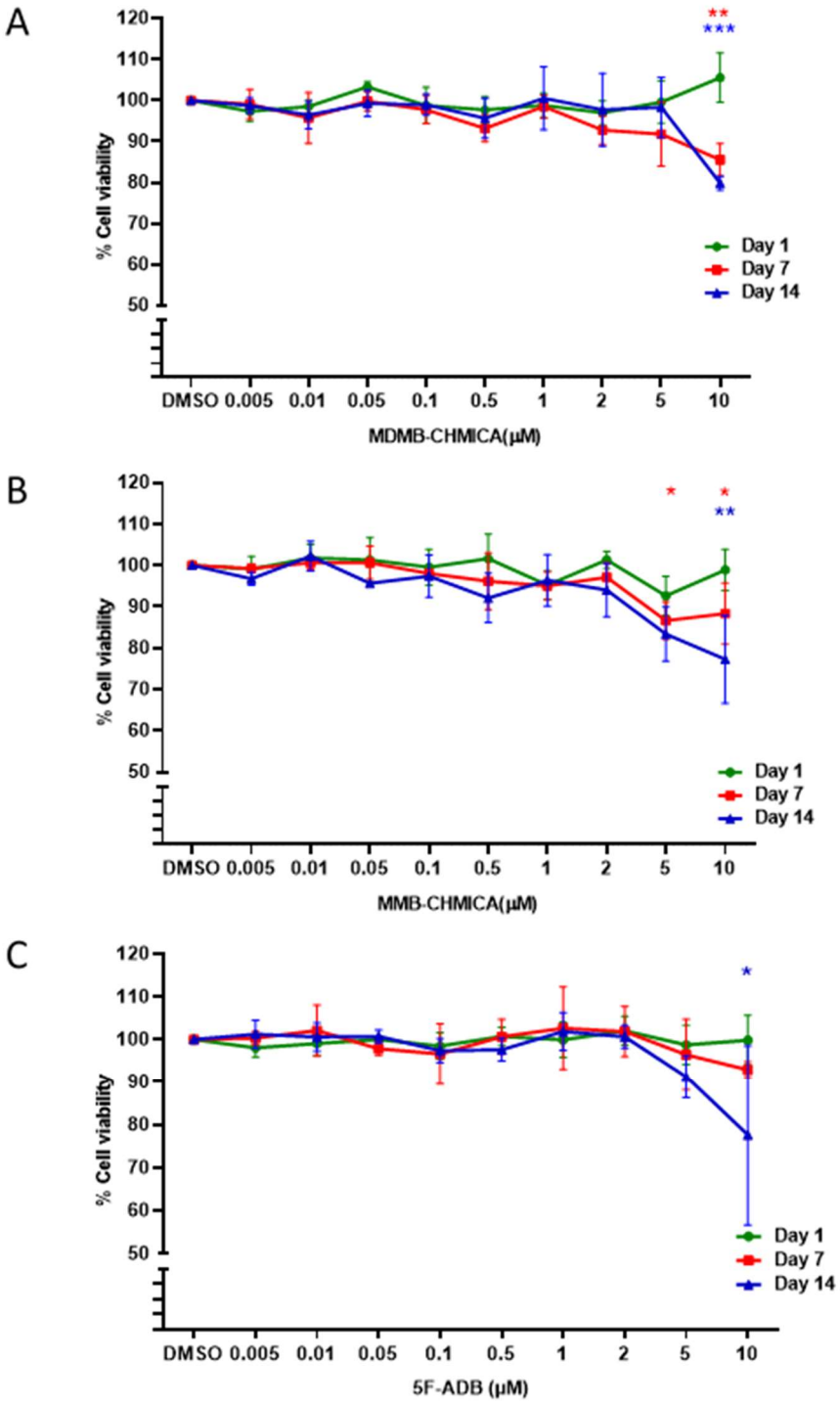


Figure 4.5 The effect of SCRAs on cell viability on immature stem cells. The results show percentage reduction in cell viability in immature forebrain cultures treated with different SCRAs. **(A)** MDMB-CHMICA, **(B)** MMB-CHMICA and **(C)** 5F-ADB (0.005 to 10 µM), using 0.1% DMSO as a control. Cytotoxicity was measured using the Alamar blue reduction assay. Data are presented as Mean ±SD from quadruplicate samples (assay repeated n=3). Statistical significance was evaluated with ANOVA at day 1, 7 and 14 time points. * represents $p < 0.05$, ** represent $p < 0.01$ and ***$p < 0.001$ when compared to DMSO treated cells.

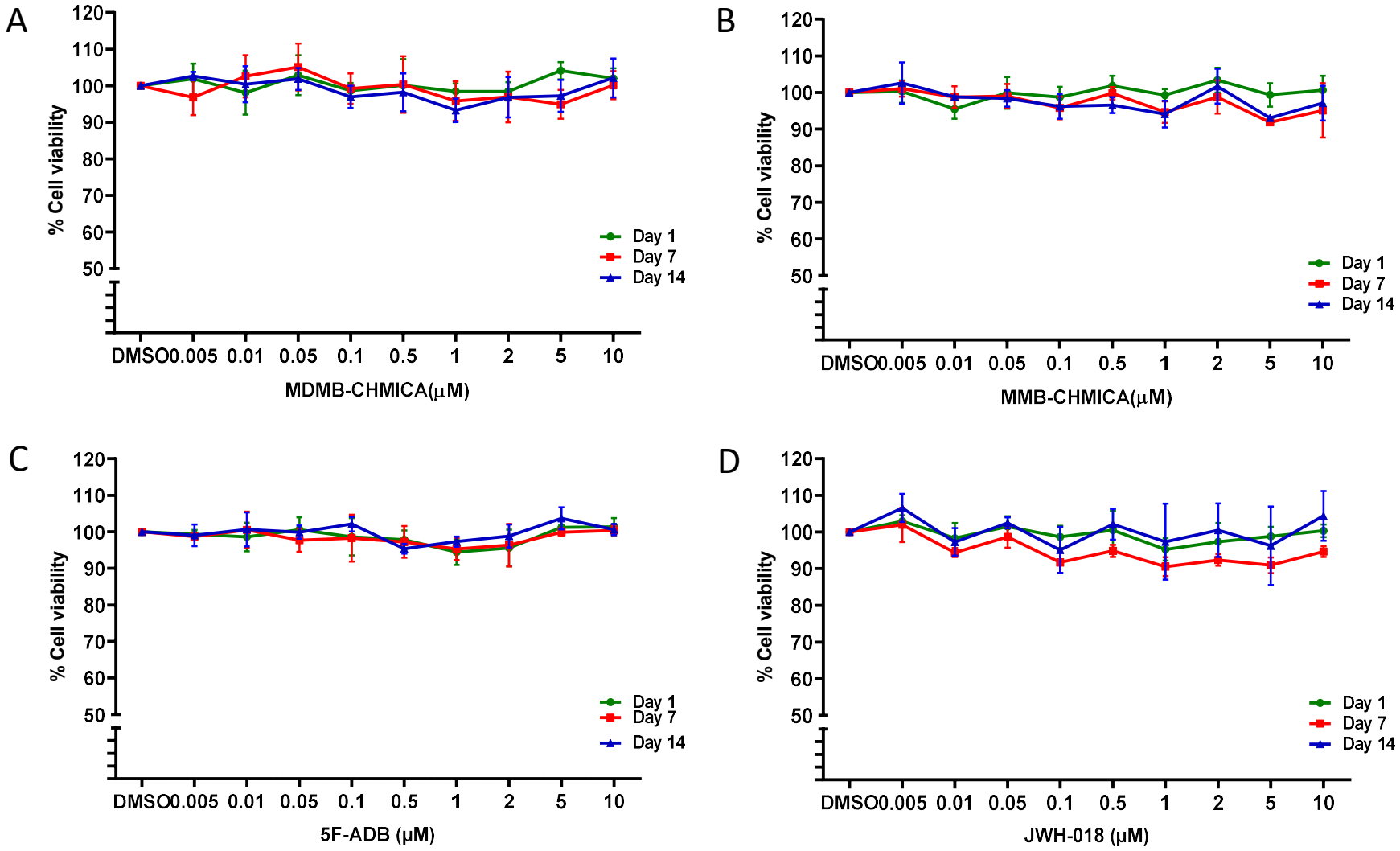


Figure 4.6 The effect of SCRAs on cell viability on mature stem cells. The results show percentage reduction in cell viability in mature forebrain cultures treated with different SCRAs. **(A)** MDMB-CHMICA, **(B)** MMB-CHMICA, **(C)** 5F-ADB and (**D**) JWH-018 (0.005 to 10 μM), using 0.1 % DMSO as a control. Cytotoxicity was measured using the Alamar blue reduction assay. Data are presented as Mean ±SD from quadruplicate samples (assay repeated n=3). Statistical significance was evaluated with ANOVA at day 1, 7 and 14 time points.

4.4.1.2 Combined exposure (poly drug use)

To explore the potential interaction of two synthetic cannabinoids, with one of the commonly co-administered conventional drugs of abuse, MDMA. Mature hNPSC were exposed to a concentration 1000-fold lower than the highest concentration tested in viability assays. The cytotoxicity of MDMA was tested first at the same concentrations range of the synthetic cannabinoids (0.005 to 10 μM) on both mature and immature cells. The results showed that MDMA reduced cell viability slightly ($p<0.05$) at a concentration range 1 to 5 μM on mature cells after 7 days of treatment as shown in Figure 4.7 A, but interestingly the cells recovered viability at day 14. However, MDMA was not toxic to immature cells at the same concentration range (Figure 4.7 B).

To determine the toxicity of the combined exposure of MDMB, 5F-ADB and MDMA the three drugs were analysed in combinations containing 1,3,10 and 20 nM of each. They were also examined in pairs as shown in Figure 4.8. The Alamar blue cell viability assay showed that the combination at these tested concentrations were not toxic to mature stem cells.

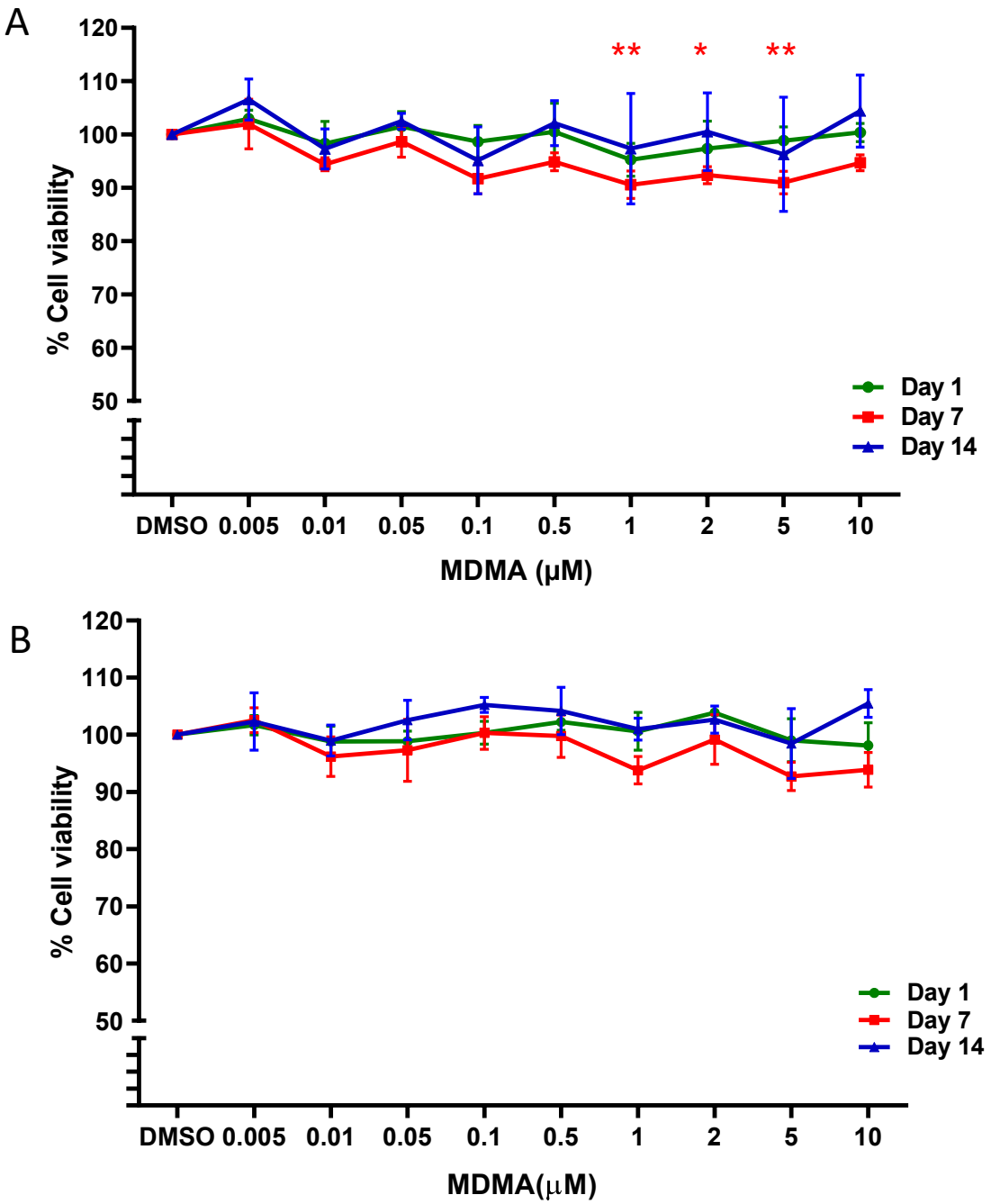


Figure 4.7 The effect of MDMA on cell viability on mature and immature stem cells. (A) The results show percentage reduction in cell viability in mature forebrain cultures treated with MDMA (0.005 to 10 µM), using 0.1% DMSO as a control. **(B)** The percentage of cell viability in immature forebrain cultures treated with MDMA (0.005 to 10 µM), using 0.1% DMSO as a control. Cytotoxicity was measured using the Alamar blue reduction assay. Data are presented as Mean ± SD from quadruplicate samples (assay repeated n=3). Statistical significance was evaluated with ANOVA at day 1, 7 and 14 time points. * represents $p < 0.05$ and ** represent $p<0.01$ when compared to DMSO treated cells.

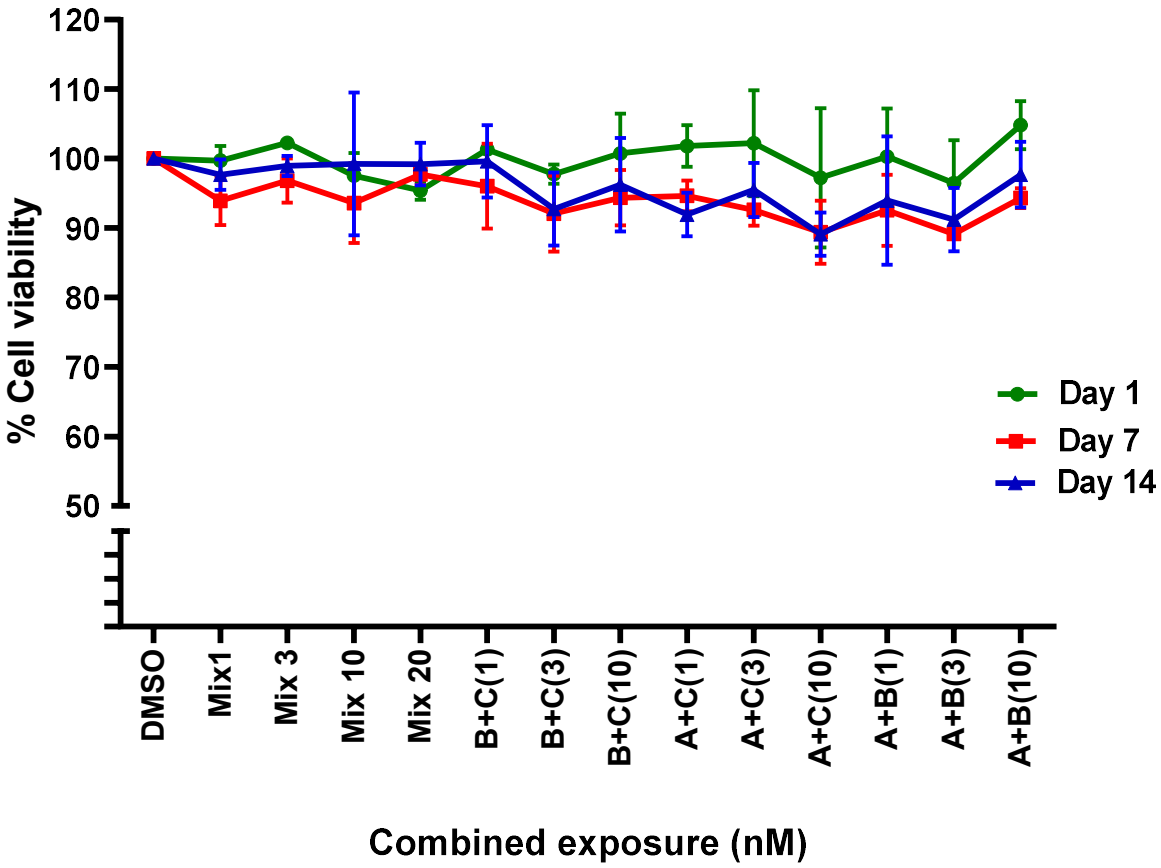


Figure 4.8 The effect of combined on cell viability on mature stem cells. The results show percentage reduction of cell viability in mature forebrain cultures treated with combination of **A**= MDMB-CHIMCA, **B**=5F-ADB and **C**=MDMA. The numbers represent concentration in nanomolar for each chemical substance. Cytotoxicity was then measured using the Alamar blue assay. Data are presented as Mean ± SD from triplicate samples (assay repeated n=3) for combined exposure. Statistical significance was evaluated with ANOVA at day 1, 7 and 14 time points.

4.4.1.3 Mephedrone and 25I-NBOMe

To compare the cytotoxic effect of SCRA with other types of NPS, two stimulants from different groups were chosen. Mephedrone (4-methyl methcathinone), a stimulant from the Cathinone family and 25I-NBOMe, a substituted phenethylamine hallucinogen.

The treatment of mature hNPSC cultured cells with mephedrone induced toxicity in a dose dependant manner after 24-hours exposure (Figure 4.9A), with a 5.6% reduction in cell viability at 2 µM (p= 0.045), increasing to 6.1% reduction in cell viability at 5 µM (p=0.025) and 7.4% reduction in cell viability at 10 µM (p= 0.006). After 7 days

exposure mephedrone reduced cell viability by 10.8% at 5 μM and 10.2% at 10 μM ($p=0.011$ and 0.017 respectively). While after 2 weeks exposure mephedrone reduced cell viability significantly at 5 μM only ($p=0.004$). Mephedrone induced acute toxicity after 24 hours of exposure, the cells seems to recover as after 14 days of exposure to mepherdrone the percentage of cells death decreased to 4.7% at 10 μM and 8.9% at 5 μM. However, the cytotoxic activity of mephedrone was not observed on immature cells after 2 weeks exposure at the same concentration range (0.005 to 10μM) (Figure 4.9 B).

25I-NBOMe did not induce significant cell death on mature cells (Figure 4.10 A), but it did induce an early death (24%) to immature cells after 24 hours of exposure at 10 μM only, and the cell viability was reduced by 85% after 7 days of exposure at 10 μM, progressing to 93% after 2 weeks of exposure ($p<0.0001$, Figure 4.10 B).

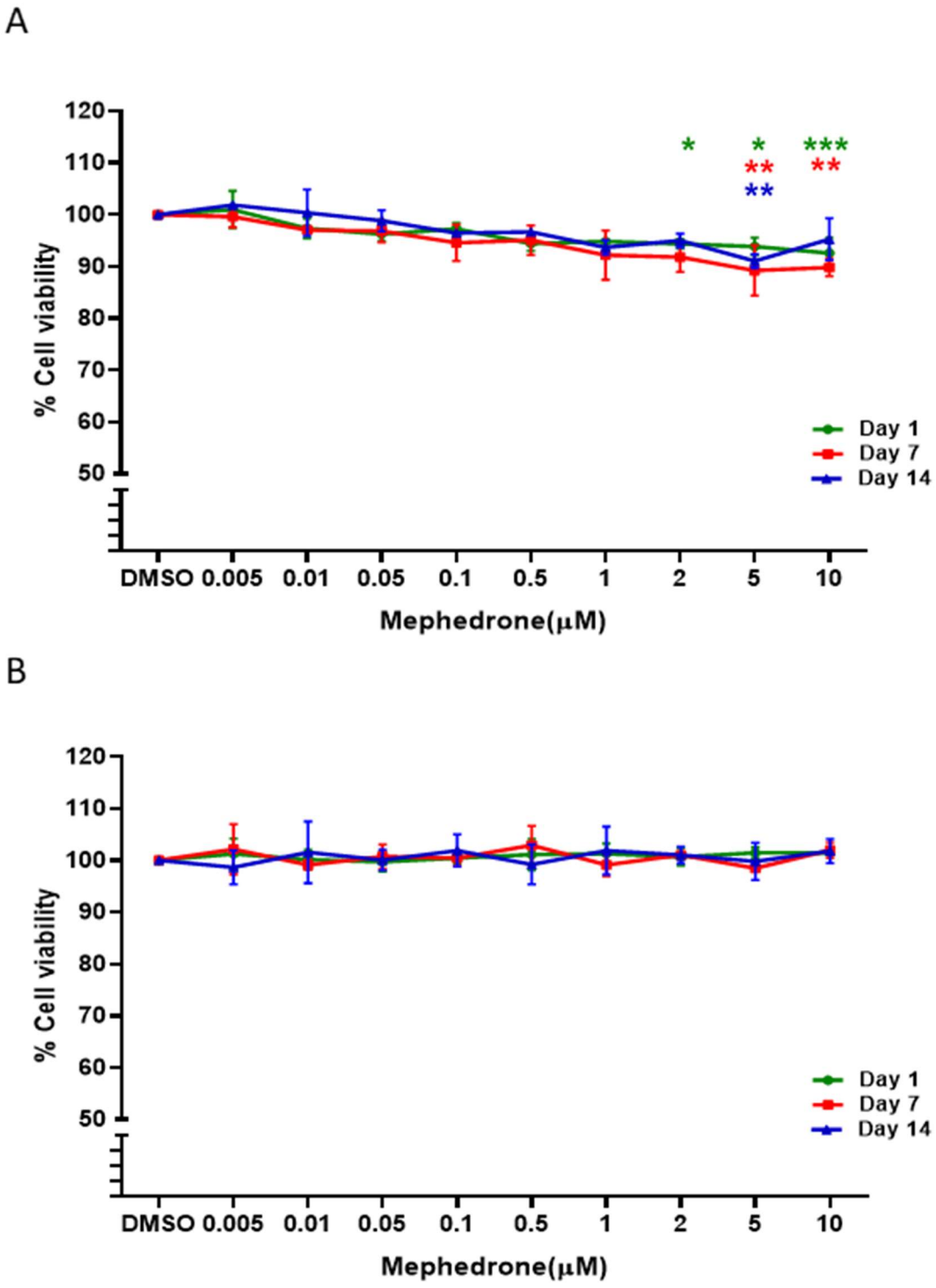


Figure 4.9 The effect of mephedrone on cell viability on mature and immature stem cells. (A) The results show percentage reduction in cell viability in mature forebrain cultures treated with mephedrone (0.005 to 10 μM), using 0.1% DMSO as a control. (B) The percentage of cell viability in immature forebrain cultures treated with mephedrone (0.005 to 10 μM) and 0.1% DMSO as a control. Data are presented as Mean ± SD from quadruplicate samples (assay repeated n=3). Statistical significance was evaluated with ANOVA at day 1, 7 and 14 time points. * represents $p < 0.05$, ** represent $p < 0.01$ when compared to DMSO treated cells.

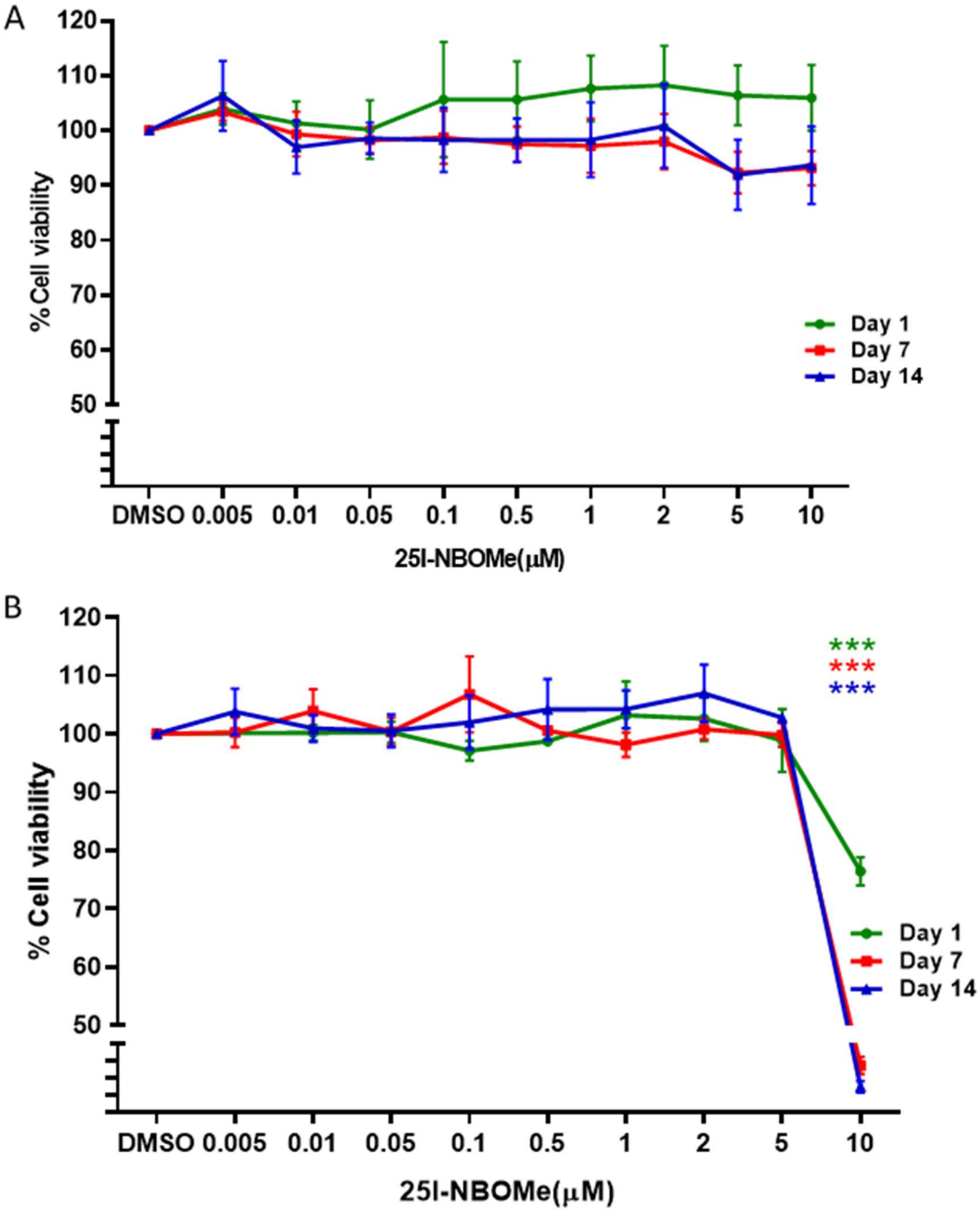


Figure 4.10 The effect of 25I-NBOMe on cell viability on mature and immature stem cells. (A) The results show percentage reduction in cell viability in mature forebrain cultures treated with 25I-NBOME (0.005 to 10 μM), using 0.1% DMSO as a control. **(B)** The percentage of cell viability in immature forebrain cultures treated with 25I-NBOMe (0.005 to 10 μM) and 0.1% DMSO as a control. Data are presented as Mean ± SD from quadruplicate samples (assay repeated n=3). Statistical significance was evaluated with ANOVA at day 1, 7 and 14 time points. * represents $p < 0.05$, ** represent $p < 0.01$ when compared to DMSO treated cells and ***$p < 0.001$ when compared to DMSO treated cells.

The results from the cytotoxicity assays for NPS have shown that different drugs have different patterns of cytotoxic effects. The SCRAs tested were toxic to immature cells but not to mature cells. In contrast to mephedrone which was toxic to mature cells but not immature cells. Whereas the novel hallucinogen 25I-NBOMe showed greatest toxicity to immature cells by killing more than 90% of the cells.

4.4.2 Gene expression changes due to NPS exposure

To gain an insight into SCRA usage at a molecular level on the human brain, MDMB-CHMICA and 5F-ADB were further investigated along with it ∆9-THC the psychoactive ingredient of the plant cannabis. These changes were assessed by changes in gene expression, protein expression and metabolic changes.

The potential gene expression changes due to NPS exposure were examined using RNA sequencing. The first step in the analysis of the gene expression data areto examine the sample distance and conduct a principal component analysis (PCA) to emphasise variations. The sample distance analysis and PCA showed that the MDMB -CHMICA, ∆9-THC and control (DMSO) samples were clustered together well in groups, suggesting that the responses to exposure between the quadruplicates were consistent (Figure 4.11). However, the 5F-ADB samples showed variance between samples which might indicate the 10 nM used was not enough to exhibit a full response or receptor desensitization.

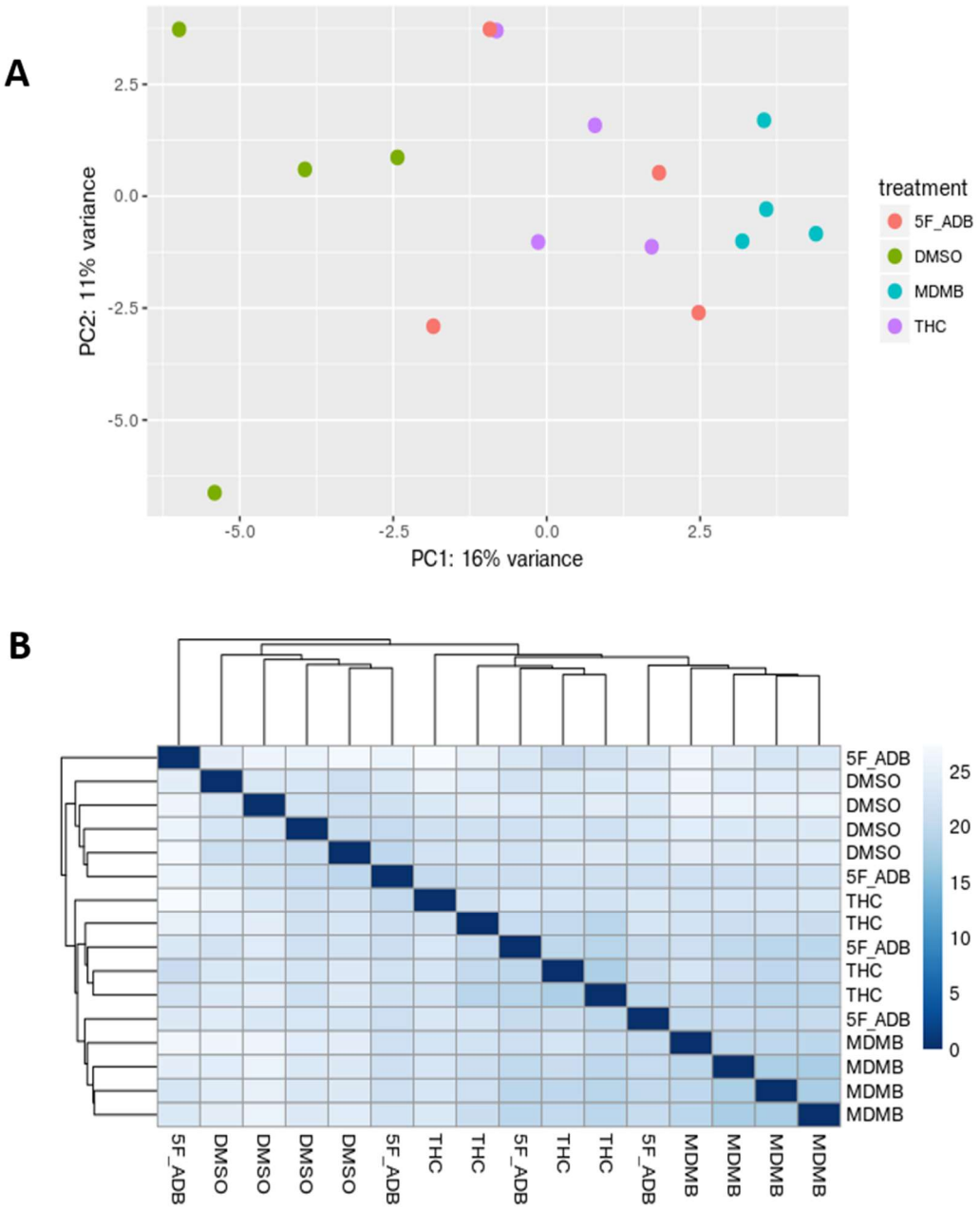


Figure 4.11 PCA and samples distance between samples. (A) The PCA plot coloured by treatment (DMSO, 5F-ADB, ∆9-THC and MDMB). (B) The heatmap shows distances between samples.

4.4.2.2 Differential Gene Expression

Differential gene expression analysis was carried out with the R package DESeq2. DESeq2 provides methods to test for differential expression by use of negative binomial generalized linear models (Lovell et al., 2019).

Genes with the absolute log 2-Fold Change higher than 1 (meaning the linear-scale fold change is greater than 2), with Benjamini-Hochberg adjusted p-values less than 0.05 have been classified as significant genes. A mean normalised count threshold of 10 has also been applied to exclude genes with very small counts across samples comparison.

To explore the effect of SCRA exposure on hNPSC derived neurones, a comparison between MDMB-CHMICA, 5F-ADB and ∆9-THC and the control cells treated with DMSO was conducted. The gene expression analysis revealed that 154 genes were significantly changed in MDMB-CHMICA treated cells, with 98 genes significantly down regulated, and 56 significantly upregulated. In 5F-ADB treated cells, 20 genes were significantly changed including 9 downregulated and 11 upregulated genes. For ∆9-THC, 50 genes were significantly changed with 22 genes were downregulated and 28 genes upregulated. These data are shown in volcano plots in Figure 4.12.

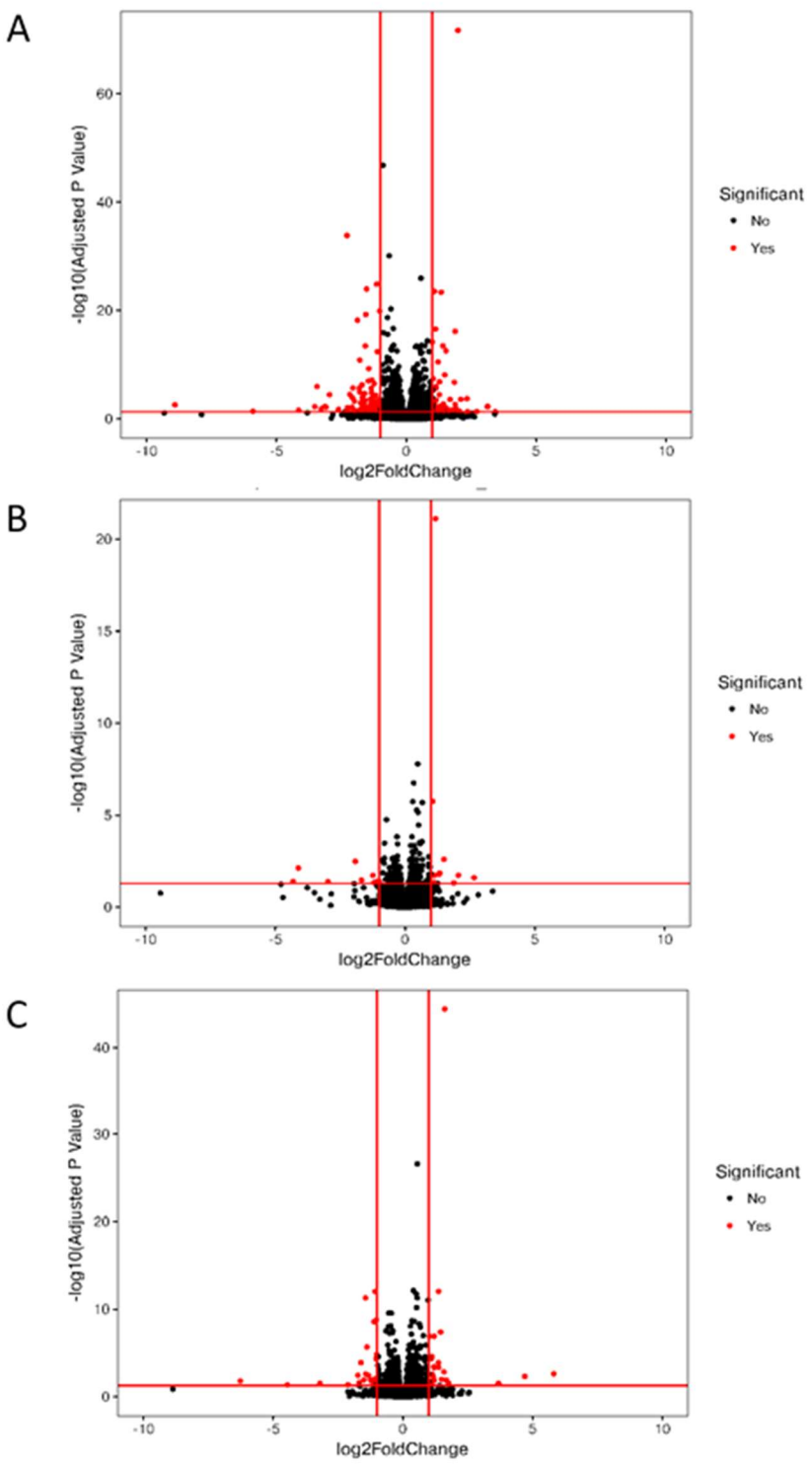


Figure 4.12 The volcano plot of the differently expressed genes. (**A)** Represents the differential expression MDMB-CHMICA treated cells versus control, **(B)** represents the of 5F-ADB treated cells differential expression of versus control and **(C)** represent differential expression of ∆9-THC treated cells versus control. Black dots indicate non-statistically significant gene changes and red dots indicated the statistically significantly differentially expressed genes.

The 20 most statistically significant genes are shown in Table 4.5 (which shows the significant genes in MDMB-CHMICA treated cells), Table 4.6 (which shows the significant genes in 5F-ADB treated cells and Table 4.7 (which shows the significant genes in ∆9-THC treated cells) arranged according to the adjusted p adjusted value (padj). The full results are shown in appendix (I).

Ensembl_gene_id	hgnc_symbol	Description	log2Fold Change	pvalue	padj	Chromoso-me name
ENSG00000210100	MT-TI	Mitochondrially encoded tRNA isoleucine	1.99	1.08E-76	2.01E-72	MT
ENSG00000261326	LINC01355	Long intergenic non-protein coding RNA 1355	-2.28	2.29E-38	1.42E-34	1
ENSG00000105708	ZNF14	Zinc finger protein 14	-1.14	4.22E-29	1.31E-25	19
ENSG00000252690	SCARNA15	Small Cajal body-specific RNA 15	-1.52	3.90E-28	1.04E-24	15
ENSG00000120738	EGR1	Early growth response 1	1.08	1.20E-27	2.78E-24	5
ENSG00000210195	MT-TT	Mitochondrially encoded tRNA threonine	1.34	1.95E-27	4.04E-24	MT
ENSG00000278864		Novel transcript	-1.02	7.32E-24	1.24E-20	17
ENSG00000233937		Uncategorized gene.	-1.56	3.31E-23	5.14E-20	5
ENSG00000273373		Novel transcript	-1.88	4.48E-22	5.96E-19	1
ENSG00000142871	CYR61	Cysteine rich angiogenic inducer 61	1.13	2.11E-20	2.46E-17	1
ENSG00000272540		Novel transcript	1.88	6.09E-20	6.67E-17	6
ENSG00000250251	PKD1P6	Polycystin 1, transient receptor potential channel interacting pseudogene 6	1.00	7.04E-18	6.24E-15	16
ENSG00000210107	MT-TQ	Mitochondrially encoded tRNA glutamine	1.41	4.27E-17	3.18E-14	MT
ENSG00000274925	ZKSCAN2-DT	ZKSCAN2 divergent transcript	-1.58	4.82E-17	3.45E-14	16
ENSG00000210077	MT-TV	Mitochondrially encoded tRNA valine	1.53	4.61E-16	2.60E-13	MT
ENSG00000271533		Novel transcript	-1.11	7.32E-16	3.78E-13	X
ENSG00000275180		Novel transcript	-1.79	3.09E-14	1.34E-11	12
ENSG00000210196	MT-TP	Mitochondrially encoded tRNA proline	1.22	7.75E-14	3.14E-11	MT
ENSG00000215493		Kelch-Like 12 (Drosophila) (KLH12) Pseudogene	-1.45	1.43E-12	4.92E-10	22
ENSG00000210112	MT-TM	Mitochondrially encoded tRNA methionine	1.48	2.83E-11	7.21E-09	MT

Table 4.5 Top 20 most differential expressed gene changes for MDMB-CHMICA treated cells. The cells were treated with 10 nM MDMB-CHMICA in comparison to DMSO treated cells (control). The negative values in log2 Fold Change values refer to the gene being downregulated and the positive values refer that the gene being upregulated.

Ensembl_gene_id	hgnc_symbol	Description	log2Fold Change	pvalue	padj	Chromosome name
ENSG00000210100	MT-TI	Mitochondrially encoded tRNA isoleucine	1.17	4.8E-26	7.8E-22	MT
ENSG00000210107	MT-TQ	Mitochondrially encoded tRNA glutamine	1.06	4.4E-10	1.8E-06	MT
ENSG00000269378	ITGB1P1	integrin subunit beta 1 pseudogene 1	1.50	4.6E-06	2.5E-03	19
ENSG00000196167	COLCA1	Colorectal cancer associated 1	-1.92	7.8E-06	3.2E-03	11
ENSG00000144045	DQX1	DEAQ-box RNA dependent ATPase 1	-4.12	3.2E-05	7.4E-03	2
ENSG00000184343	SRPK3	SRSF protein kinase 3	1.33	7.6E-05	1.4E-02	X
ENSG00000196218	RYR1	ryanodine receptor 1	1.31	1.0E-04	1.6E-02	19
ENSG00000183018	SPNS2	sphingolipid transporter 2	1.05	1.2E-04	1.7E-02	17
ENSG00000171700	RGS19	regulator of G protein signalling 19	2.05	1.3E-04	1.8E-02	20
ENSG00000081479	LRP2	LDL receptor related protein 2	1.13	1.4E-04	1.8E-02	2
ENSG00000108231	LGI1	leucine rich glioma inactivated 1	-1.24	1.3E-04	1.8E-02	10
ENSG00000150667	FSIP1	fibrous sheath interacting protein 1	2.66	2.2E-04	2.4E-02	15
ENSG00000109255	NMU	Neuromedin U	-1.68	3.3E-04	3.3E-02	4
ENSG00000255326		Nnovel transcript	-1.00	4.0E-04	3.7E-02	11
ENSG00000099725	PRKY	Protein kinase, Y-linked, pseudogene	-4.32	4.7E-04	4.0E-02	Y
ENSG00000118972	FGF23	Fibroblast growth factor 23	-1.09	4.8E-04	4.0E-02	12
ENSG00000163694	RBM47	RNA binding motif protein 47	-2.97	4.9E-04	4.0E-02	4
ENSG00000181790	ADGRB1	Adhesion G protein-coupled receptor B1	1.11	5.6E-04	4.4E-02	8
ENSG00000172339	ALG14	ALG14, UDP-N-acetylglucosaminyltransferase subunit	-1.20	5.9E-04	4.5E-02	1
ENSG00000121101	TEX14	Testis expressed 14, intercellular bridge forming factor	1.88	6.5E-04	4.7E-02	17

Table 4.6 Top 20 most differential expressed gene for 5F-ADB treated cells. The cells were treated with 10 nM 5F-ADB in comparison to DMSO treated cells (control). The negative values in log 2Fold Change values refer to the gene being downregulated and the positive values refer that the gene being upregulated.

Ensembl_gene_id	hgnc_symbol	description	log2FoldChange	pvalue	padj	Chromosome name
ENSG00000210100	MT-TI	Mitochondrially encoded tRNA isoleucine	1.61	2.37E-49	3.86E-45	MT
ENSG00000210107	MT-TQ	Mitochondrially encoded tRNA glutamine	1.37	2.78E-16	9.03E-13	MT
ENSG00000252690	SCARNA15	small Cajal body-specific RNA 15	-1.07	2.28E-16	9.03E-13	15
ENSG00000104435	STMN2	Stathmin 2	-1.44	2.33E-15	5.05E-12	8
ENSG00000079102	RUNX1T1	RUNX1 translocation partner 1	-1.02	1.39E-12	1.62E-09	8
ENSG00000261326	LINC01355	long intergenic non-protein coding RNA 1355	-1.11	2.75E-12	2.63E-09	1
ENSG00000210112	MT-TM	Mitochondrially encoded tRNA methionine	1.45	6.09E-11	3.96E-08	MT
ENSG00000210077	MT-TV	Mitochondrially encoded tRNA valine	1.21	2.31E-10	1.21E-07	MT
ENSG00000214402	LCNL1	Lipocalin like 1	1.03	2.29E-10	1.21E-07	9
ENSG00000187122	SLIT1	Slit guidance ligand 1	-1.39	5.31E-09	2.01E-06	10
ENSG00000167202	TBC1D2B	TBC1 domain family member 2B	-1.01	4.95E-08	1.44E-05	15
ENSG00000263826		Isoform 2 EIF4A2	1.12	8.79E-08	2.34E-05	3
ENSG00000151090	THRB	Thyroid hormone receptor beta	-1.02	1.77E-07	3.73E-05	3
ENSG00000272540		Antisense To TUBB	1.10	2.20E-07	4.32E-05	6
ENSG00000269399		Novel Transcript	-1.03	2.33E-07	4.51E-05	19
ENSG00000108231	LGI1	Leucine rich glioma inactivated 1	-1.61	7.70E-07	0.000123	10
ENSG00000249859	PVT1	Pvt1 oncogene (non-protein coding)	1.37	7.57E-07	0.000123	8
ENSG00000210156	MT-TK	Mitochondrially encoded tRNA lysine	1.19	3.01E-06	0.000363	MT
ENSG00000153902	LGI4	Leucine rich repeat LGI family member 4	1.38	3.62E-06	0.000419	19
ENSG00000210117	MT-TW	Mitochondrially encoded tRNA tryptophan	1.20	4.31E-06	0.00047	MT

Table 4.7 Top 20 most differential expressed gene for ∆9-THC treated cells. The cells were treated with 10 nM ∆9-THC in comparison to DMSO treated cells (control). The negative values in log 2 Fold Change values refer to the gene being downregulated and the positive values refer that the gene being upregulated.

It is noteworthy, that the most significantly changed group of genes across the 3 groups were mitochondrial tRNA genes (MT-tRNA). All of these were upregulated with fold changes between 1.17 and 1.99. In addition, mitochondrially encoded tRNA isoleucine (MT-TI) was the highest significantly changed gene in the 3 groups. In MDMB-CHMICA treated cells, there were 9 MT-tRNA significantly changed genes, whereas there were 2 and 6 MT-tRNA significantly changed gene in 5F-ADB and ∆9-THC treated cells respectively. There were 4 common genes that were significantly changed among 3 groups (Table 4.8).

hgnc_ symbol	Description	MDMB-CHMICA		5F-ADB		∆9-THC	
		log2Fold Change	*padj*	*log2Fold Change*	*padj*	*log2Fold Change*	*padj*
MT-TI	mitochondrially encoded tRNA isoleucine	1.99	2.01E-72	1.17	7.80E-22	1.61	3.86E-45
MT-TQ	mitochondrially encoded tRNA glutamine	1.41	3.18E-14	1.06	1.80E-06	1.37	9.03E-13
LGI1	leucine rich glioma inactivated 1	-1.35	1.25E-03	-1.24	1.80E-02	-1.61	1.23E-04
DQX1	DEAQ-box RNA dependent ATPase 1	-3.28	1.29E-02	-4.12	7.40E-03	-3.2	2.80E-02

Table 4.8 The common genes changed among MDMB-CHMICA, 5F-ADB and ∆9-THC.

Although, there were only 4 changes that were commonly changed in all 3 treatment groups, there were greater overlaps in gene expression changes between treatment groups when analysed in pairs (Figure 4.13). The direction of changes in all genes across the 3 groups follow the same patterns in term of activation or inhibition. The full lists of genes and the overlapping genes are shown in appendix (I).

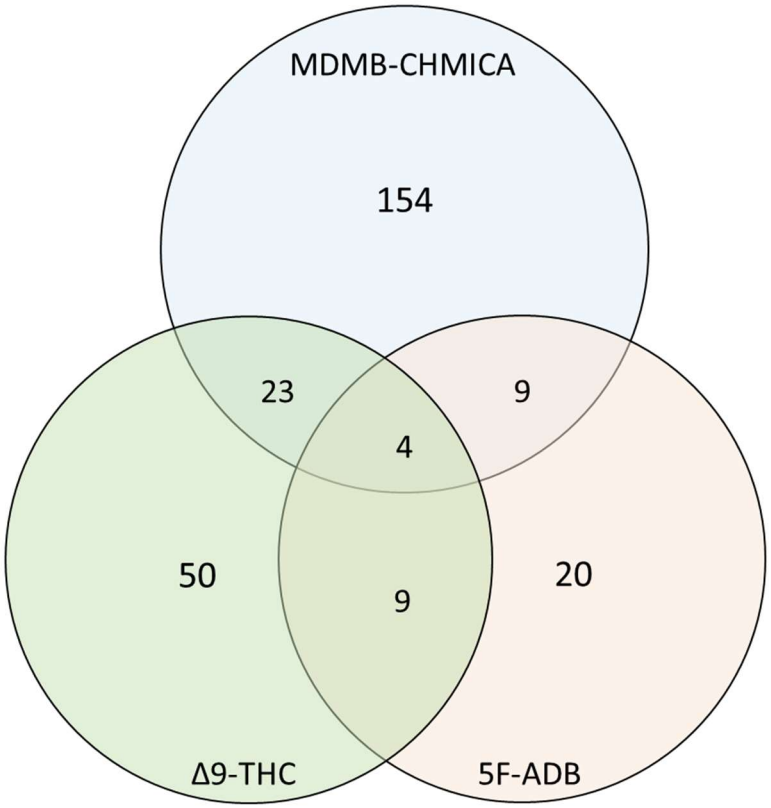


Figure 4.13 Diagram indicating the number of differentially expressed genes identified in MDMB-CHMICA, 5F-ADB and ∆9-THC, including the number of genes that overlapped between exposed groups.

4.4.2.3 Ingenuity Pathway Analysis (IPA)

To explore, the canonical pathways, functional gene networks, and biological functions, all the expressed gene sets were analysed by IPA software (Ingenuity Pathways Analysis, Redwood City, USA; (Qiagenbioinformatics.com, 2019). The p value of 0.05 was set as a cut off value for further analysis. The p value is an arithmetic value used to arrange networks according to how relevant they are to the genes in the input dataset. Nevertheless, it may not imply of the significance or the quality of the pathway. The values take into consideration the number of focus genes in the pathway and the extent of the network to estimate how related this network is to the original list of focus genes. IPA has a library of canonical pathways and the canonical pathway analysis recognises the most significant in the input data set by comparing it with the library.

In order to interrogate the particular canonical pathways modified with the treated cells, IPA was conducted on the MDMB-CHMICA, 5F-ADB and ∆9-THC treated cells versus DMSO treated cells (control). IPA identified that there were 34 canonical pathways significantly changed in MDMB-CHMICA treated cells, 25 pathways for cells exposed to 5F-ADB and 17 pathways for ∆9-THC exposure at $p<0.05$. The canonical pathway analysis predicts the activity of pathways either activated or inhibited or where no prediction can be made. Table 4.9 showed the activated and inhibited pathways after exposure to MDMB-CHMICA, 5F-ADB and ∆9-THC. The full lists of canonical pathways are shown in appendix (I).

MDMB vs DMSO	5F-ADB vs DMSO	∆9-THC vs DMSO
Inhibited Canonical pathways	***Inhibited Canonical pathways***	***Inhibited Canonical pathways***
Sumoylation Pathway		Wnt/β-catenin Signalling
Sirtuin Signalling pathways		EIF2 Signalling
Chondrotin Sulfate degradation		Ephrin B signalling
		Aryl hydrocarbon receptor signalling
		D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis
		D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis
Activated Canonical pathways	***Activated Canonical pathways***	***Activated Canonical pathways***
EIF2 Signalling	p53 Signalling	Superpathway of Inositol Phosphate Compounds
Notch Signalling	Actin Cytoskeleton Signalling	
mTOR Signalling	Neuropathic Pain Signalling In Dorsal Horn Neurons	
BMP signalling pathway	NGF Signalling	
TGF-β Signalling		
Superpathway of Inositol Phosphate Compounds	Adrenomedullin signalling pathway	
Thrombin Signalling		
Wnt/β-catenin Signalling		
Phospholipase C Signalling		
IL-8 Signalling		
Protein Kinase A Signalling		
Mouse Embryonic Stem Cell pluripotency		

Table 4.9 Canonical pathways significantly changed in MDMB-CHMICA, 5FADB and ∆9THC treated cells.

The pathway analysis revealed that the main molecular and cellular function affected in MDMB-CHMICA treated cells includes gene expression, protein synthesis, cellular development, cellular growth and proliferation and RNA post-translational modification. The overlapped canonical pathways which were detected as significant are displayed in Figure 4.14.

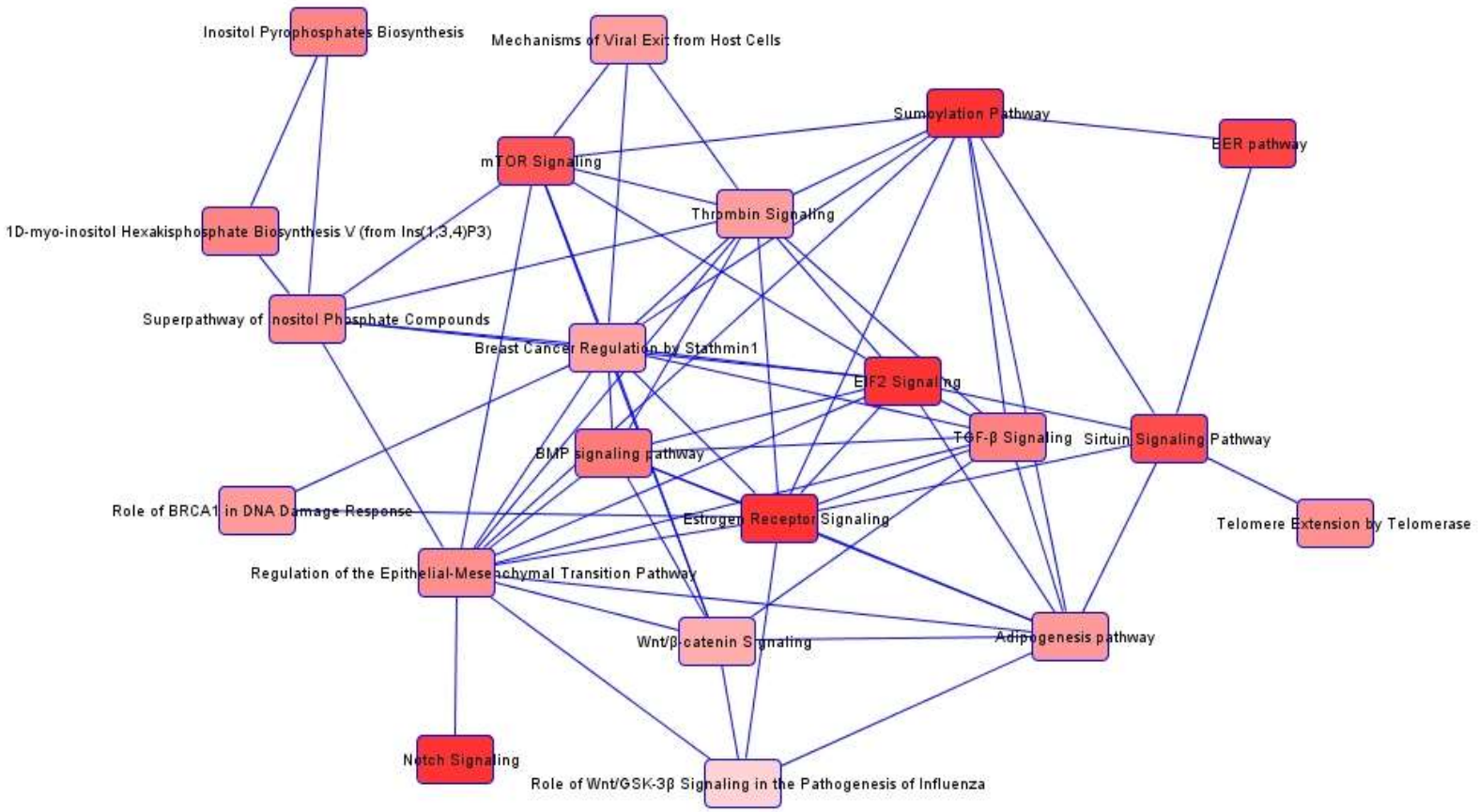


Figure 4.14 Overlapping canonical pathways maps altered pathways and representing shared biology among them in MDMB-CMICA versus control treated cells. RNAseq dataset based on pathway analysis using the Ingenuity Pathway Analysis software**.** Mature neuronal cells treated with 10 nM MDMB-CHMICA. Each link corresponds to connected pathways shared one or more genes in common. The brighter the red colour correspond the larger the –log p values.

The MDMB-CHMICA IPA analysis showed that the SUMOylation pathway (Figure 4.15) and sirtuin pathway Figure 4.16). SUMOylation is a process in which a member of the small ubiquitin-like modifier (SUMO) family of proteins is conjugated to the lysine residues in target proteins as part of post-translational modification. It is involved in many biological processes for example transcription, DNA repair, nuclear transport and chromosome segregation (Schorova and Martin, 2016, Gareau and Lima, 2010). It is also a main element in many extra nuclear neuronal mechanisms and has been involved in broad spectrum of neuropathological circumstances such as Huntington's disease and Neuronal intra-nuclear inclusion disease (NIID) (Martin et al., 2007). Sirtuin are NAD^+-dependent histone deacetylases enzymes controlling many metabolic pathways in prokaryotes and eukaryotes. The seven sirtuin members play an important role in many biological processes such as cell survival, senescence, proliferation, apoptosis, DNA repair, cell metabolism, and cellular response to stress (van de Ven et al., 2017). The canonical analysis showed also that EIF2 (Figure 4.17), Notch, mTOR signalling (Figure 4.18), BMP and TGF had been activated in MDMB-CHMICA exposed cells. The EIF2 (eukaryotic initiation factor 2) protein plays a vital role in protein synthesis regulation, through its involvement in the unfolded protein response (UPR) (Cao and Kaufman, 2012). Under stress EIF2 either alleviates cellular injury or induces apoptosis as an alternative (Wek et al., 2006). Notch is a signalling pathway that plays an essential role in the specification of cell fates that take place over local cell interactions in different tissues and organisms. It plays an important role in neural development (Artavanis-Tsakonas et al., 1999). mTOR (mechanistic target of rapamycin) pathway has a vital role in controlling protein synthesis and ultimately proliferation and cell growth (Laplante and Sabatini, 2012).

The BMP signalling pathway (Bone morphogenetic proteins) was initially recognized as osteo inductive elements in bone extracts, but is now known to play significant roles in a broad range of procedures during the creation and maintenance of multiple parts of the body including cartilage, bone, muscle, kidney, and blood vessels (Katagiri and Watabe, 2016). The TGF-B (transforming growth factor β) family play an important role in the regulation of cell proliferation and survival together with other related family member such as BMPs proteins (Zhang et al., 2017). From the changes in pathways, MDMB-CHMICA exposure tend to affect gene expression, metabolic diseases and protein synthesis as shown in (Figure 4.19).

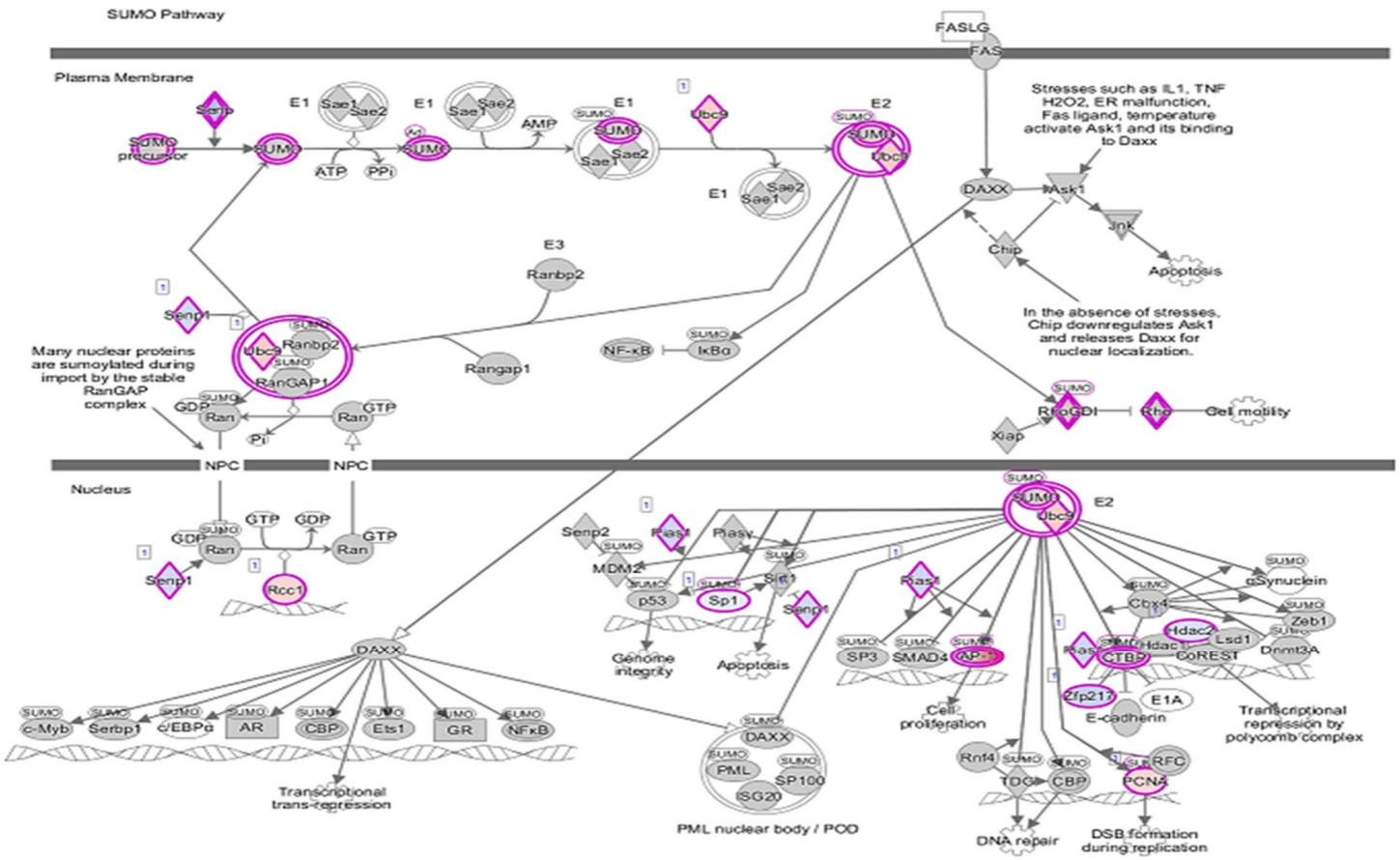


Figure 4.15 Effects on SUMOylation pathway in MDMB-CHMICA treated cells. The SUMOylation pathway was inhibited by MDMB-CHMICA in mature stem cells**.**Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

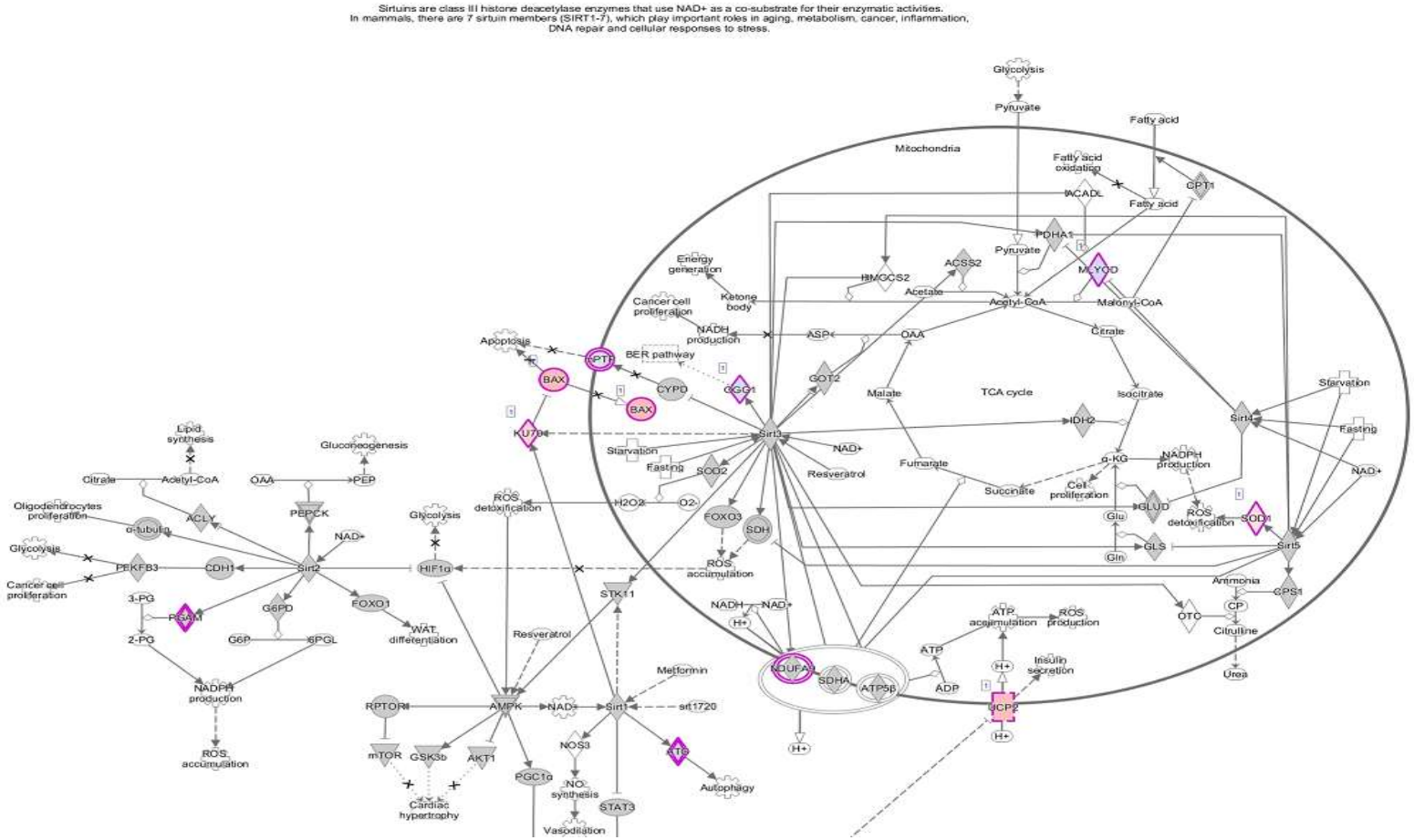


Figure 4.16 Effect on Sirtuin pathway in MDMB-CHMICA treated cells. The Sirtuin pathway was inhibited by MDMB-CHMICA in mature stem cells**.**Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

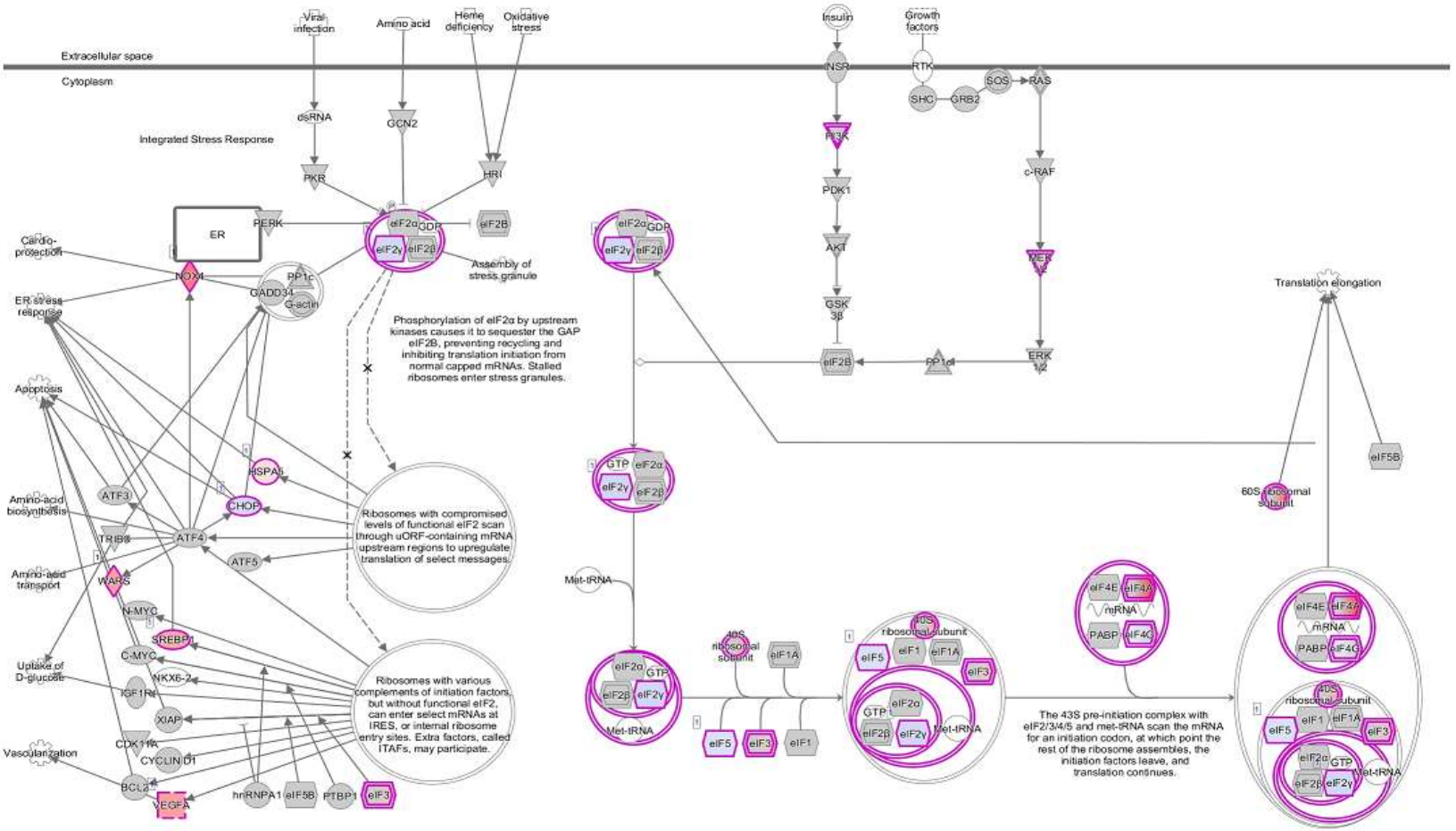


Figure 4.17 Effect on EIF2 signalling pathway in MDMB-CHMICA treated cells. EIF2 was activated by MDMB-CHMICA in mature stem cells.Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

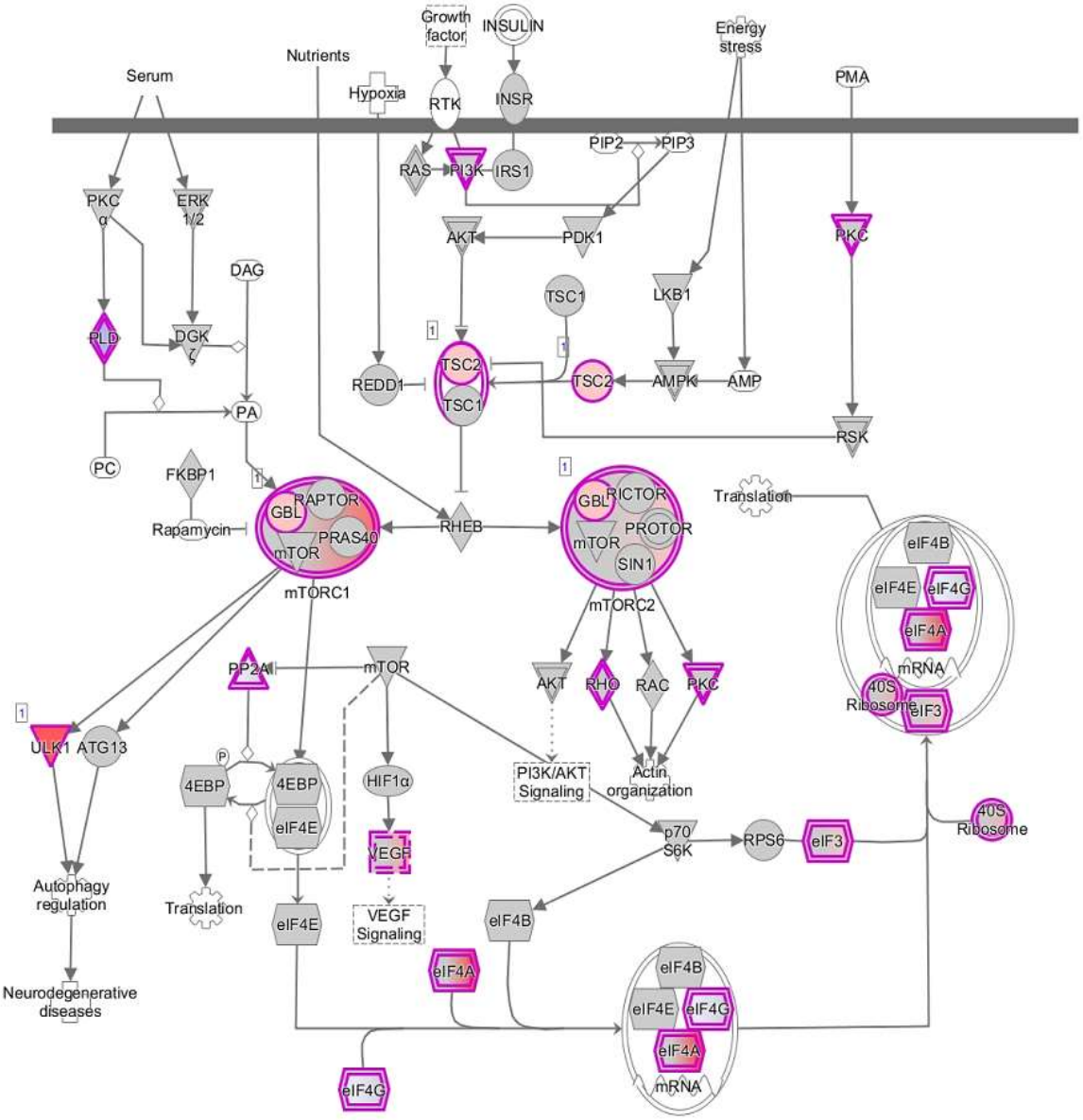


Figure 4.18 Effect on mTOR pathway in MDMA-CHMICA treated cells. mTOR was activated by MDMB-CHMICA in mature stem cells**.** Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

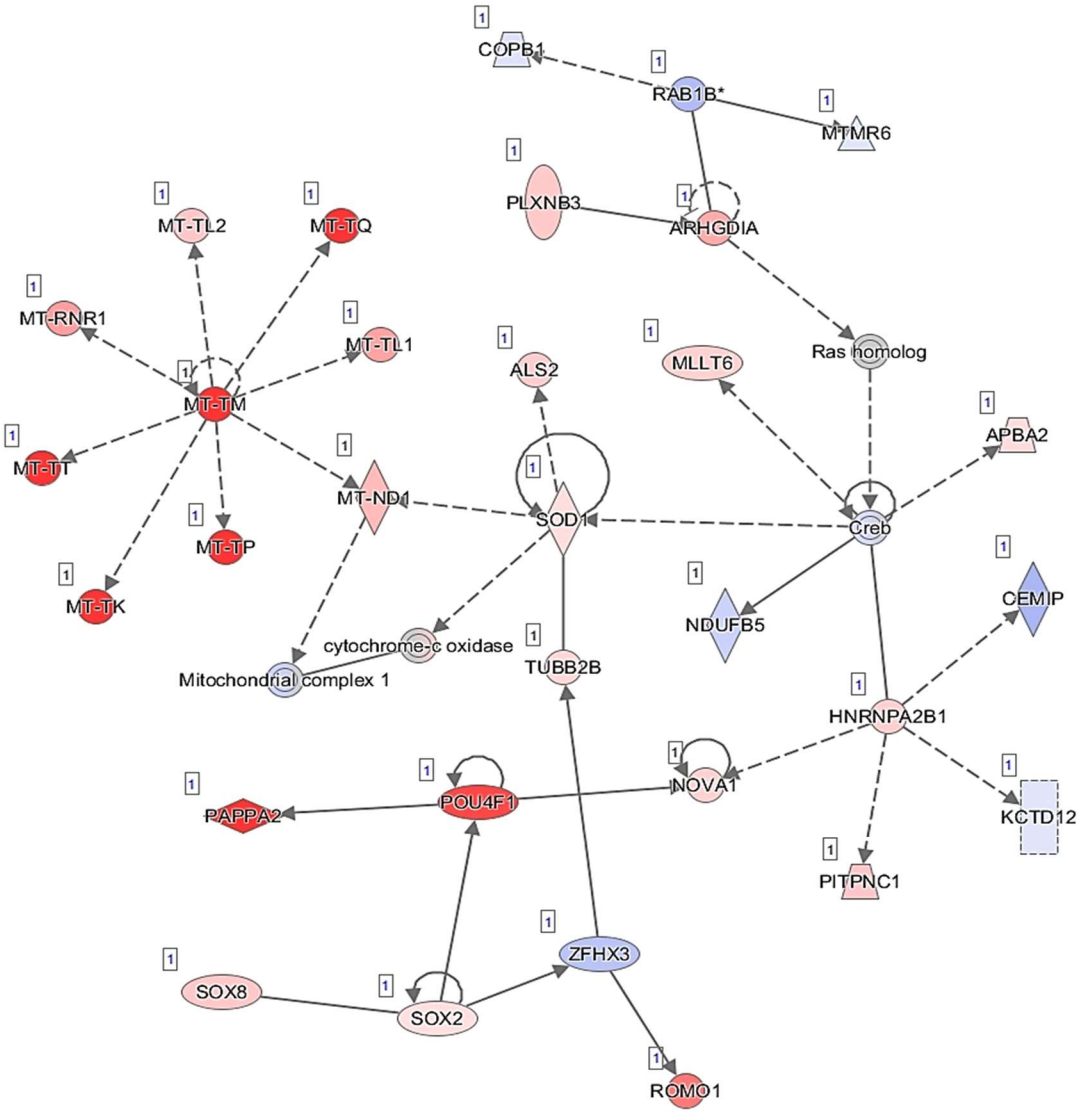


Figure 4.19 Gene- gene network generated by IPA software depicting the Gene Expression, Metabolic Disease, Protein Synthesis network in MDMB-CHMICA treated cells. The red colour represented upregulated genes and the blue colour representing predicted inhibition. Solid lines indicated direct interaction and dashed line indicated indirect interaction.

In cells exposed to 5F-ADB, the pathway analysis revealed that the main molecular and cellular function affected in 5F-ADB treated cells includes protein synthesis, gene expression, cell cycle, cellular movement and cell death and survival. The overlapped canonical pathways which detected as significant are displayed in Figure 4.20.

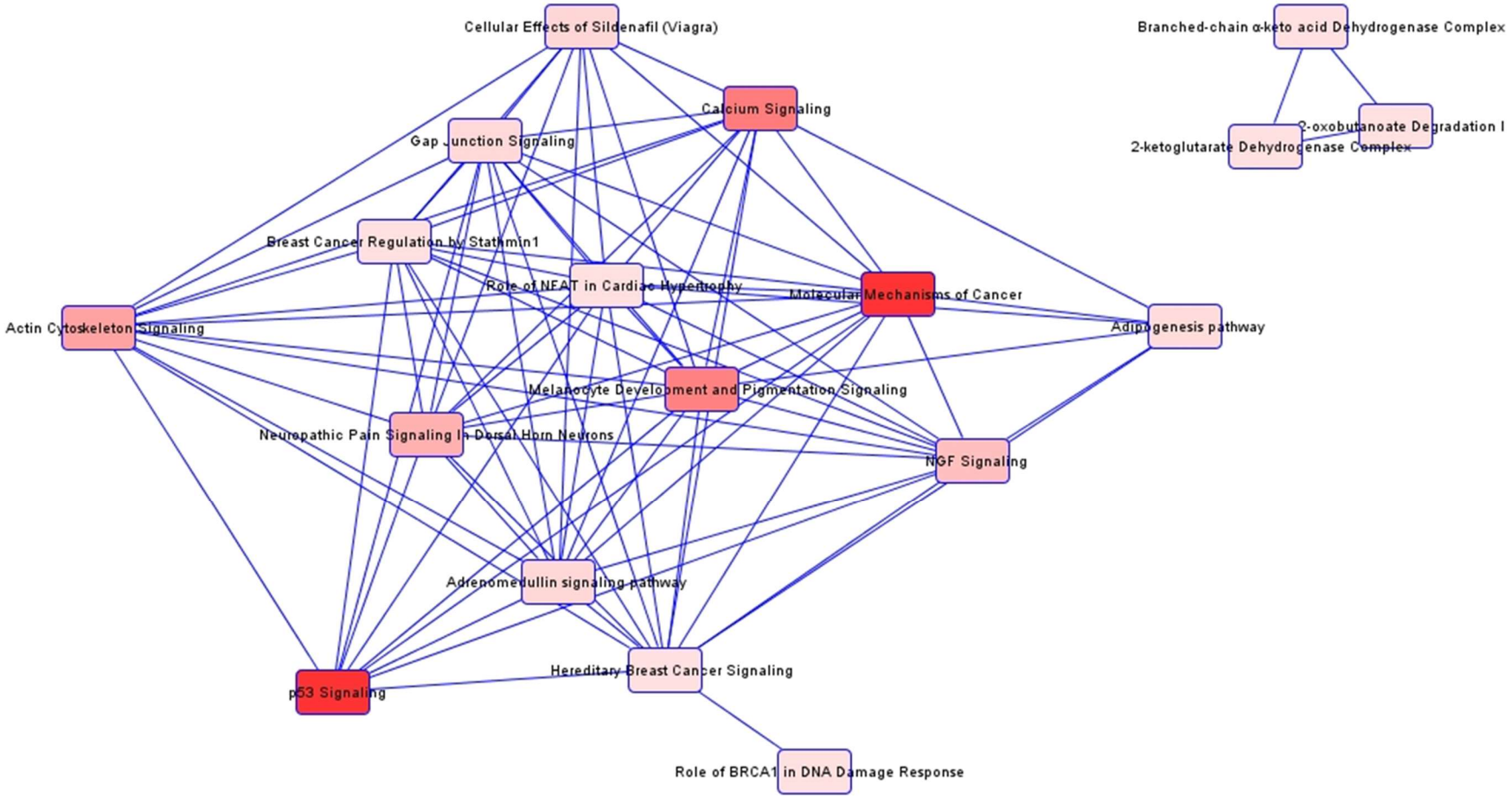


Figure 4.20 Overlapping canonical pathways maps altered pathways and representing shared biology among them in 5F-ADB versus control treated cells. RNAseq dataset based on pathway analysis using the Ingenuity Pathway Analysis software**.** Mature neuronal cells treated with 10 nM 5F-ADB. Each link corresponds to connected pathways shared one or more gene in common. The brighter the red colour correspond the larger the log p value.

The IPA analysis indicated that p53, actin cytoskeleton signalling pathway, neuropathic pain signalling in dorsal horn neurons, nerve growth factor (NGF) and adrenomedullin signalling pathway signalling have been activated in 5F-ADB treated cells.

The p53 (Figure 4.21) is a tumour suppressor gene in human cancers; activated p53 induces apoptosis by transactivating pro-apoptotic genes and direct binding to anti-apoptotic mitochondrial proteins to successfully provoke apoptosis (Ranjan and Iwakuma, 2016). The actin cytoskeleton signalling pathway (Figure 4.22) regulates many biological function and acts as a mediator of cellular motility and changes in cell shape during the cell cycle in consequence to extracellular stimuli. It also organises the cytoplasm and produces mechanical forces within the cell. As well as acting as a mediator initiator of apoptosis signalling (Desouza et al., 2012, Schmidt and Hall, 1998). Finally, NGF (Nerve growth factor) is essential for maintenance, growth, and survival of neurons. In particular sympathetic and sensory neuron which undergo apoptosis in the lack of NGF (Kristiansen and Ham, 2014). It can be noticed that the activated pathways in 5F-ADB treated cells are related to increases in apoptosis and consequently cell death.

From the changes in pathway analysis, 5F-ADB affects gene expression, metabolic disease and protein synthesis as shown in Figure 4.23.

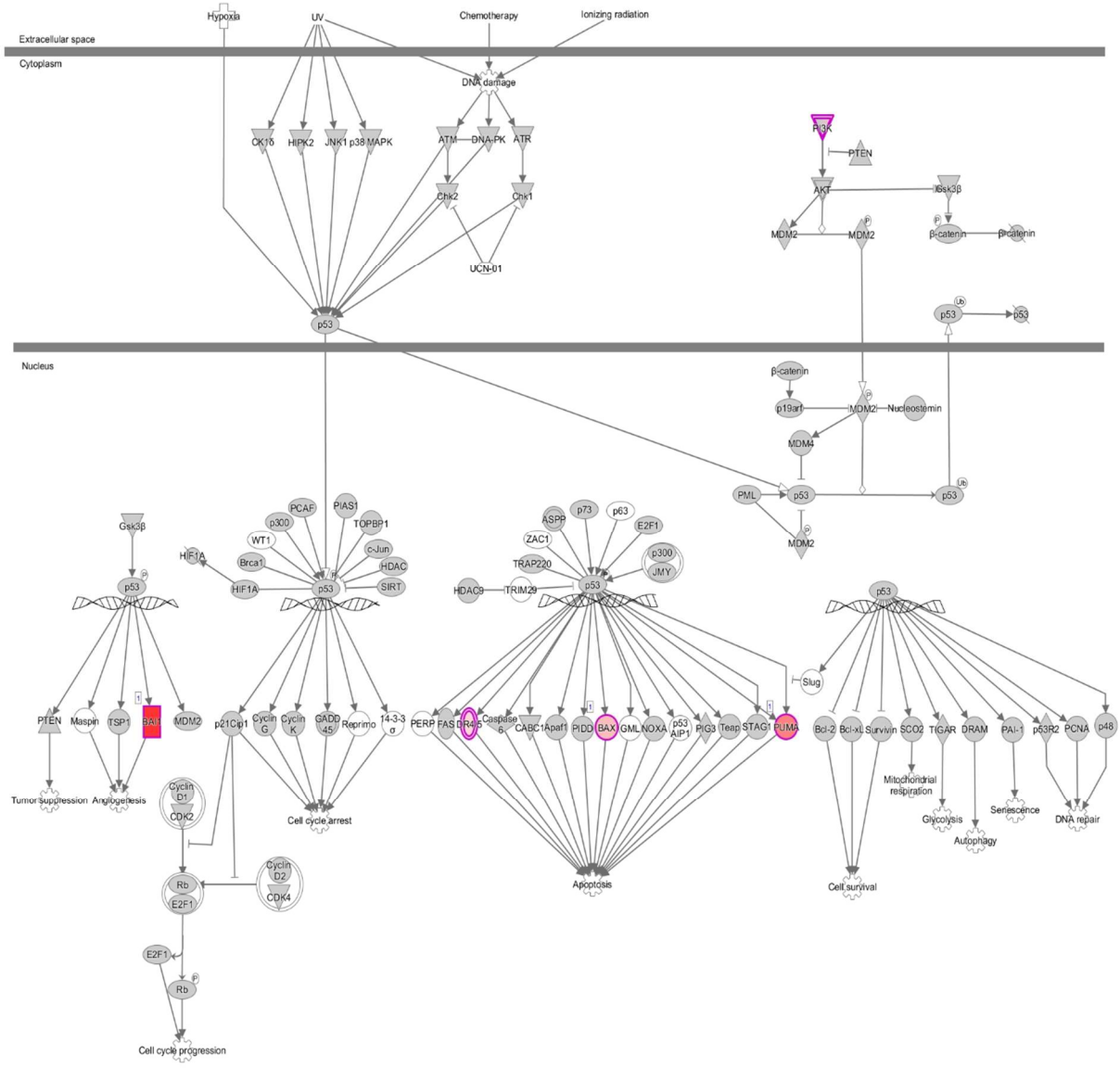


Figure 4.21 Effect on P53 pathway in 5F-ADB treated cells. P53 pathway was activated by 5F-DDB in mature stem cells. Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

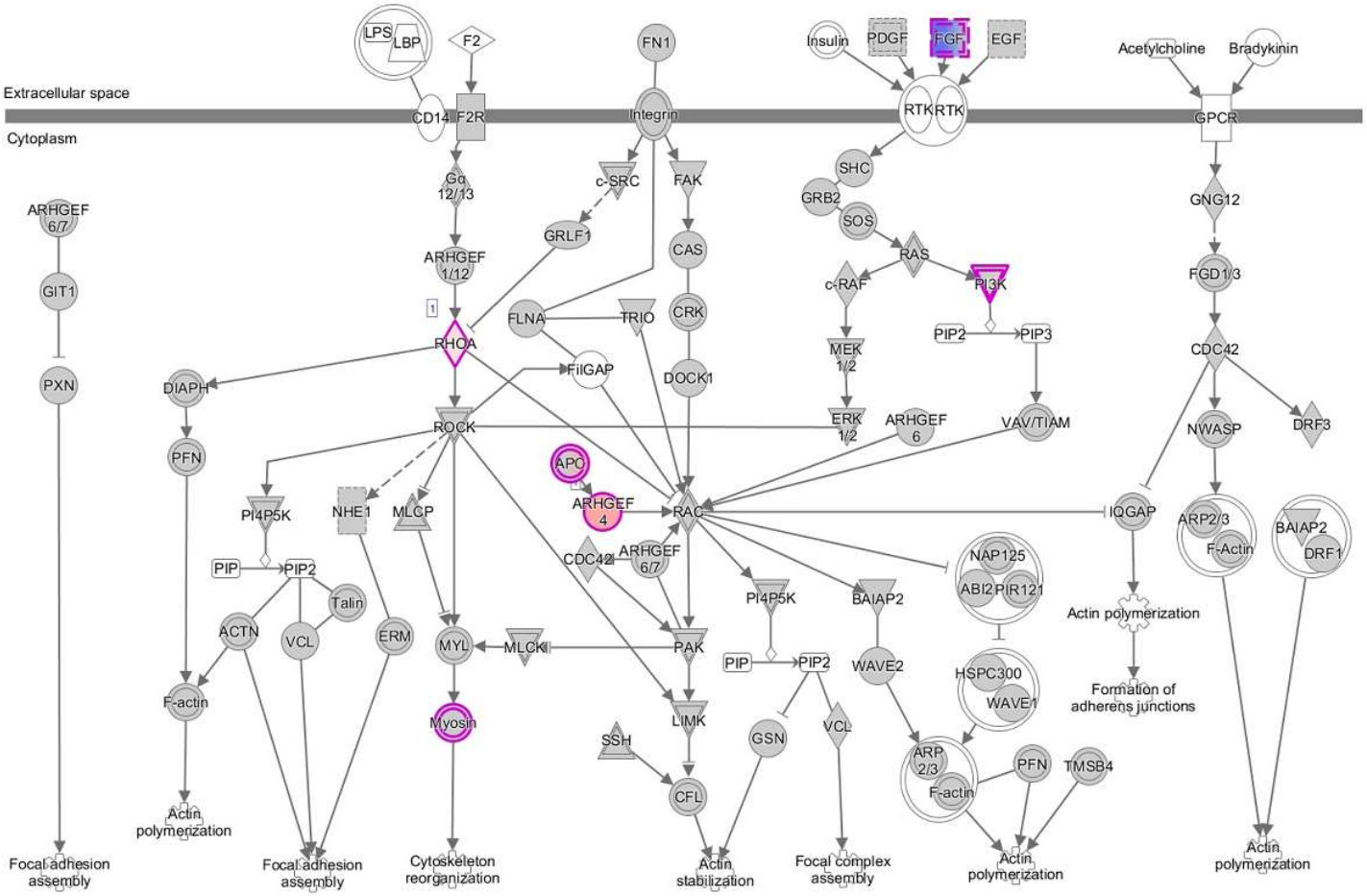


Figure 4.22 Effect on Actin cytoskeleton signalling pathway in 5F-ADB treated cells. Actin cytoskeleton signalling pathway was activated by 5F-ADB in mature stem cells. Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity pathway analysis.

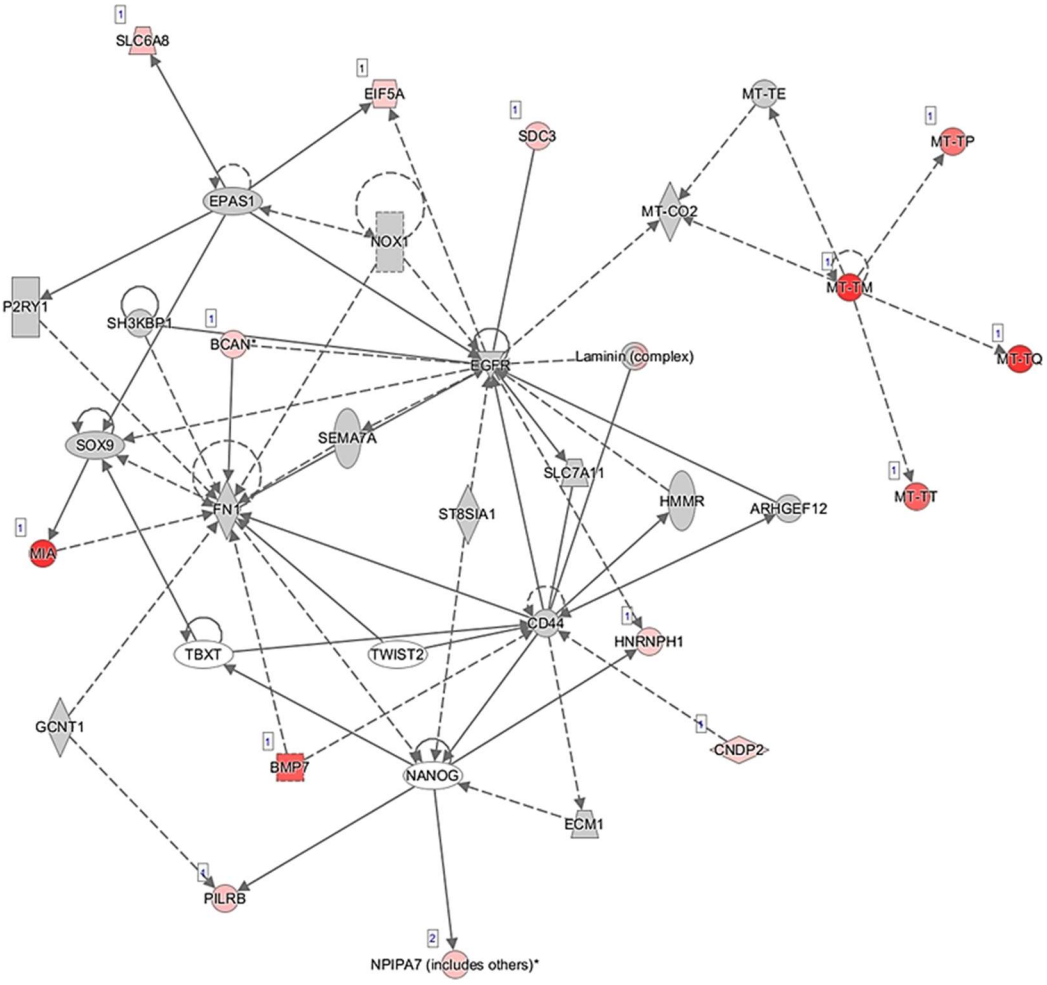


Figure 4.23 Gene- gene network generated by IPA software depicting the Gene Expression, Metabolic Disease, Protein Synthesis network in 5F-ADB treated cells. The red colour represented upregulated genes and the blue colour representing predicted inhibition. Solid lines indicated direct interaction and dashed line indicated indirect interaction.

In cells exposed to ∆9-THC, the pathway analysis revealed that the main molecular and cellular function affected in ∆9-THC treated cells includes cellular development; cellular growth and development; cellular assembly and organisation; cellular function and maintenance and gene expression. The overlapped canonical pathways which detected as significant are displayed in (Figure 4.24).

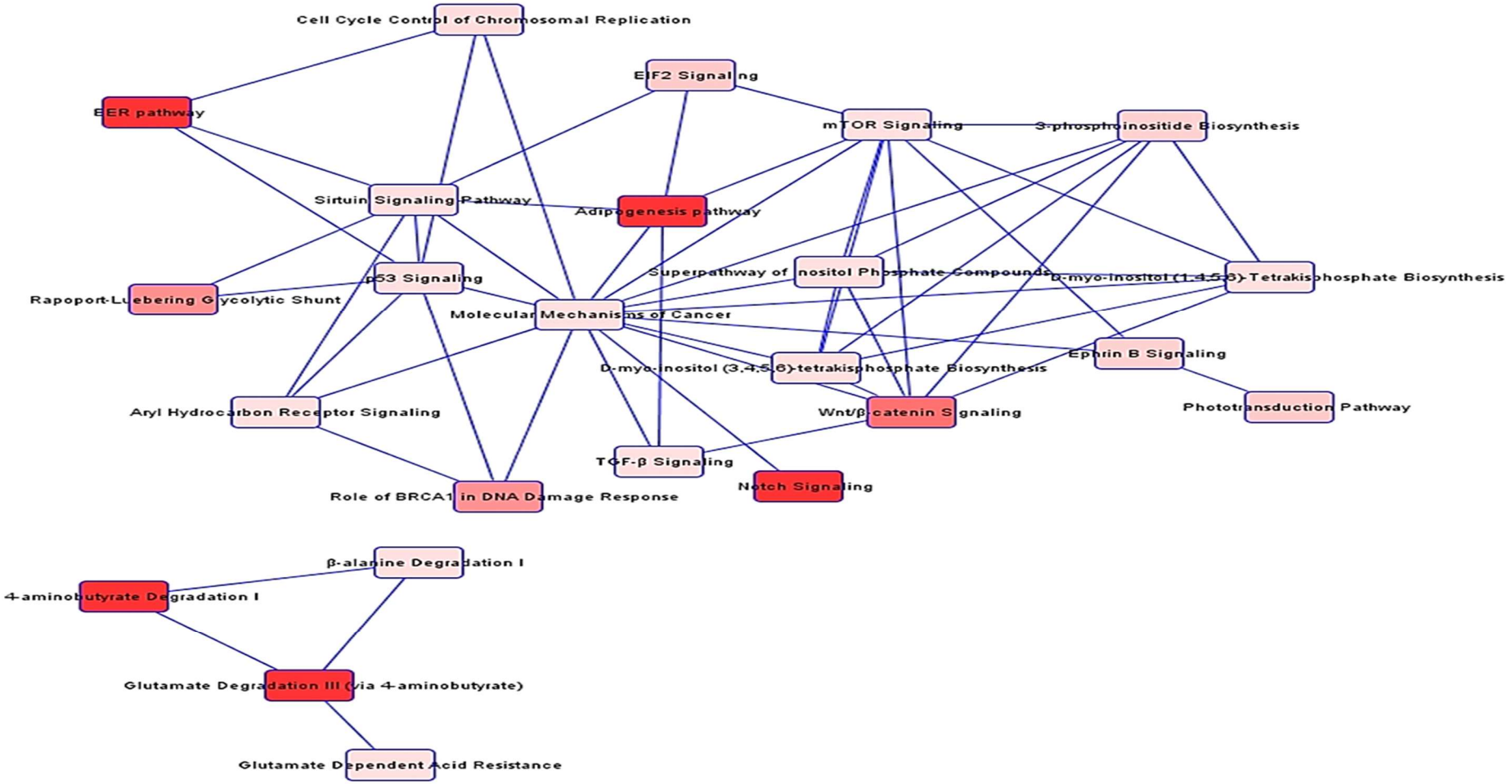


Figure 4.24 Overlapping canonical pathways maps altered pathways and representing shared biology among them in ∆9-THC treated cells versus control treated cells. RNAseq dataset based on pathway analysis using the Ingenuity Pathway Analysis software. Mature neuronal cells treated with 10 nM 5F-ADB. Each link corresponds to connected pathways shared one or more genes in common. The brighter the red colour correspond the larger the –log p value.

The IPA analysis indicated that Wnt/β-catenin signalling (Figure 4.25) and EIF2 (Figure 4.26), ephrin B signalling (Figure 4.27) and aryl hydrocarbon receptor signalling (Figure 4.28) have been inhibited in ∆9-THC treated cells.

Wnt/β-catenin signalling plays a role in cell proliferation regulation, differentiation and cell fate determination, it acts through transmitting the signal across the cell membrane. As a consequence, Wnt mutations are often associated with birth defects in humans, cancer and other illnesses such as neurodegeneration and osteoporosis (Kim et al., 2013). Ephrin B is involved in regulation of many physiological process, such as regulation of cytoskeletal rearrangements, formation of topographic maps and controls cell migration in neurons, allowing the cells to reach their final destination (Kania and Klein, 2016). The Aryl hydrocarbon receptor signalling has a role in xenobiotic metabolism as a regulator of metabolising enzymes such as cytochrome P450s. Also, it is involved in the regulation of gene expression and plays a role in immunity regulation and cellular differentiation (Esser and Rannug, 2015, Gutiérrez-Vázquez and Quintana, 2018).

From the changes in pathway analysis, ∆9-THC affects neurological disease, organismal injury and abnormalities and psychological disorders as shown in Figure 4.29.

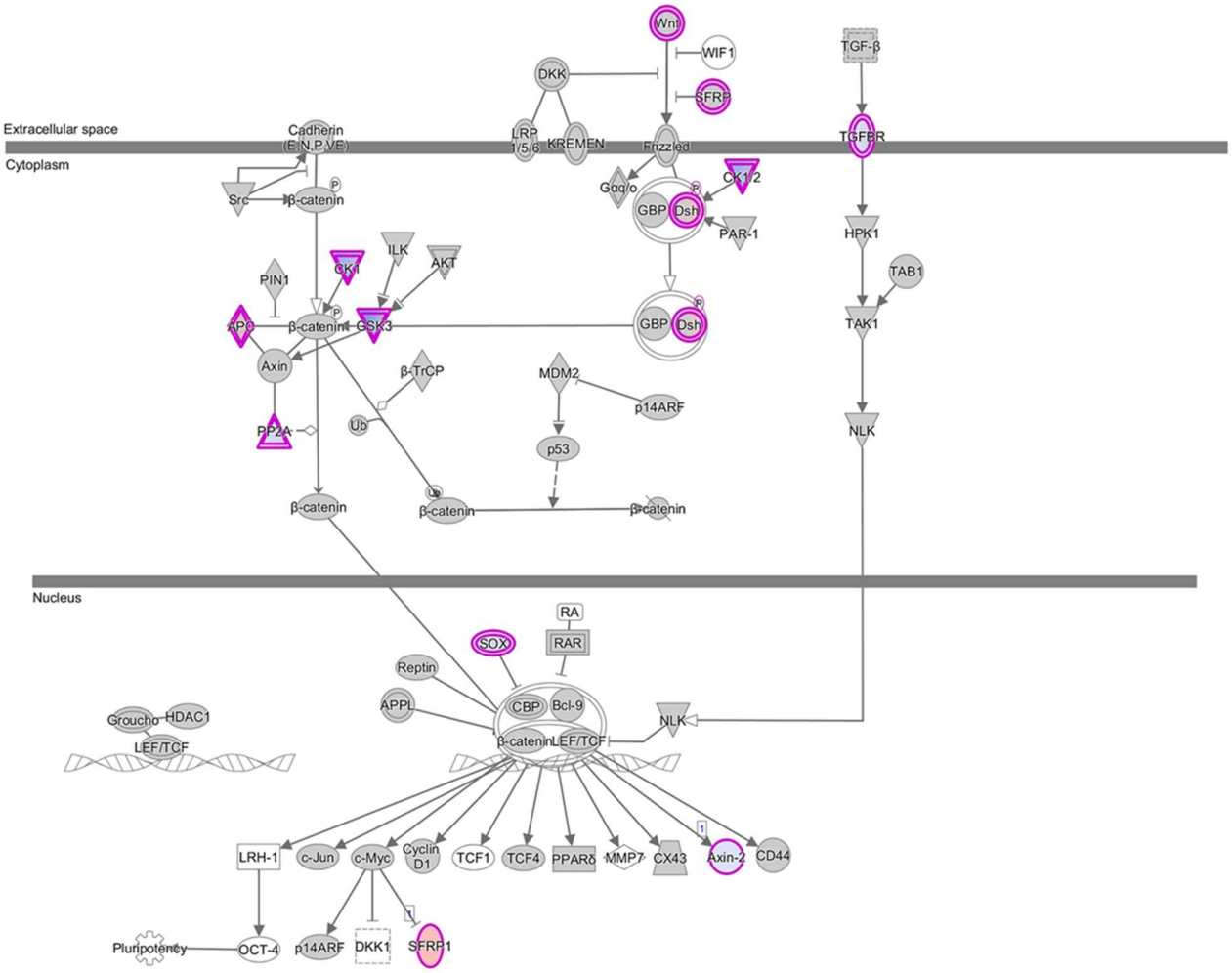


Figure 4.25 Effect on Wnt/β-catenin Signalling pathway in ∆9-THC treated cells. The Wnt/β-catenin was inhibited by ∆9-THC in mature stem cells. Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

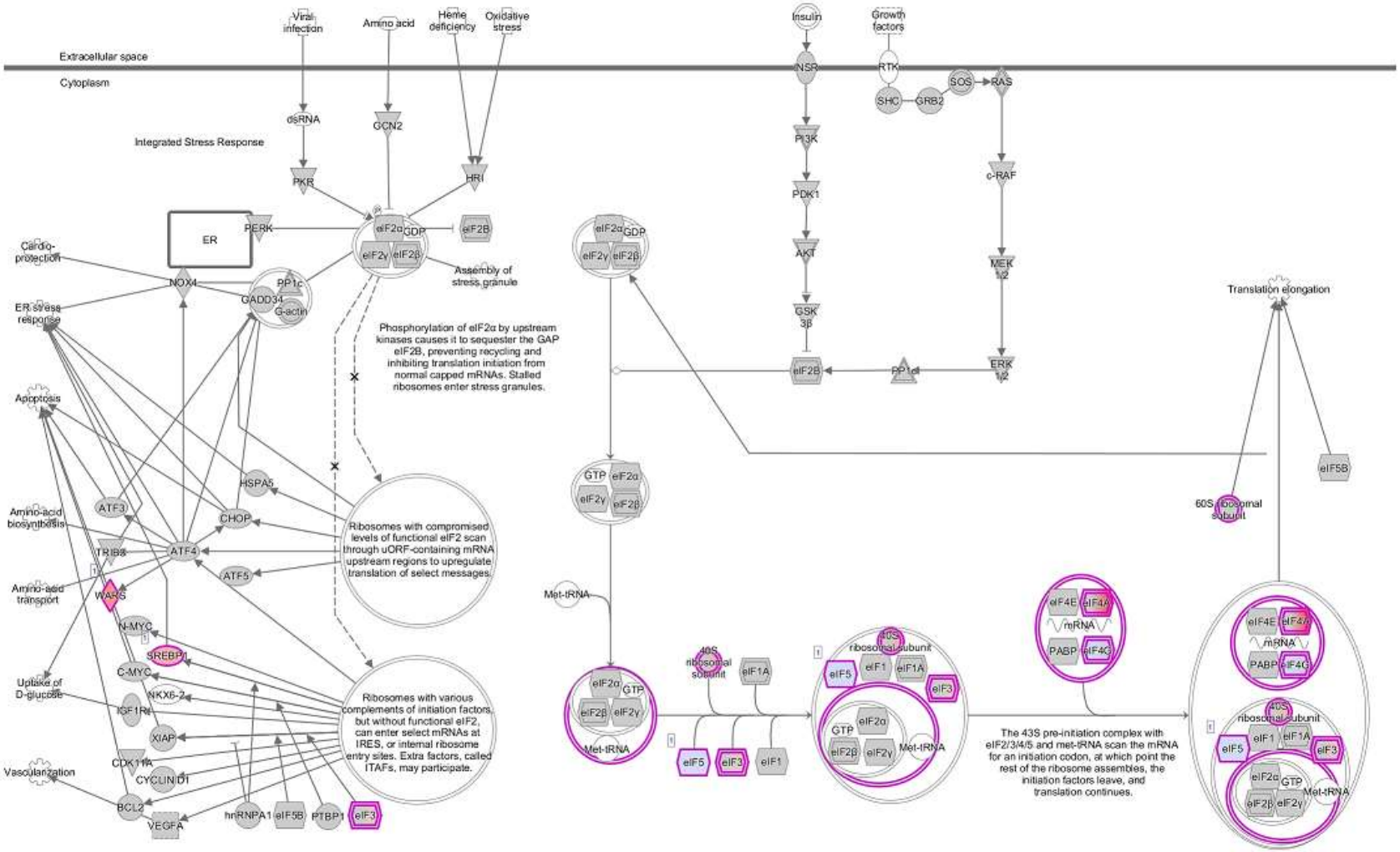


Figure 4.26 Effect on EIF2 signalling pathway in ∆9-THC treated cells. The EIF2 signalling pathway was inhibited by ∆9-THC in mature stem cells. Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

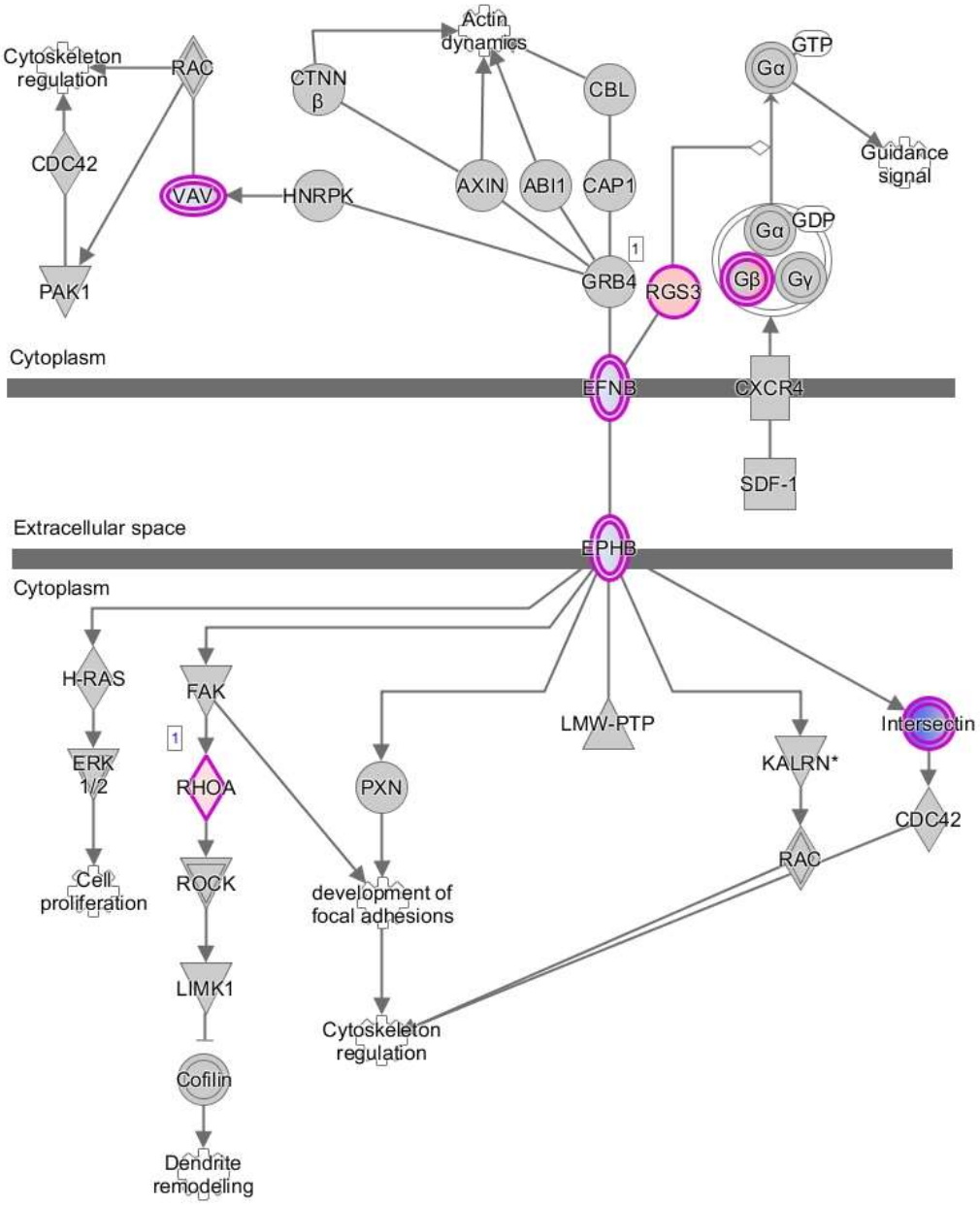


Figure 4.27 Effect on ephrin B Signalling pathway in ∆9-THC treated cells. The ephrin B Signalling pathway was inhibited by ∆9-THC in mature stem cells. Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

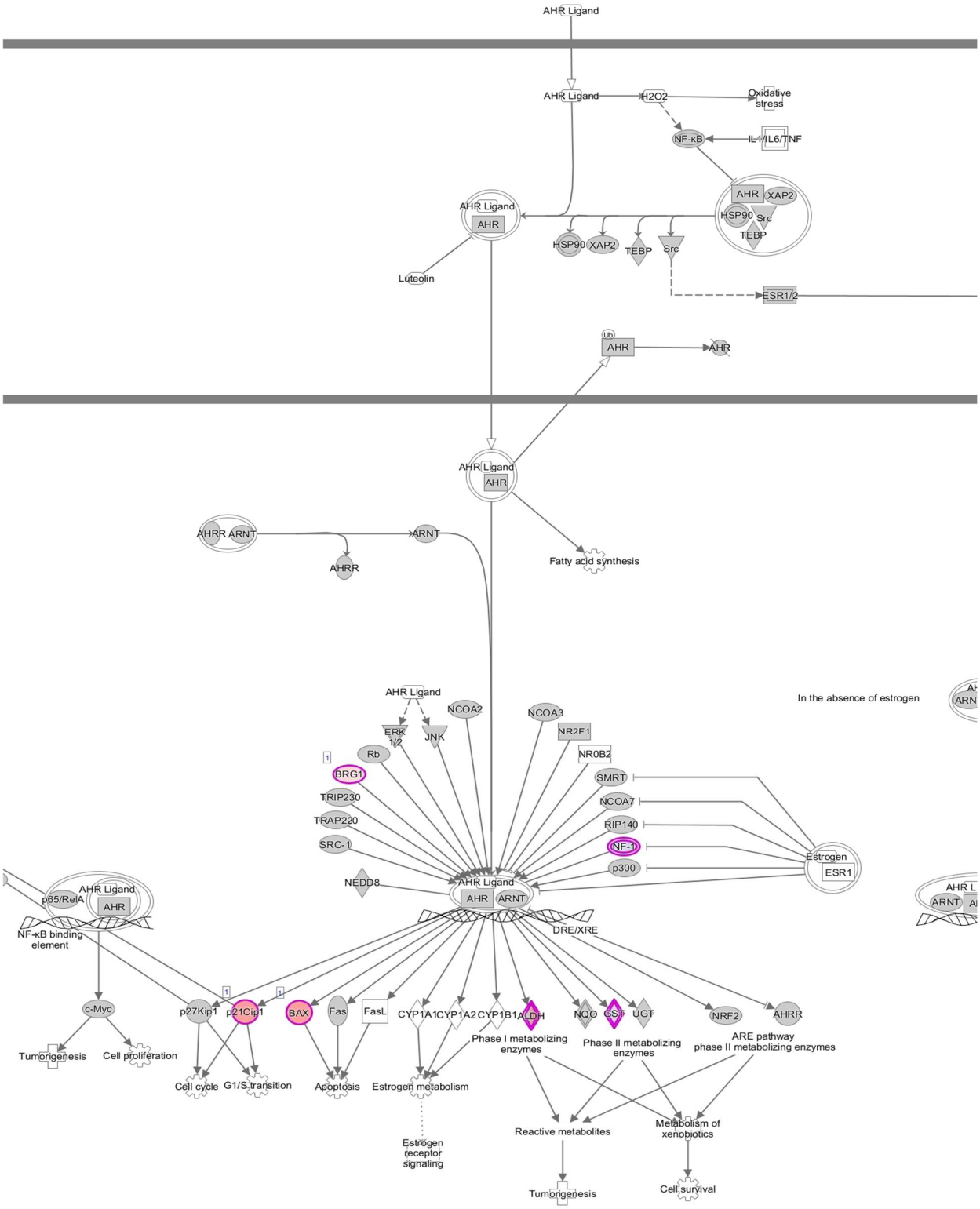


Figure 4.28 Effect on Aryl hydrocarbon receptor signalling pathway in ∆9-THC treated cells. The Aryl hydrocarbon receptor signalling pathway was inhibited by ∆9-THC in mature stem cells. Genes that are highlighted in red are considered upregulated and the one highlighted in blue are downregulated. Canonical pathways were identified and depicted by Ingenuity Pathway Analysis software.

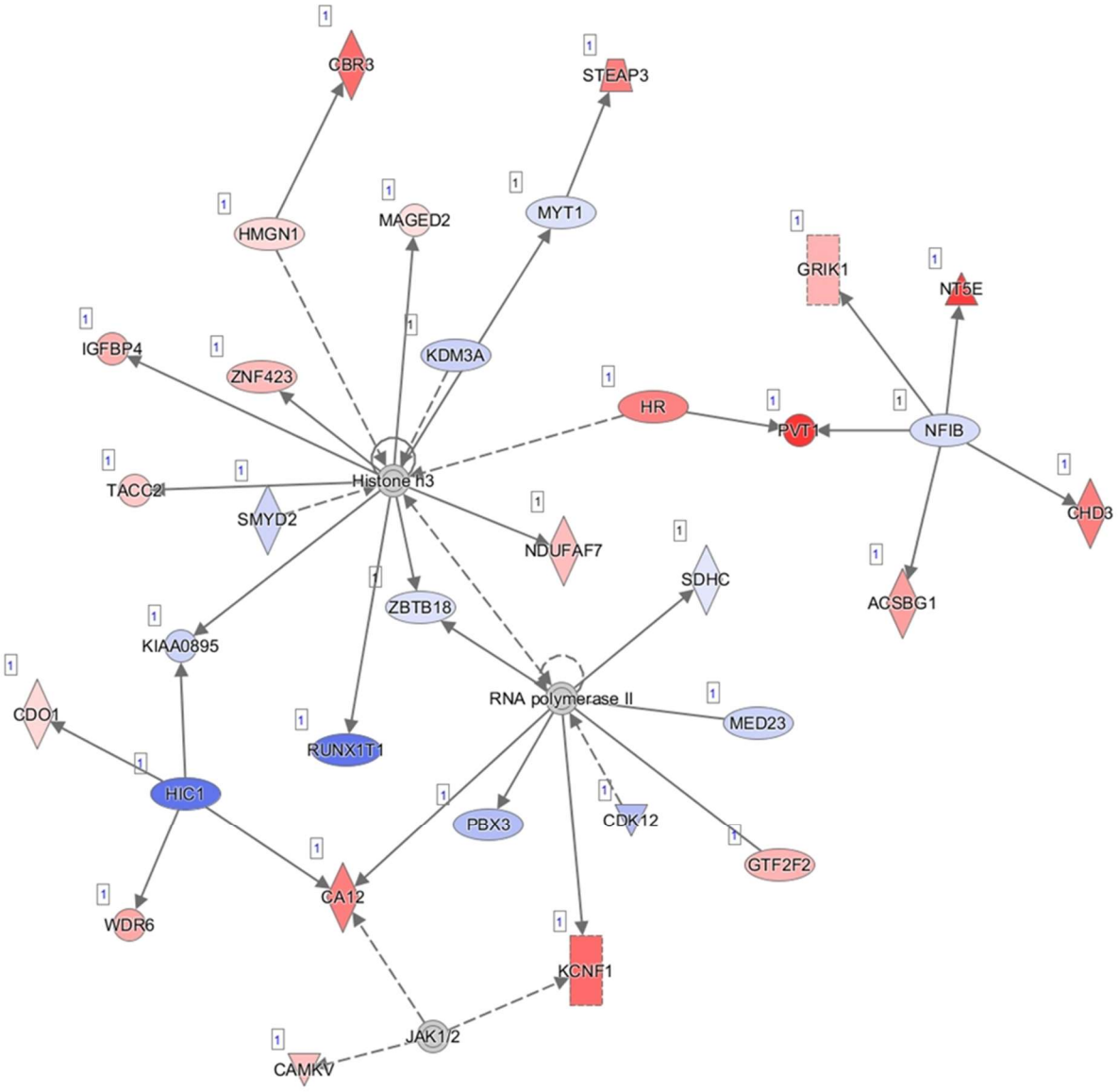


Figure 4.29 Gene- gene network generated by IPA software depicting the Gene Expression, neurological disease, organismal injury and abnormalities psychological disorders in ∆9-THC treated cells. The red colour represented upregulated genes and the blue colour representing predicted inhibition. Solid lines indicated direct interaction and dashed line indicated indirect interaction.

The RNA sequencing analysis suggests that the tested SCRAs and ∆9-THC modulate gene expression at the molecular level that could lead to serious side effects, the most prominent effect being the induction of ER stress. MDMB-CHMICA induced more changes (154 genes) in comparison with 5F-ADB (20 genes) and ∆9-THC (50 genes) which might suggest that MDMB-CHMICA is potentially induce more changes at cellular level and hence might be more harmful, albeit having the same toxic effect at

10 µM (Figure 4.6). However, 5F-ADB response was inconsistence (Figure 4.11) that would affect the statistical gene changes analysis and the higher effective concentration used (17 times the EC50 of 5F-ADB at CB1 receptor) might be responsible for triggering more cell death pathways than MDMB-CHMICA. Therefore, it was not possible to draw a conclusive comparison between 5F-ADB and MDMB-CHMICA. The increases in MT tRNA expression across the 3 groups indicates that the cells were stressed. In addition, the difference in response can be seen as in EIF2 signalling pathway activated in MDMB-CHMICA treated cells it was inhibited in ∆9-THC treated cells.

4.4.3 Proteomic changes due to NPS exposure

To investigate the functional significance of the changes in protein level after chronic exposure to SCRAs and ∆9-THC, western blotting was performed on mature neuronal cells treated with 10 nM of MDMB-CHMICA, 5F-ADB and ∆9-THC for 2 weeks.

4.4.3.1 CB1 receptor expression in hNPSC cells

CB1 receptor expression was investigated because it is the receptor via which cannabinoids produce their major psychoactive effects. Western blot analysis of CB1 showed 3 bands at the expected molecular weights at 53 kDa (CB1), 49 kDa (CB_{1b}) and 46 kDa (CB_{1a}) as shown in Figure 4.30. The isoform CB_{1a} was the only isoform analysable due to low fluorescent signal of the other isoforms. There were no statistical differences in CB_{1a} expression between the control and treated cells in all exposures ($p>0.05$), with percentage changes ranging from a 7% decrease in MDMB-CHMICA treated cells to 3% and 19% increases in expression with ∆9-THC and 5F-ADB respectively.

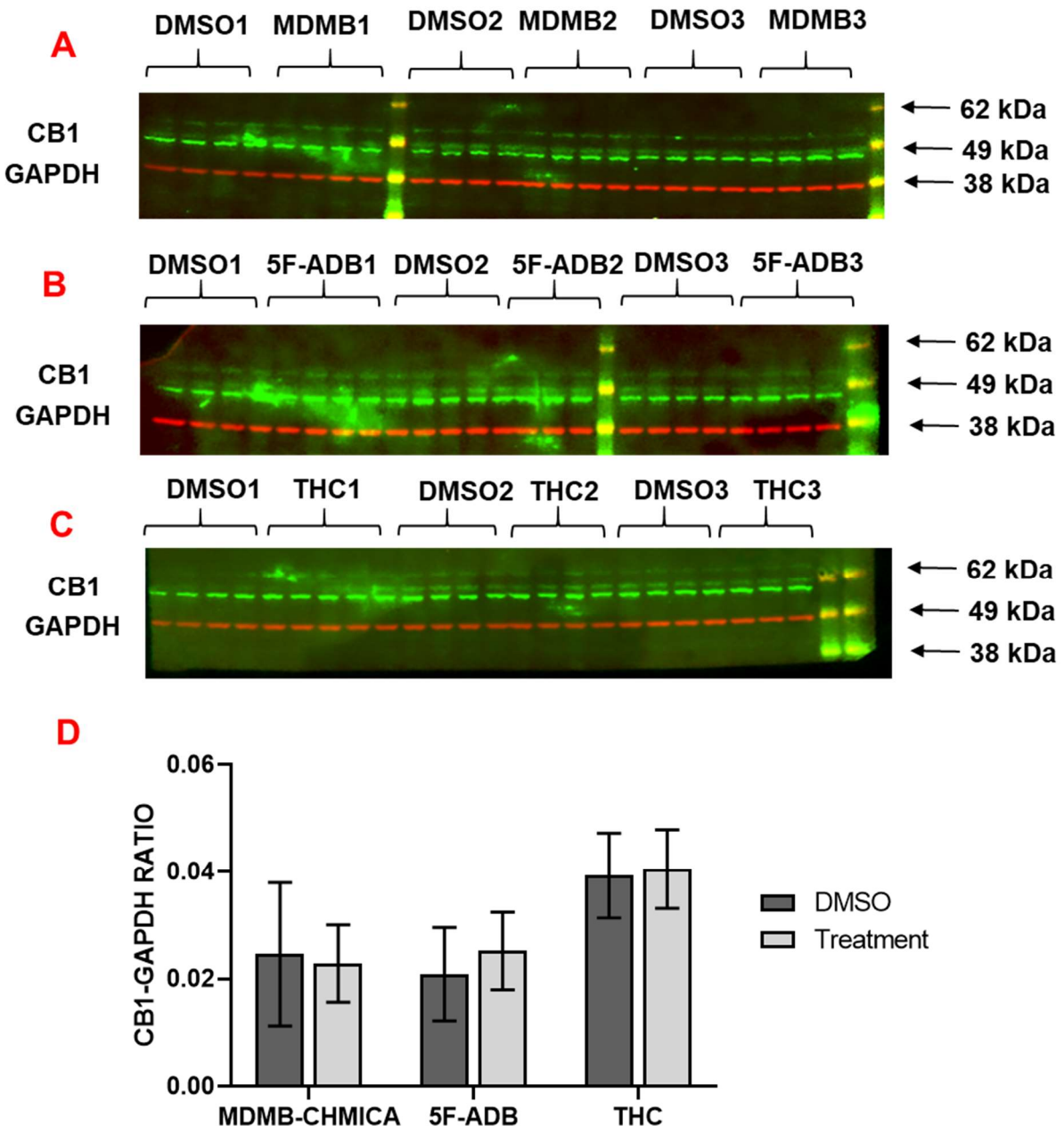


Figure 4.30 Expression of the CB1a isoform receptor in mature cells treated with SCRA and ∆9- THC. Western blot for the expression of CB1 receptor in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

4.4.3.2 Neuronal and astrocytic marker expression

To assess the effect of exposure to MDMB-CHMICA or 5F-ADB on neurons and astrocytes, the proteins β III – tubulin and glial fibrillary acidic protein (GFAP) were examined respectively. The cytoskeletal protein β III - tubulin is expressed in neuronal cells, but not glial cells (Sullivan, 1988). GFAP is the astrocytic marker glial fibrillary

acidic protein (GFAP) which is noted to increase in gliosis as a response to brain injury or stress (Nolte et al., 2001).

Western Blot analysis showed a single band at approximately 50 kDa for β III -tubulin, as shown in Figure 4.31 and this was not significantly different between treatment groups and control ($p>0.05$).

Two bands were shown for GFAP between 40-50 kDa (Figure 4.32), the top and bottom band were analysed and are subsequently referred to as GFAP isoform 1 and 2, respectively. GFAP isoform 1 & 2 were not statically different for MDMB-CHMICA or 5F-ADB treated cells compared to controls ($p>0.05$). Following ∆9-THC treatment, however isoform 1 expression was significantly increased by 20% ($p= 0.003$) but there was no significant effect for GFAP isoform 2 for the same cells ($p=0.27$). Similarly, the sums of the 2 isoforms showed that total GFAP expression was higher in ∆9-THC treated cells compared to controls ($p=0.003$) but there was no effect with MDMB-CHMICA or 5F-ADB.

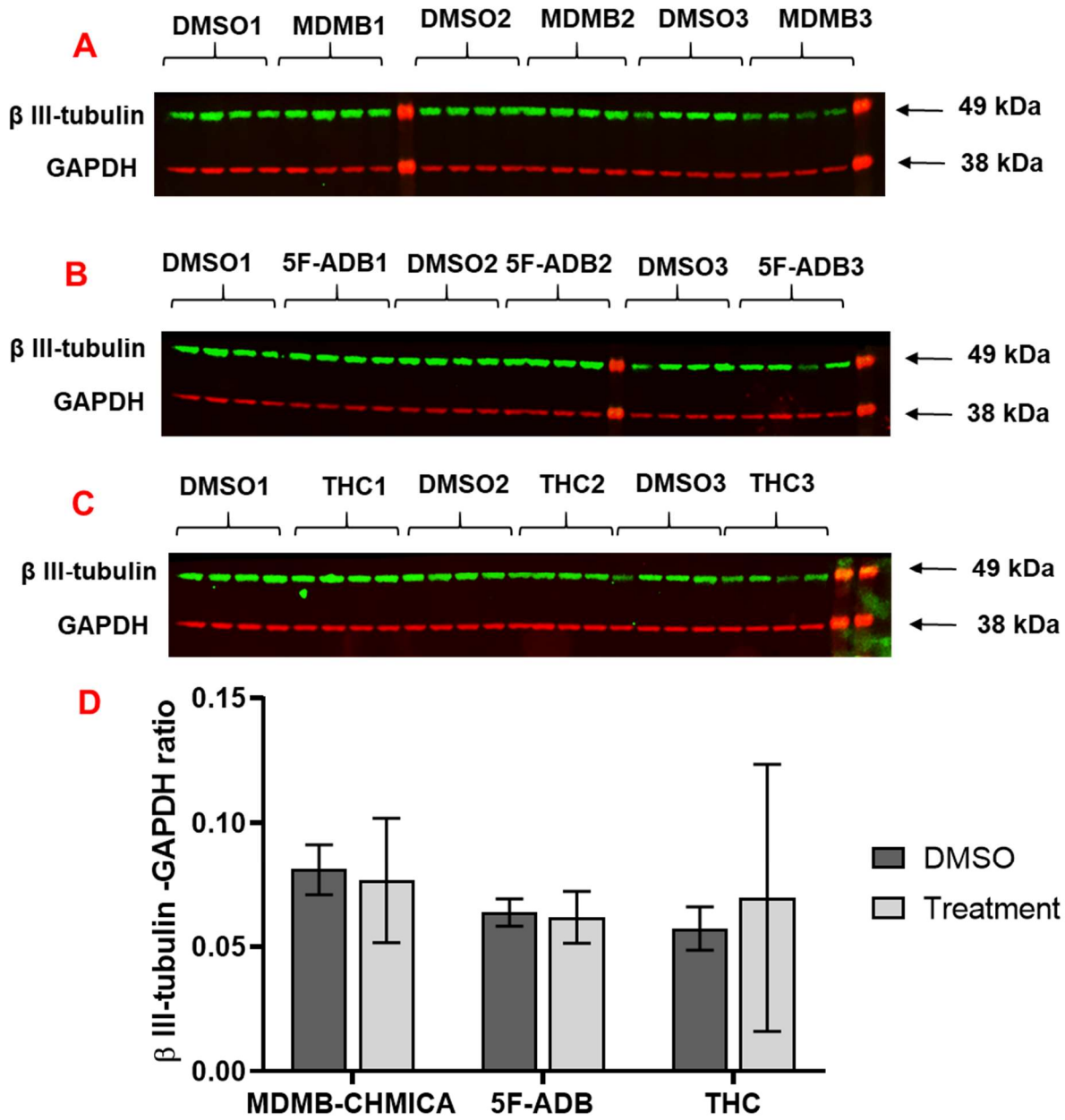


Figure 4.31 Expression of βIII-tubulin in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of β-tubulin in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

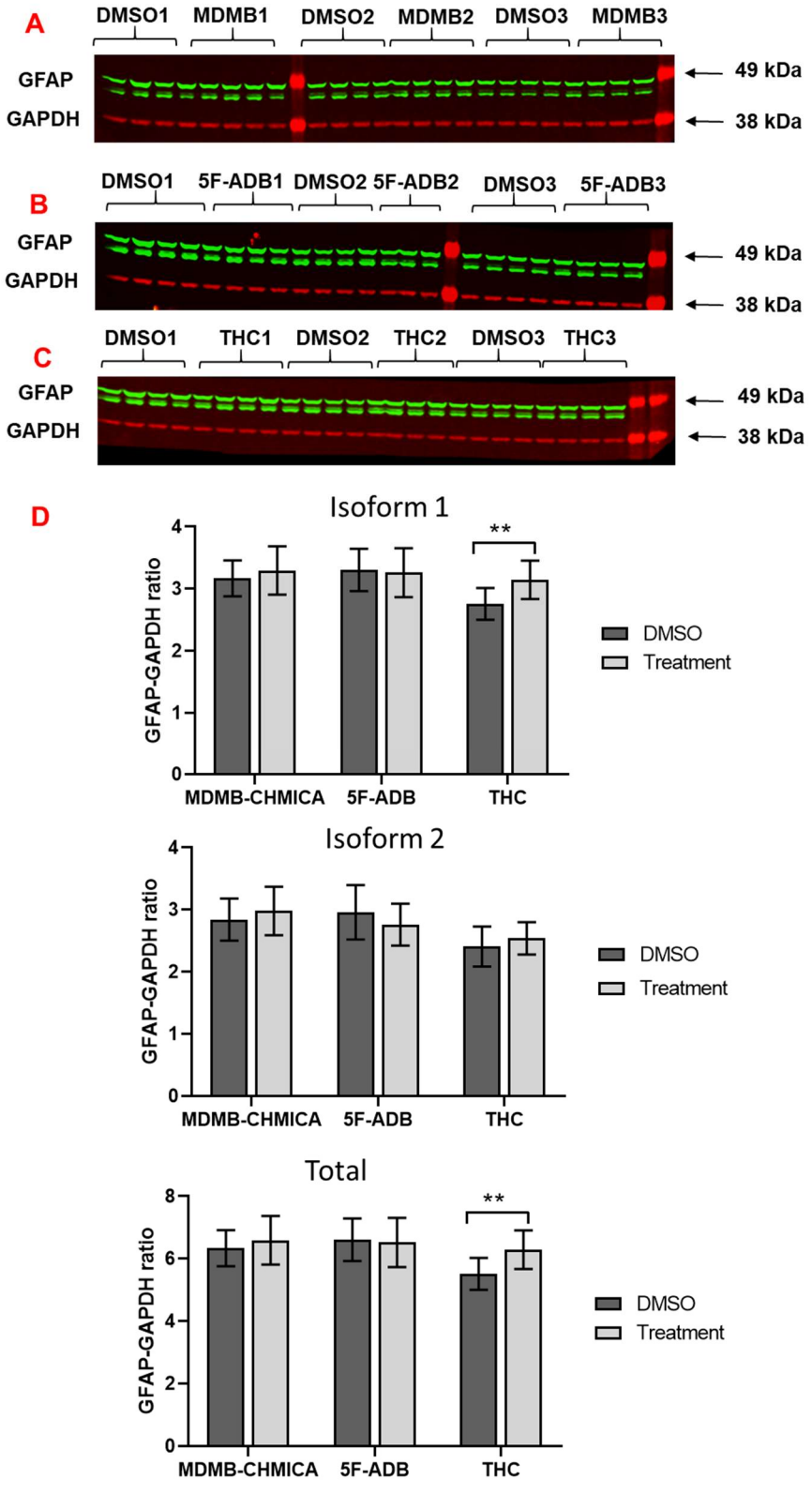


Figure 4.32 Expression of GFAP in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of GFAP in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test. ** represent P<0.01.

4.4.3.3 Mitochondrial markers expression

The differential gene expression analysis showed that the most significant changes were in mitochondrial tRNA (MT-tRNA) across SCRA and ∆9-THC. MT-tRNA are 22 genes that are encoded by MT-DNA which are required for protein synthesis in the mitochondria (Suzuki et al., 2011). Mitochondrial proteins are necessary to produce the key machinery of the oxidative phosphorylation system. This is especially important in organs with high energy consumption, such as the brain. Defects in MT-tRNA genes lead to mitochondrial dysfunction and numerous pathologies such as brain malformation and many metabolic defects (Kanungo et al., 2018, Bohnsack and Sloan, 2018). Mutations in MT-tRNA affect the mitochondrial respiratory chain because the mutation will lead to no, or incorrect, amino acid integration into complex I, III, IV, and V proteins. The most affected proteins will be those that are dependent on the affected MT-tRNA. In addition, MT-tRNA are often positioned adjacent to a subunit gene, and the mutation may influence the polycistronic transcript processing that might affect respiratory complexes (Triepels et al., 2001).

The most significant change in the 3 treatment groups affected mitochondrially encoded tRNA isoleucine (MT-TI), and mutations in this gene are related to complex IV deficiency. Therefore, analysis of potential mitochondrial protein changes focused respiratory complexes I and IV.

Mitochondrial complex I NADH-ubiquinone oxidoreductase subunit A9 (NDUFA9) and NADH-ubiquinone oxidoreductase subunit (NDUFS1) which are located in the inner membrane of the mitochondria were investigated. They are responsible for electron transfer to the respiratory chain from nicotinamide adenine dinucleotide (NADH) (Pagniez-Mammeri et al., 2012).

Complex I NDUFA9 (Figure 4.33) showed a single band at approximately 37 kDa with no significant differences between the treated cells and DMSO treated cells (controls) in the MDMB-CHMICA and 5F-ADB treated cells. There was a 16% reduction in NDUFA9 expression in ∆9-THC treated cells with respect to control, but this was not statistically significant ($p>0.05$).

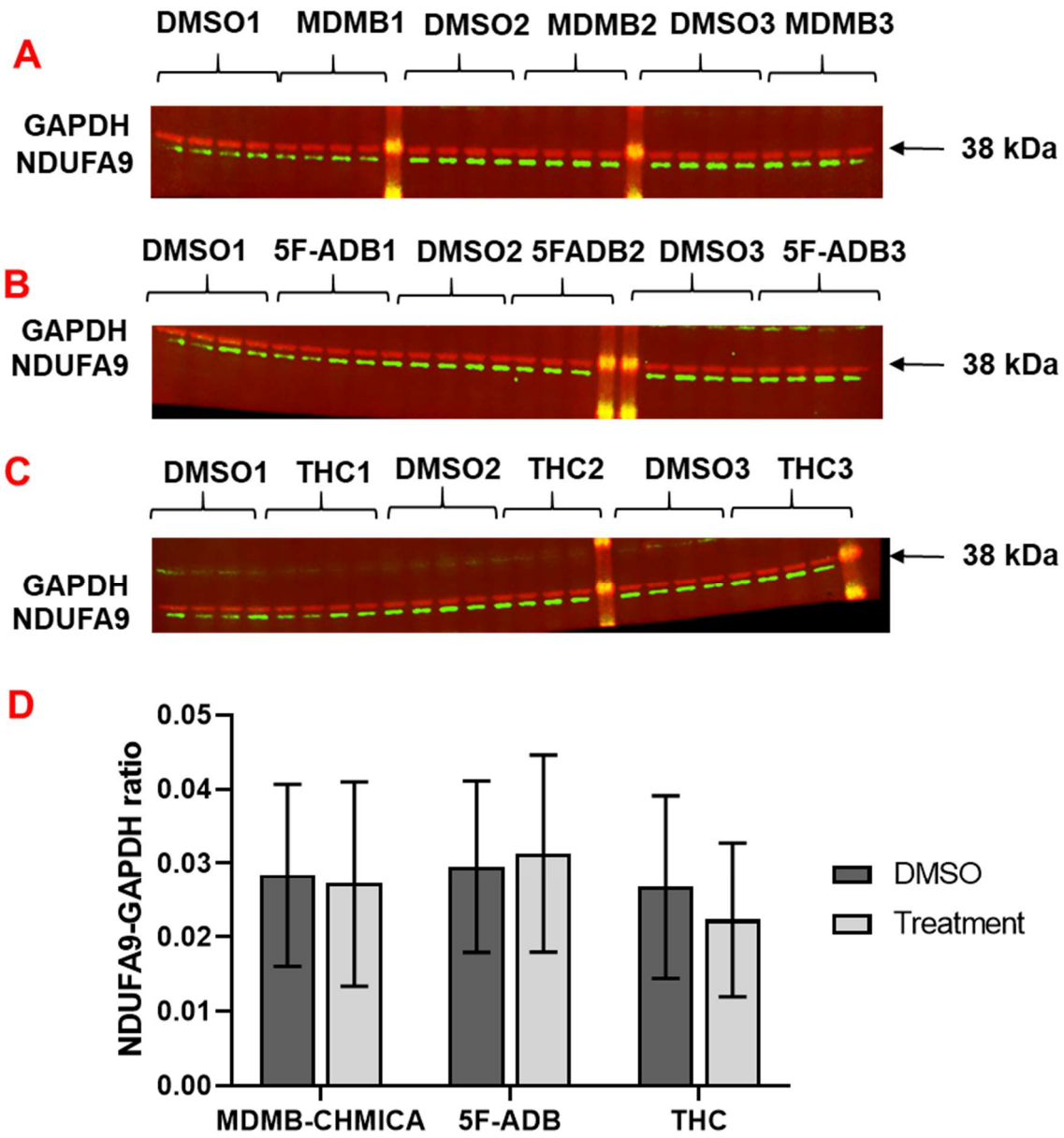


Figure 4.33 Expression of NDFA9 in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of NDFA9 in mature neuronal treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

Western Blot analysis showed a single band at approximately 75kDa for NDUFS1 and this was not significantly different between treated cells and control cells, as shown in Figure 4.34.

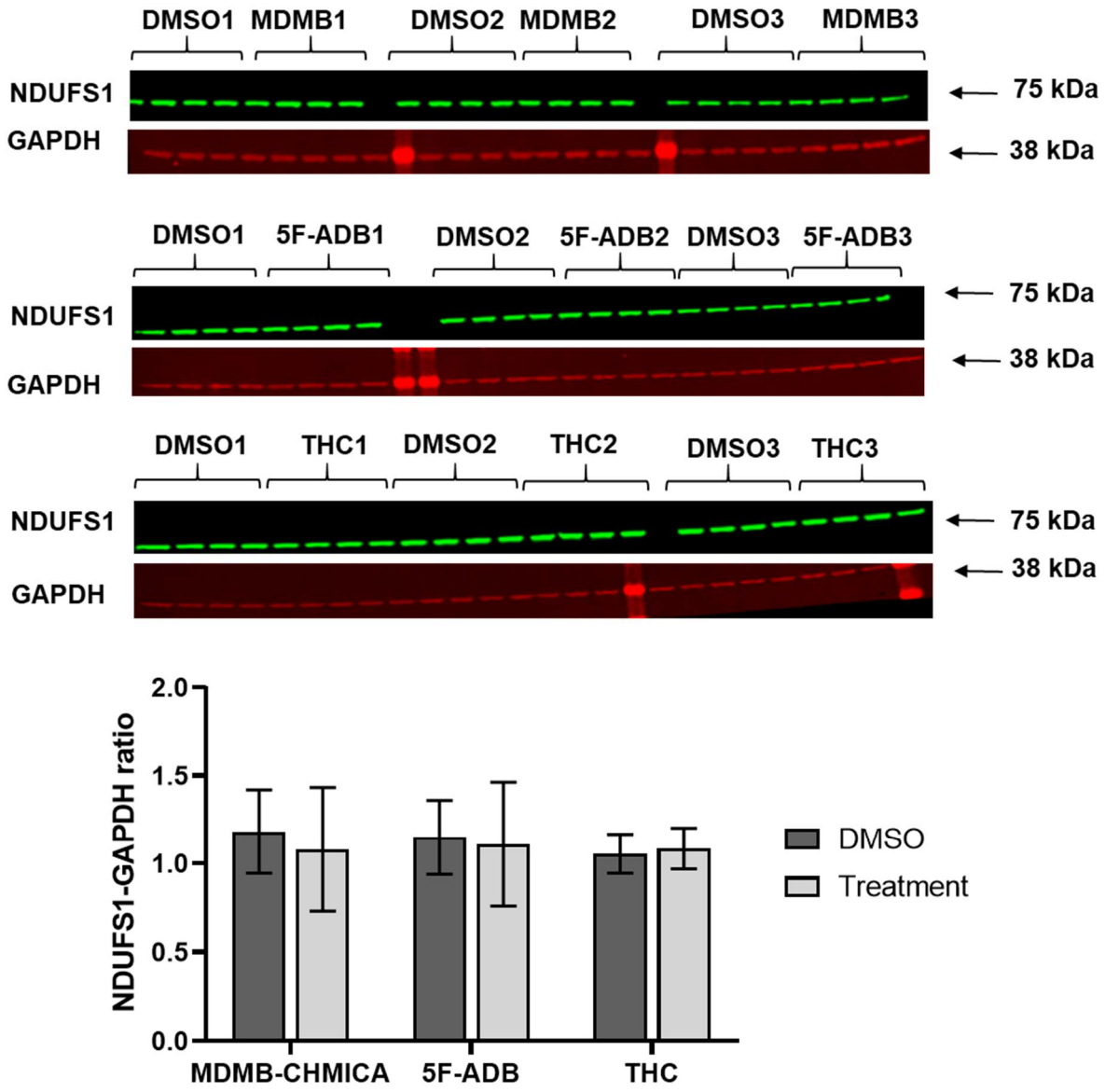


Figure 4.34 Expression of NDUFS1 in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of NDFS1 in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

Cytochrome c oxidase subunit 5a (CoxVa) and mitochondrially encoded cytochrome c oxidase I (MTCO1) subunits of the electron transport chain of the mitochondria (complex IV) were also verified using western blotting.

Western blotting analysis detected COX Va at approximately 14 kDa. COX Va was 30% significantly higher in MDMB-CHMICA treated cells compared to control (p=

0.028), while no significant change was observed in 5F-ADB and ∆9-THC treated cells compared to control (Figure 4.35).

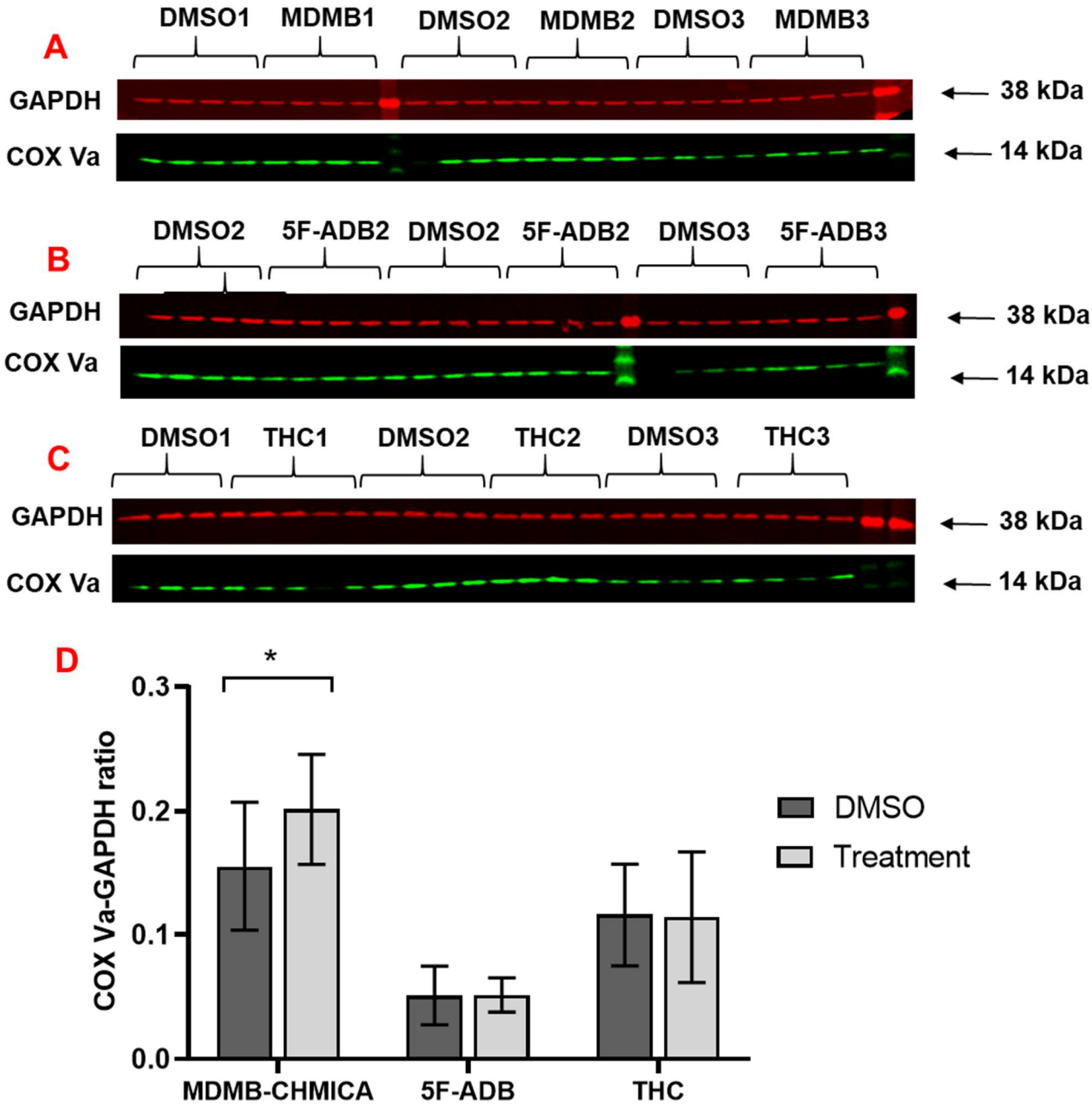


Figure 4.35 Expression of COX Va in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of COX Va in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and THC **(C)** for 14 days. Data presented as Mean ± SD quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test. * represents $p<0.05$.

MTCO1 was demonstrated as single band at approximately 33 kDa. MTCO1 was 14% higher in MDMB-CHMICA treated cells compared to control, but this change was not statistically significant (P>0.05). There was also no significant difference in MTCO1 expression among the 5F-ADB and ∆9-THC with respect to control as shown in Figure 4.36.

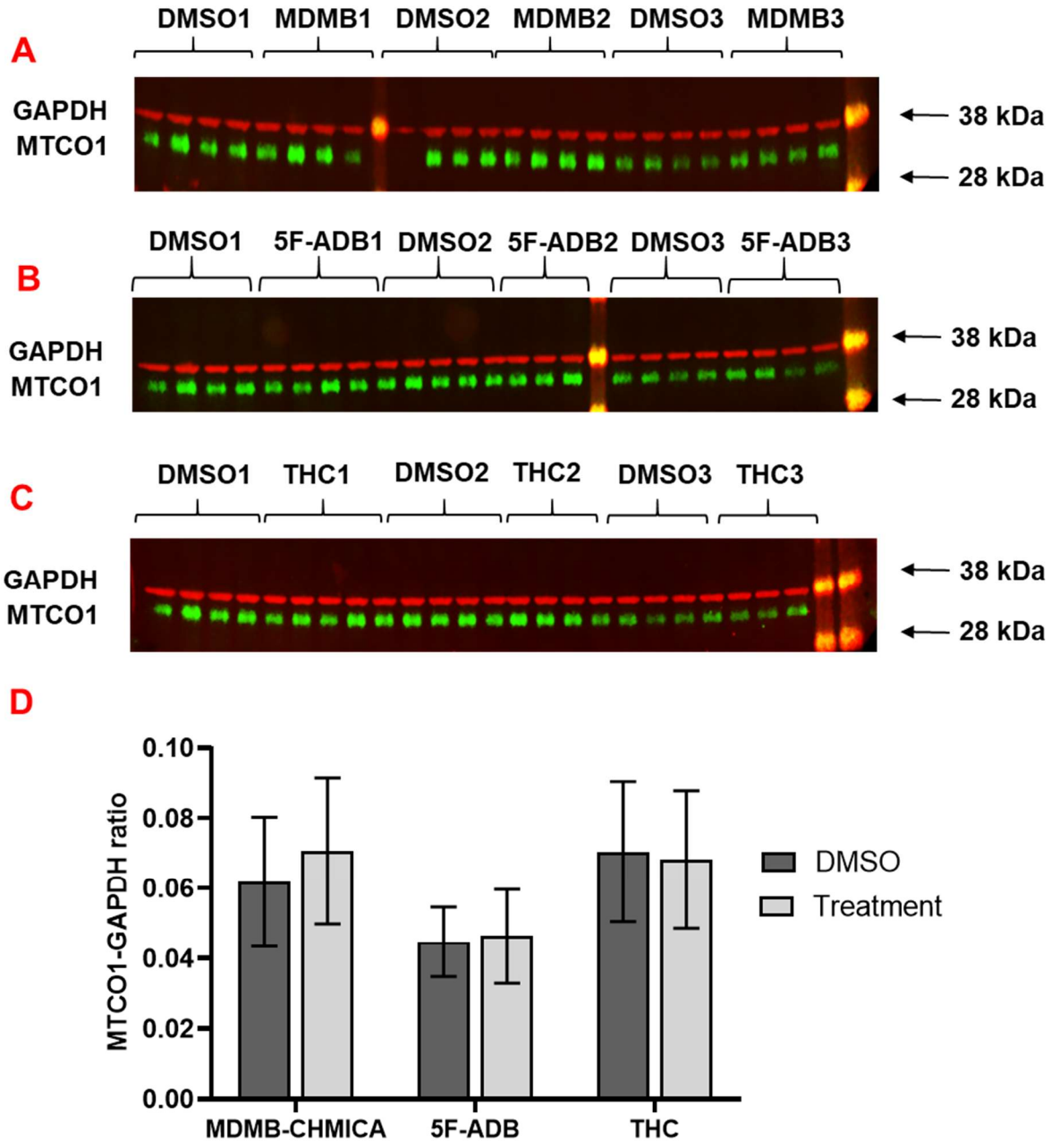


Figure 4.36 Expression of MTCO1 in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of MTCO1 in mature neuronal treated with 10 nM of MDMB-CHMICA (A), 5F-ADB (B) and ∆9-THC (C) for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) (D). Statistical significance was evaluated by independent sample t-test.

Voltage-dependent anion channel 1 (VDAC1) was assessed by western blotting. VDAC1 is located in the mitochondrial outer membrane and plays an important role in controlling calcium homeostasis and apoptosis (Shoshan-Barmatz et al., 2018). A single band at approximately 30 kDa was demonstrated for VDAC1. There was no significant differences between MDMB-CHMICA, 5F-ADB and ∆9-THC with respect to control ($p>0.05$), but there was a trend of increment with synthetic cannabinoids MDMB-CHMICA and 5F-ADB of 11% ($p=0.07$) and 14% ($p=0.09$), respectively, while with ∆9-THC it was less than 1% increment as shown in Figure 4.37.

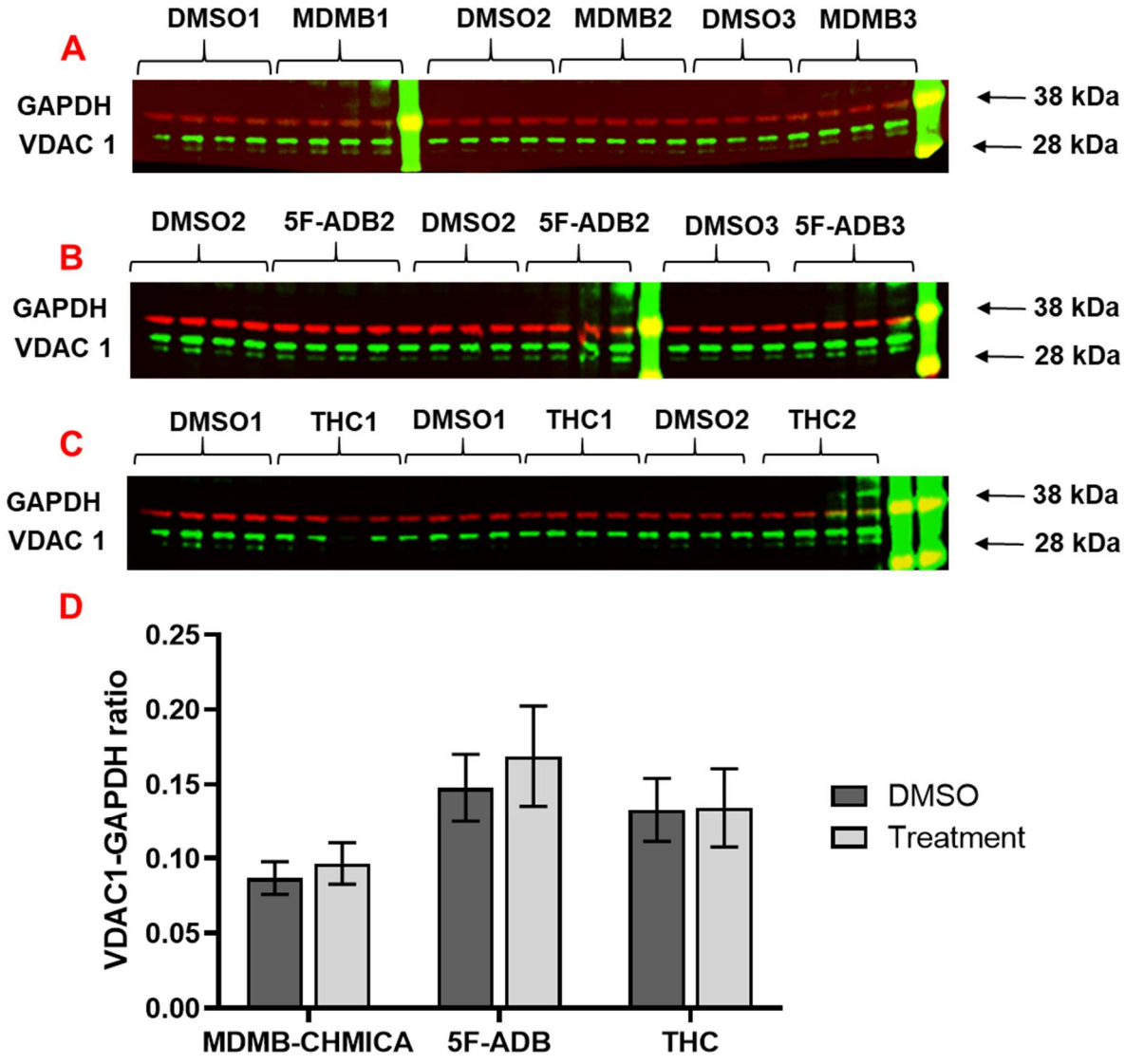


Figure 4.37 Expression ofVDAC1 in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of VDAC1 in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A),** 5F-ADB **(B)** and ∆9-THC for 14 days **(C)**. Data presented as Mean ± SD quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

4.4.3.4 Endoplasmic- reticulum (ER) stress markers

The IPA analysis showed that the cells exposed to 10 nM of MDMB-CHMICA, 5F-ADB and ∆9-THC have increased protein synthesis acting through different pathways such as the inhibition of SUMOylation pathway, the activation of EIF2 signalling and Notch signalling. The increase in protein synthesis may trigger ER stress. To investigate the effects on ER stress, the markers binding immunoglobulin protein (BiP), ER resident protein disulphide isomerase (PDI) and C/EBP homologous (CHOP) were examined.

BiP which acts as a chaperone positioned in the ER lumen and interacts with newly synthesised proteins and plays an important role in the protein folding process during ER response (Ni and Lee, 2007), was demonstrated as a single band at approximately 62 kDa. There were no significant differences in BIP expression among the 3 treatment group ($p>0.05$) despite a 12% increase in BIP protein expression in MDMB-CHMICA treated cells, as shown in Figure 4.38.

ER resident protein disulphide isomerase (PDI), which has an essential role in protein folding quality control (Bottomley et al., 2001), was demonstrated as single band at 55 kDa. There were no significant differences in PDI expression among the 3 treatment groups ($p>0.05$) (Figure 4.39). Unfortunately, CHOP antibody did not work when tested by western.

The extracellular signal-regulated protein kinases 1 and 2 phospho (ERK1/2), which mediate cell proliferation and cell death (Mebratu and Tesfaigzi, 2009), were also investigated. Western blotting analysis showed 2 bands at approximately 42 and 44 kDa (Figure 4.40); the top bands were too weak to be analysed and these correspond to phospho-ERK2. The bottom band which corresponds to phospho-ERK1, was analysed. Phospho-ERK1 was not statistically significant in the 3 treatment groups with respect to DMSO vehicle treated cells ($P>0.05$).

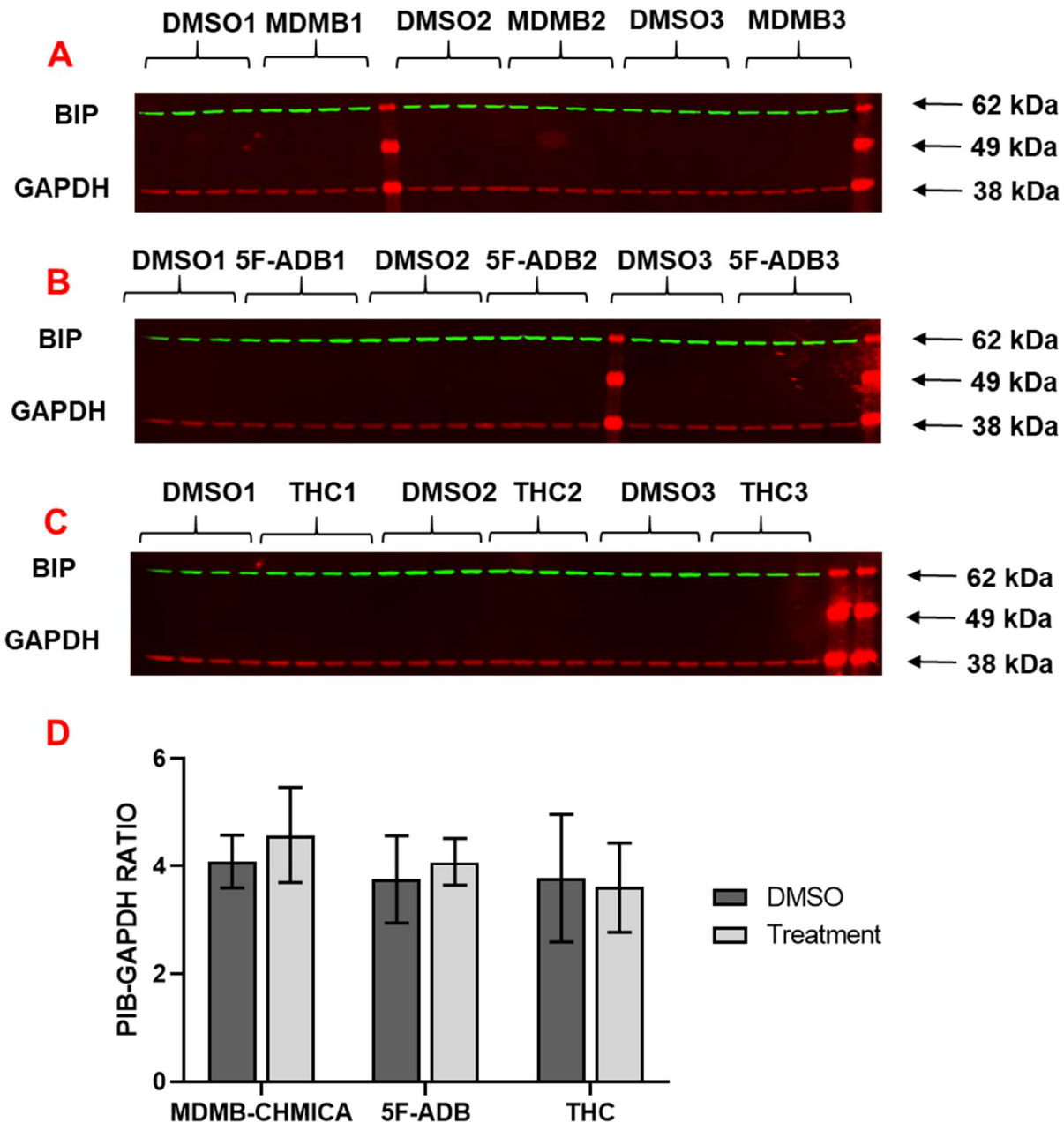


Figure 4.38 Expression of BIP in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of BIP in mature neuronal cells treated with 10 nM of MDMB-CHMICA (A), 5F-ADB (B) and ∆9-THC (C) for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) (D). Statistical significance was evaluated by independent sample t-test.

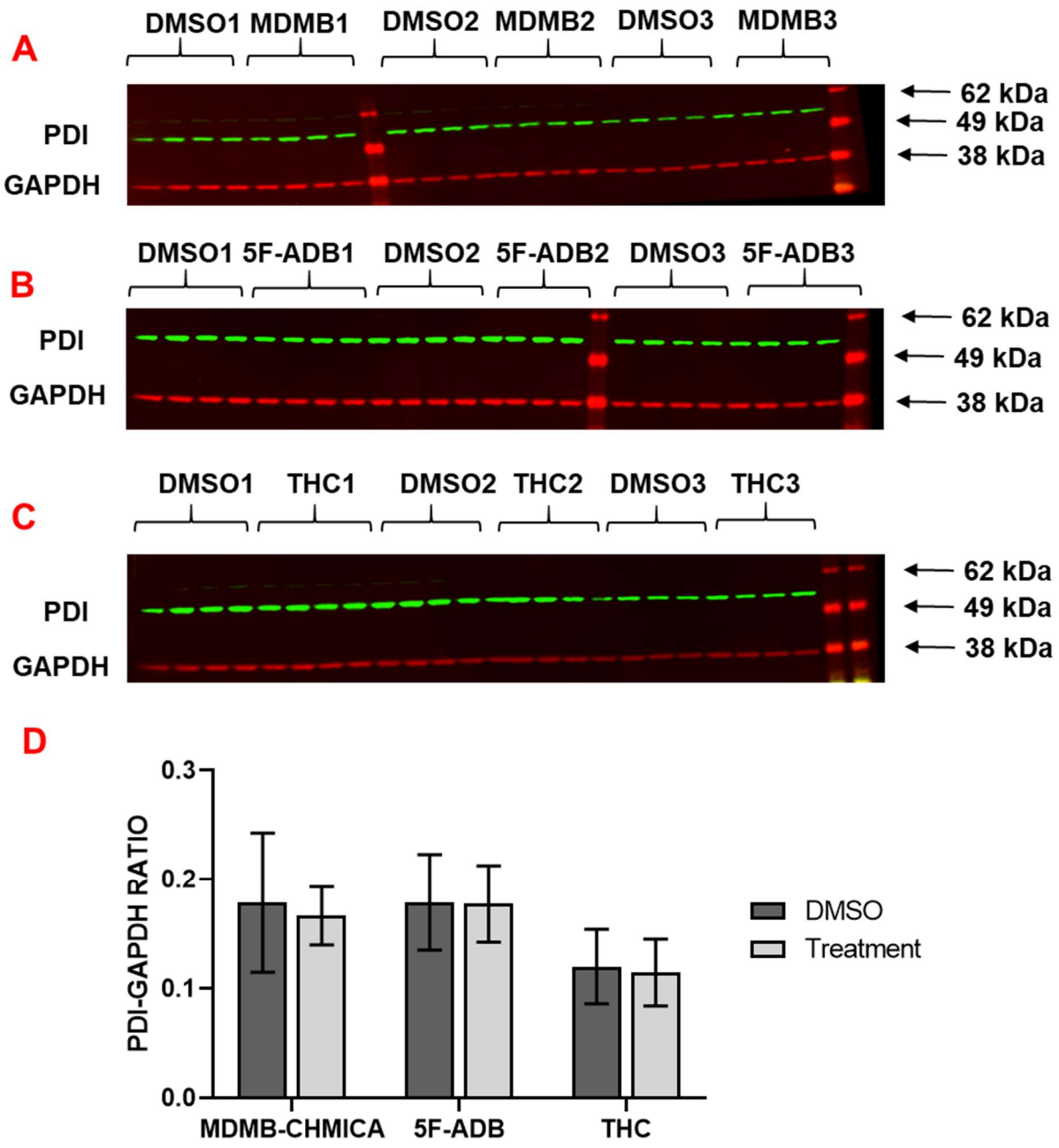


Figure 4.39 Expressionof PDI in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of PDI in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

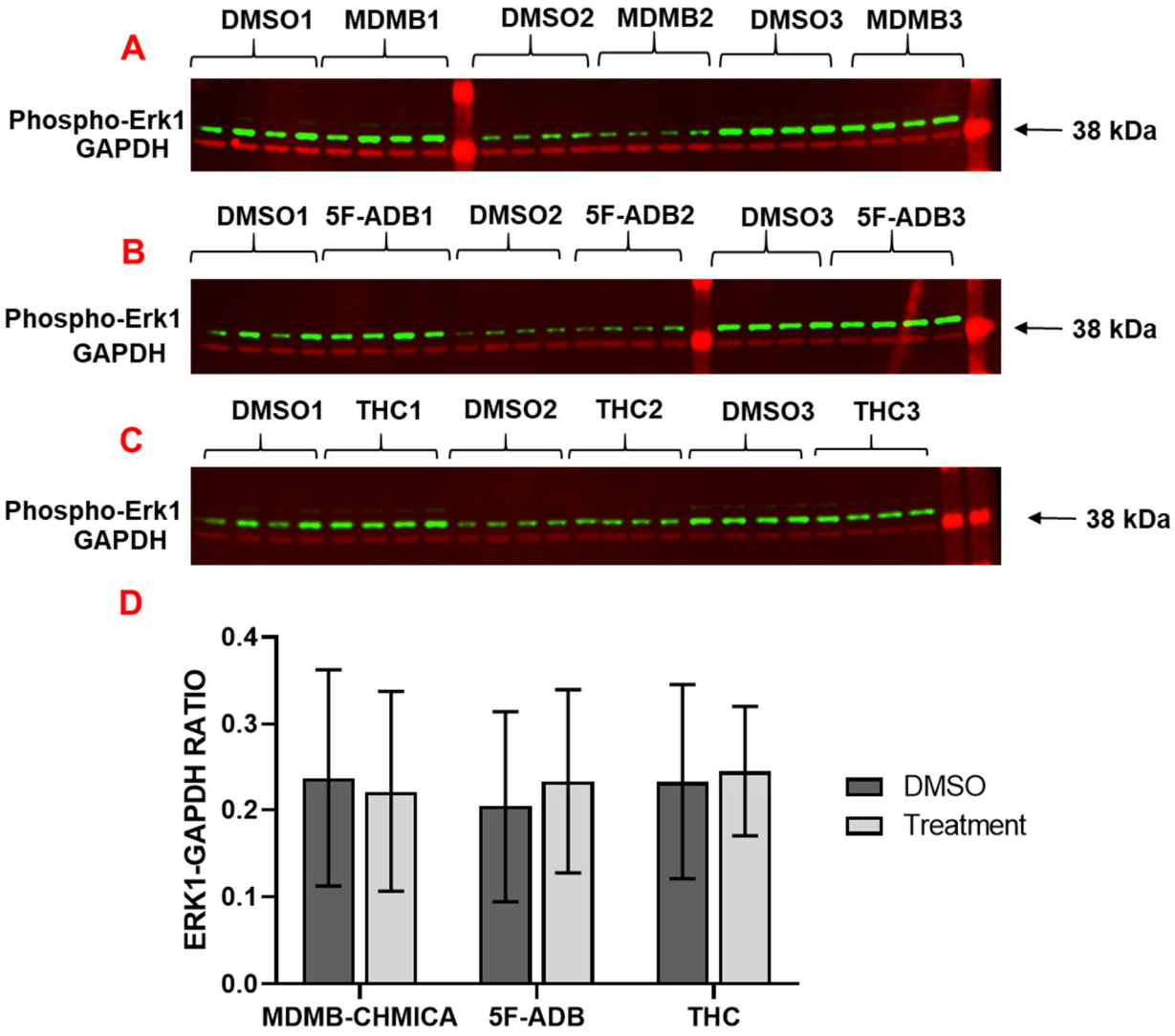


Figure 4.40 Expression of phospho-Erk1 in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of ERK1 in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A),** 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

4.4.3.5 Metabolic enzymes

The RNA sequencing analysis demonstrated that exposure to cannabinoids can have an effect on the levels of metabolising enzymes. In MDMB-CHMICA treated cells the enzymes CYP17A1 and CYP27B1 were 2-3 times upregulated with p value of 0.04 and 0.03 respectively (appendix I). In ∆9-THC treated cells CYP17A1 was 4 times upregulated (p =0.004) and the enzyme glutathione S-transferase mu 4 (GSTM4) was slightly upregulated (p= 0.04). Therefore, it was decided to investigate potential changes in metabolising enzymes, since these substances might be metabolised into toxic metabolites or affect the metabolizing enzymes involved in biotransformation of other substances.

NADPH-cytochrome P450 reductase (CYPOR) is an endoplasmic reticulum enzyme responsible for the metabolism of drugs and xenobiotic through its role in supplying electrons to microsomal P450 and other proteins such as heme oxygenase (Pandey and Flück, 2013). CYPOR was demonstrated as a single band at approximately 70 kDa (Figure 4.41). There was no significant difference in CYPOR expression among the 3 treatment group in comparison to DMSO vehicle treated cells ($p>0.05$).

Cytochrome P450 3A4 (CYP3A4) and Cytochrome P450 2E1 (CYP2E1) are phase I metabolising enzymes that are involved in xenobiotic and drugs metabolism (Rendic and Carlo, 1997). UDP-glucuronosyltransferase 1-1 (UGT1A1) and UDP-glucuronosyltransferase 1-6 (UGT1A6) are phase II metabolising enzymes that are responsible of detoxification of many drugs and hormones by conjugation with glucuronide to increase water solubility ad enhance excretion (Tukey and Strassburg, 2000). CYP3A4, CYP2E1, UGT1A1 and UGT1A6 enzymes were tested but, unfortunately it did not work due to poor quality antibodies.

Glutathione-S-transferases (GSTs) are a group of phase II metabolising enzymes that are involved in the metabolism of xenobiotic by catalysing the conjugation of glutathione via a sulfhydryl group to a substrate to make the compound more water soluble (Boyer, 1989). GSTP1 was demonstrated as a single band at approximately 23 kDa (Figure 4.42). GSTP1 expression decreased in MDMB-CHMICA, 5F-ADB and ∆9-THC treated cells compared to control. ∆9-THC treated cells showed a significant 39% reduction ($p= 0.007$), but the reduction of 8% was not significant for both MDMB-CHMICA and 5F-ADB ($p>0.05$).

The Aryl hydrocarbon receptor (AHR), which is a transcription factor that regulates the gene expression of xenobiotic metabolising enzymes for example cytochrome P450 family 1 (Nebert, 2017), was investigated as AHR pathway was inhibited in ∆9-THC exposed cells. AHR was demonstrated as a single band at approximately 96kDa (Figure 4.43). There was no significant difference in AHR expression among the 3-treatment group in comparison to DMSO vehicle treated cells ($p>0.05$).

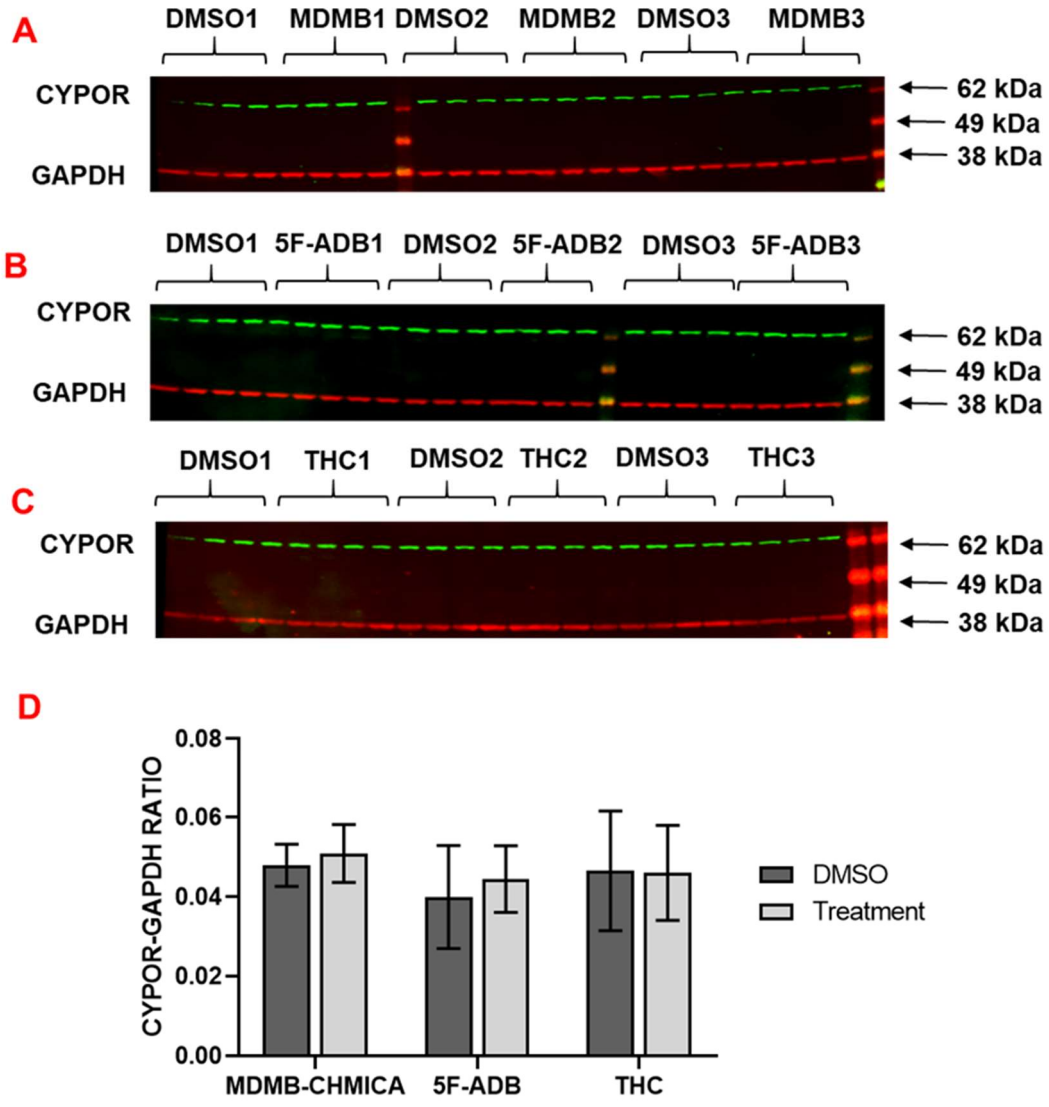


Figure 4.41 Expression of CYPOR in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of CYPOR in mature neuronal cells treated with 10 nM of MDMB-CHMICA (A), 5F-ADB (B) and ∆9- THC(C) for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) (D). Statistical significance was evaluated by independent sample t-test.

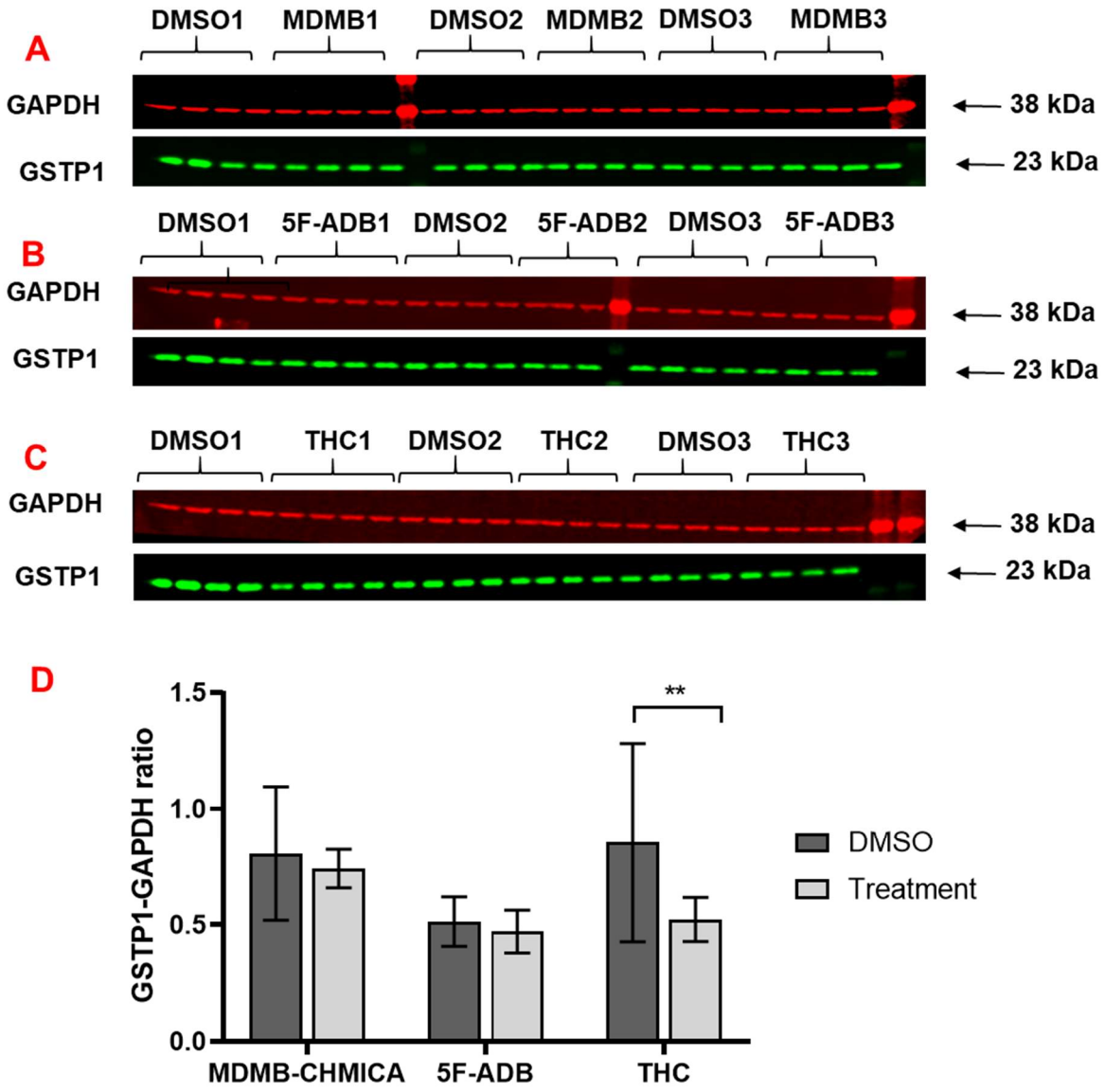


Figure 4.42 Expression of GSTP1 in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of GSTP in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9- THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test. ** represents $p<0.01$.

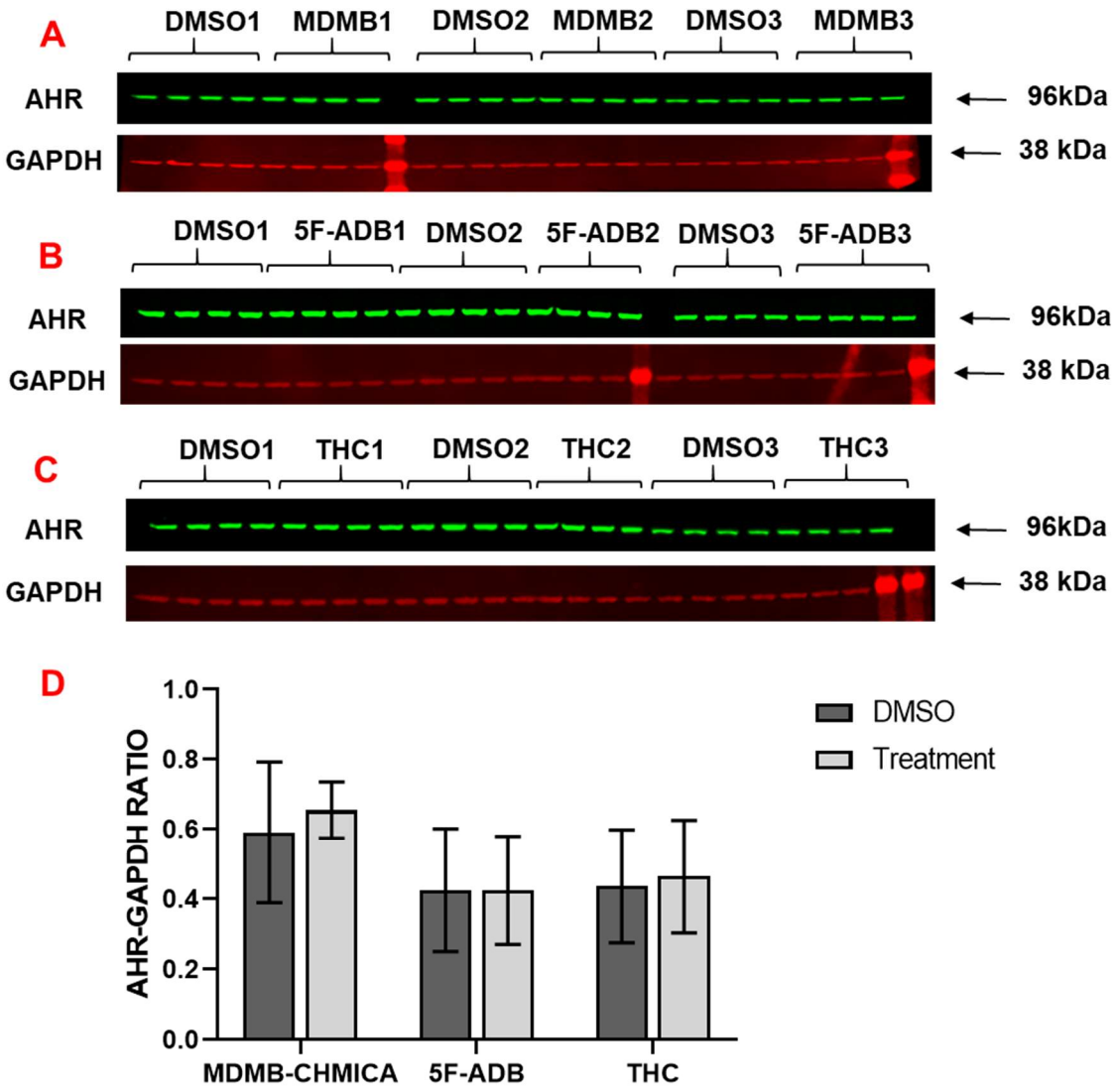


Figure 4.43 Expression of AHR in mature cells treated with SCRA and ∆9-THC. Western blot for the expression of AHR in mature neuronal cells treated with 10 nM of MDMB-CHMICA **(A)**, 5F-ADB **(B)** and ∆9-THC **(C)** for 14 days. Data presented as Mean ± SD from quadruplicate samples (assay repeated n=3) **(D)**. Statistical significance was evaluated by independent sample t-test.

4.4.4 Neurotransmission

There is little known about the direct effect of SCRA on other neurotransmitters. Therefore, the effect of the 3 synthetic cannabinoids (MDMB-CHMICA, MMB-CHMICA and 5F-ADB) as well as ∆9-THC, MDMA, and the combined exposure of MDMA and SCRA was investigated. Since the serotonergic system might contribute to SCRA adverse effects. 5-HT_{2A} receptor and monoamine enzymes (MAO) were investigated.

The 5-HT_{2A} receptor is distributed in the CNS and has a role in cognitive impairment in mental disorders such as schizophrenia and depression and mediates the psychoactive effects of the phenylisopropylamine hallucinogens (Kroeze and Roth, 1998, Titeler et al., 1988).

The 5-HT_{2A} was demonstrated as a single band at approximately 50 kDa (Figure 4.44). There was no significant difference in 5-HT_{2A} expression among the 6 treatment groups in comparison to DMSO vehicle treated cells ($p>0.05$). It is noteworthy that there was a 10-15 % reduction in 5-HT_{2A} protein expression across the 3 SCRA treatment groups and the mixed exposure cells, whereas ∆9-THC treated cells showed 1% reduction only.

MAO A and MAO B were tested; MAO A antibody was not reactive, with no bands detected by western blotting, while MAO B demonstrated a band at approximately 59 kDa as shown in Figure 4.45. There was a general increase in MAO B expression among all the treated cells (more than 10%); the mixed exposure treated cells (MDMB-CHMICA, 5F-ADB and MDMA) showed the highest increase of up to 26% compared with control but was not statistically significant ($p>0.05$).

Dopamine- and cAMP-regulated neuronal phosphoprotein (DARRP 32), which is a protein phosphatase inhibitor and acts as a key target for dopamine neuronal signalling (Fernandez et al., 2006), was also tested.

DARRP 32 was demonstrated as four bands between 42 and 32 kDa (Figure 4.46). All the bands were analysed and will subsequently be referred as DARRP 32 isoforms 1, 2, 3 and 4 respectively. There were no significant difference in DARRP 32 expression among the 6 treatment groups in comparison to DMSO vehicle treated cells ($p>0.05$).

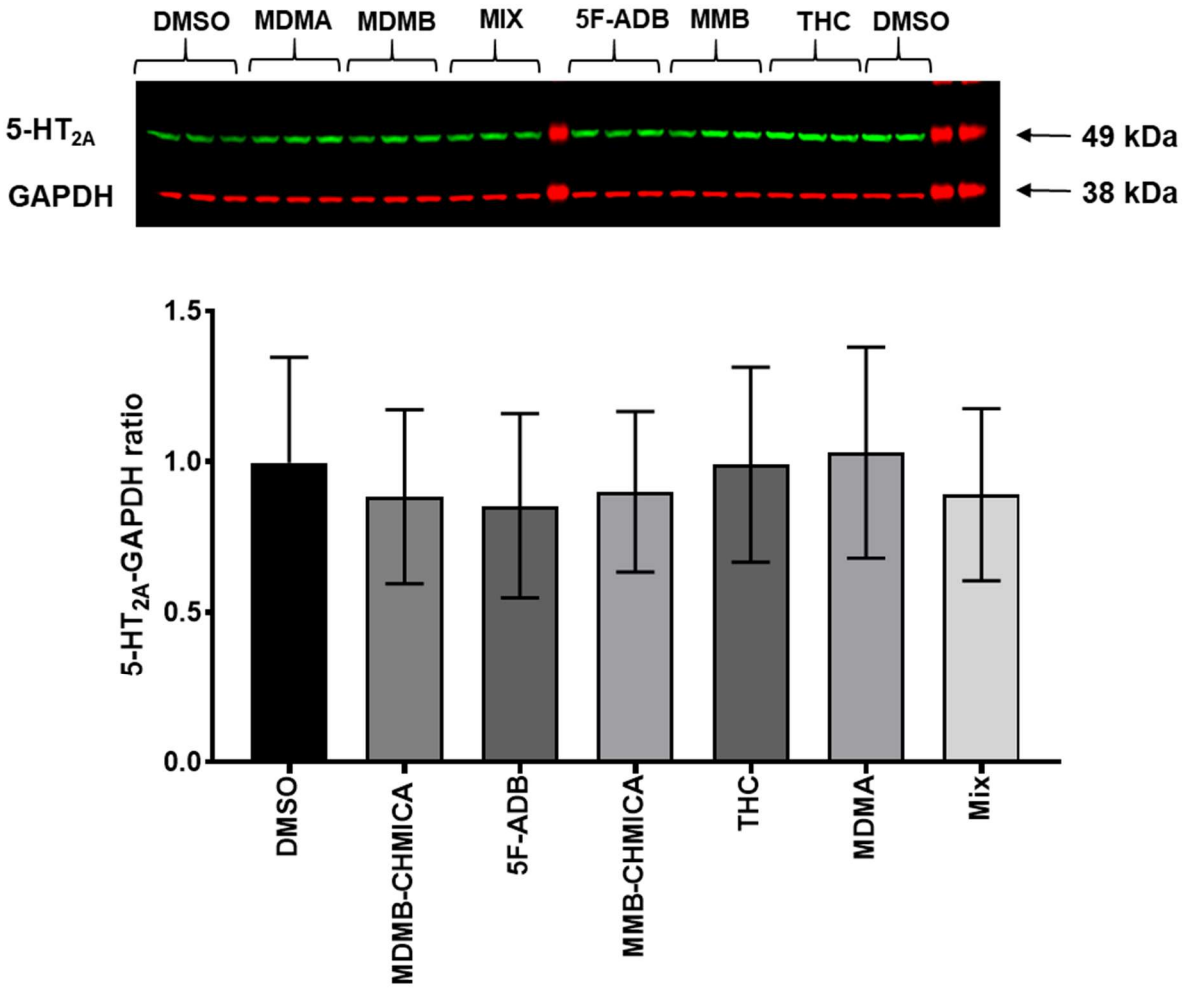


Figure 4.44 Expression of SR2A receptor in mature cells treated with SCRA, ∆9-THC MDMA and mixed exposure. Western blot for the expression of SR2A receptor in mature hNPSC cells treated with separately to 10 nM of 10 nM MDMB-CHMICA, 5F-ADB, 10 nM MMB-CHMICA, 10 nM ∆9-THC, 10 nM MDMA (ecstasy) and to a combination of SCRA and MDMA (3 nM 5F-ADB combined with 3 nM MDMB-CHMICA and 3nM MDMA) for 14 days. Data presented as mean ± SD from triplicate samples (assay repeated n=3). Statistical significance was evaluated by independent sample t-test.

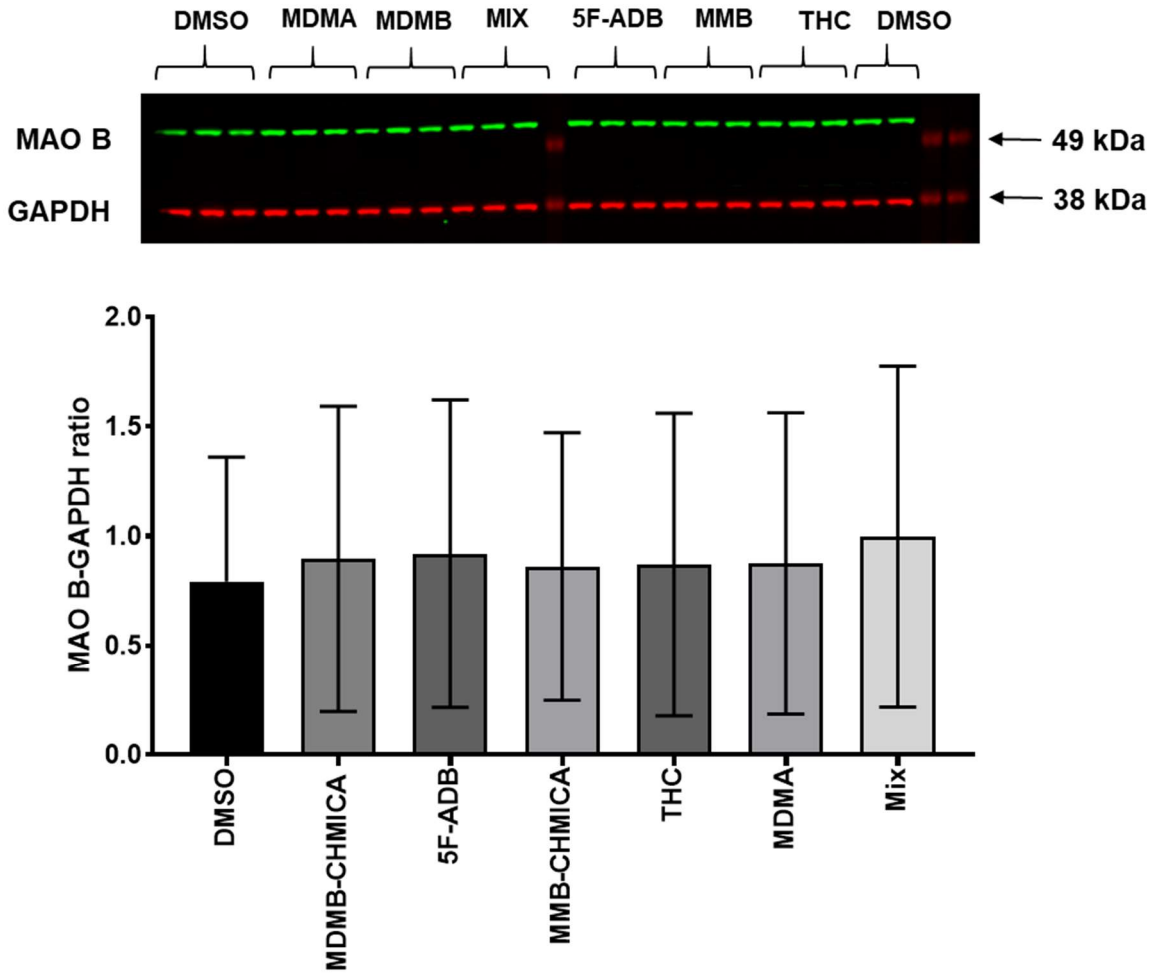


Figure 4.45 Expression of MAO B in mature cells treated with SCRA, ∆9-THC ecstasy and mixed exposure. Western blot for the expression of MAO B in mature hNPSC cells treated with separately to 10 nM of 10 nM MDMB-CHMICA, 5F-ADB, 10 nM MMB-CHMICA, 10 nM ∆9-THC, 10 nM MDMA (ecstasy) and to a combination of SCRA and MDMA (3 nM 5F-ADB combined with 3 nM MDMB-CHMICA and 3nM MDMA) for 14 days. Data presented as Mean ± SD from triplicate samples (assay repeated n=3). Statistical significance was evaluated by independent sample t-test.

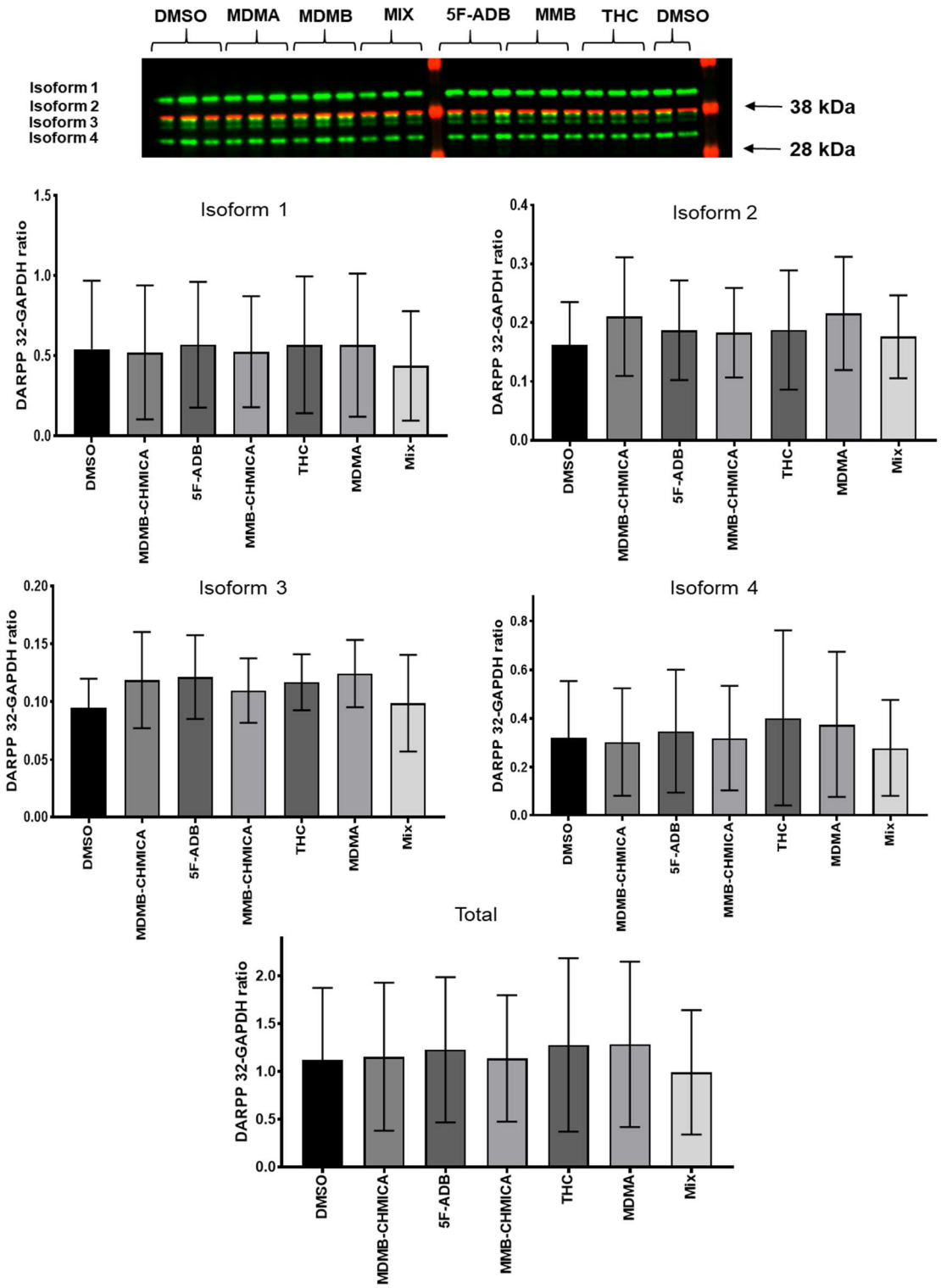


Figure 4.46 Expression of DARRP 32 in mature cells treated with SCRA, ∆9-THC ecstasy and mixed exposure. Western blot for the expression of DARRP 32 in mature hNPSC cells treated with separately to 10 nM of 10 nM MDMB-CHMICA, 5F-ADB, 10 nM MMB-CHMICA, 10 nM ∆9-THC, 10 nM MDMA (ecstasy) and to a combination of SCRA and MDMA (3 nM 5F-ADB combined with 3 nM MDMB-CHMICA and 3nM MDMA) for 14 days.. Data presented as Mean ± SD from triplicate samples (assay repeated n=3). Statistical significance was evaluated by independent sample t-test.

4.4.5 Preliminary metabolism experiments

The RNAseq results showed changes in some cytochrome metabolising enzymes such as CYP17A1 and western blotting showed a significant reduction in GSTP1 protein expression. Therefore, the elucidation of the role of SCRA in inhibiting or activating metabolising enzymes is important from a clinical and toxicological point of view. To examine this a commonly prescribed and routinely examined painkiller (paracetamol) was examined for its metabolism profile after cells were exposed to cannabinoids. Therefore, paracetamol was used to examine if cannabinoid use can affect the metabolism of commonly used pharmaceuticals. Paracetamol is metabolised through both phase I and phase II pathways as Figure 4.47 briefly illustrates (Mazaleuskaya et al., 2015).

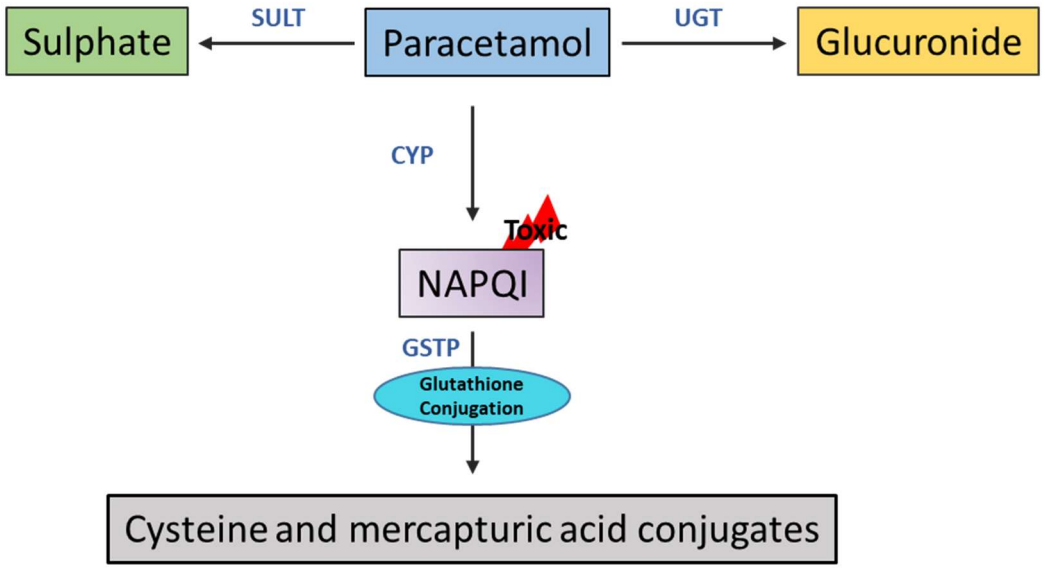


Figure 4.47 Summary of paracetamol metabolism. Where SULT is Sulfotransferase enzymes, UGT is UDP-glucuronosyltransferase, CYP is cytochrome P450 and NAPQI is N-acetyl-p-benzoquinone imine, the paracetamol toxic metabolite.

The analysis aimed to measure paracetamol concentration and the paracetamol metabolites sulphate, glucuronide and glutathione conjugates in both culture media and in cell lysate. Unfortunately, complete results were not obtained due to technical issues with the spectrometry unit due to blockage of the liquid chromatography column. Therefore, only paracetamol concentration in cell culture media was measured.

The preliminary data for paracetamol showed reductions in paracetamol concentration in MDMB-CHMICA, 5F-ADB and ∆9-THC treated cells compared to non –cannabinoids treated cells (control).

The paracetamol concentration in the media of non-treated cells was higher than the treated cells both immediately after cannabinoid exposure and after 48 hours wash out as shown in Table 4.10. Paracetamol concentration was decreased by 32% in MDMB-CHMICA, 80.5% in 5FADB and 85% in ∆9-THC treated cell culture media, in cells exposed to paracetamol immediately after cannabinoids treatment. However, it was only statistically significant in ∆9-THC exposed cells in comparison to non-cannabinoid treated cells (p= 0.028) (Figure 4.48). Paracetamol concentration was decreased by 78.9% in MDMB-CHMICA, 77.8% in 5FADB and 88.1% in ∆9-THC treated cell culture media, in cells exposed to paracetamol after 48 hours (wash-out) after cannabinoids treatment. However, it was only statistically significant in ∆9-THC exposed cells in comparison to non-cannabinoid treated cells (p= 0.024) (Figure 4.48).

	Paracetamol concertation in different treated cell media (ng/ml)			
Type of exposure	DMSO	MDMB-CHMICA	5F-ADB	∆9-THC
Immediately after Cannabinoid exposure	201	136	39	30
After 48 hours (wash-out)	185	39	41	22

Table 4.10 Paracetamol concentrations in culture media after exposure to MDMB-CHMICA, 5F-ADB and∆9-THC. Paracetamol concentration was measured either directly after the cells been exposed to cannabinoids for 14 days or after 48 hours wash out period.

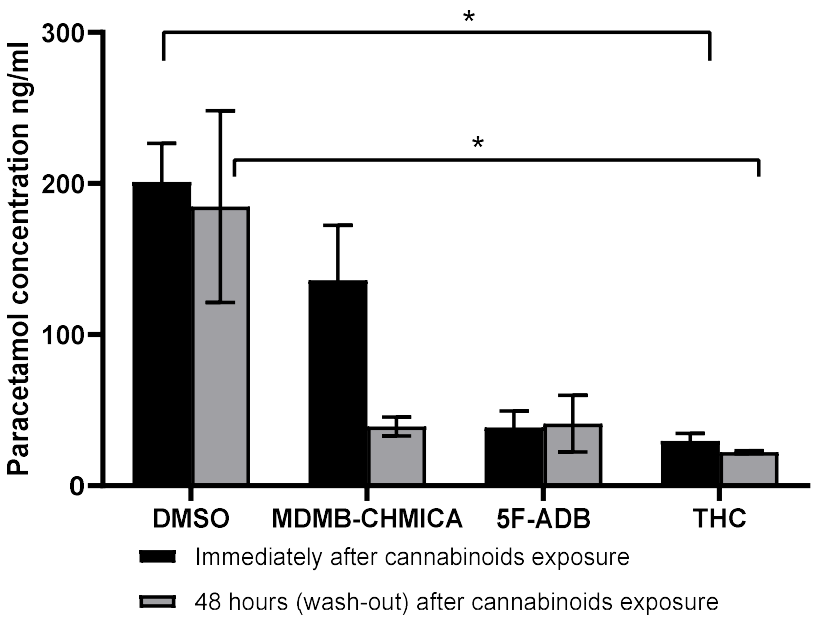


Figure 4.48 level of paracetamol concentration in culture medium of mature cells treated with SCRA and ∆9-THC. Paracetamol concentration in culture medium of mature hNPSC cells treated with 10 nM of MDMB-CHMICA, 5F-ADB and ∆9-THC for 14 days, then treated with 1µg/ml paracetamol either immediately after cannabinoid exposure or 48 hour (wash-out) after cannabinoids exposure. Statistical significant was evaluated by Kruskal-Wallis test. * represents $p < 0.05$.

The decreases in paracetamol concentration in all 3 treatment groups might suggest that cannabinoids increase paracetamol metabolism, with the highest effect been seen in ∆9-THC. The reduction in 5F-ADB was greater than for MDMB-CHMICA but slightly less than the reduction with ∆9-THC. The concentration of paracetamol was lower in cells exposed to paracetamol after 48 hours wash out period of cannabinoids except for 5F-ADB which only marginally increased.

4.5 Discussion

The objective of this study was to investigate the potential neurotoxicity of SCRAs and try to characterise the mechanisms that might be involved, using human neuronal stem cells.

4.5.1 Cytotoxicity assay

This study investigated the neurotoxicity of SCRAs utilising a human stem cell brain model. Since SCRA are psychoactive substances and their major site of action is the human brain, the human neuronal stem cell model provides a good model to simulate the effects of SCRA neurotoxicity. The SCRAs and other comparative NPS were tested on both neuronal progenitor cells and mature neuronal (differentiated) cells. The neuronal progenitor cells are classed as an immature model of the human brain (developing). They were used to model the potential effects of NPS, in particular SCRAs use by young individuals or by potential breast feeding or pregnant women where there is the possibility of passive exposure. The mature (differentiated) neuronal model is used to study the response of the adult brain to NPS exposure.

The human neuronal precursor stem cell model (hNPSC) line N1997 used in this study offers advantage of using human cells over using animal cells. Hence, avoiding the need to extrapolate results across species (Breier et al., 2010, Scott et al., 2013). In addition, other human cell lines have many limitations such as HEK293 lack neuronal phenotype; SHSY5Y cell lines (that is derived from human neuroblastoma cells) are hard to differentiate into mature dopaminergic state (Schlachetzki et al., 2013). Moreover, hNPSC cells has been used successfully as a model to investigate the cytotoxicity of other compounds and to study brain development & neurodegeneration (Nisar et al., 2015, Kurzawa-Akanbi et al., 2012, Madgwick et al., 2015). Although hNPSC display and maintain functional features close to brain cells, it has some limitations. hNPSC is a 2-dimensional model which does not reflect the *in vivo* where the vascular perfusion continuously supplies nutrient and remove metabolic waste products. Also, it lacks the interaction between the cell and its matrix, and between adjacent cells (Haycock, 2011). Furthermore, differentiation into specific types of neurons with high purity might vary between experiments (Zhao and Moore, 2018). The immature and

mature stem cells were exposed daily to the tested compounds, as many users used it regularly on a daily bases (Cooper, 2016, Macfarlane and Christie, 2015).

The toxicity studies show that MDMB-CHMICA, 5F-ADB and MMD-CHMICA were toxic to immature cells at 5-10 μM after 7-14 day of exposure, with MMB-CHMICA showing higher toxicity. The cytotoxicity of these SCRAs at 5-10 μM which is more than 1000 time higher the range of their binding affinities to both CB1 and CB2 receptors (0.59-10 nM for CB1 and 7.5-71 nM for CB2 receptors). i.e all receptors sites will be occupied, and the maximum effect will occur. In addition to the other potential binding with non-CB receptors, transporters and metabolising enzyme. Thus, the high binding affinities of SCRAs when compared to ∆9-THC can be responsible for unusual severe effects due to CB receptors overstimulation.

The differences in cytotoxicity might be attributed to the differences in SCRA potencies to CB1 receptor and its potential interaction with other neurotransmitter or activation/inhibition of signalling pathways on the brain.

These finding are in line with Tomiyama et al. (2014) who proposed that the cytotoxicity may depend upon the role of CB1 receptor rather than the chemical structure of SCRA (Tomiyama and Funada, 2014).

The results obtained from the Alamar Blue on the mature neuronal model assay in the presences of JWH-018 did not show a significant reduction in cell viability, suggesting that JWH-018 is not cytotoxic over the range of concentrations tested. These results are consistent with those of Couceiro et al. (2016), who demonstrated that JWH-018 did not decrease cell viability in SH-SY5Y and HEK293T cell lines. Similarly, Koller et al. (2013) investigated the toxicological profiles of some SCRAs including JWH-018 at concentrations up to 100 μM and 24 hours incubation, using MCF-7 (breast) and TR146 (buccal) cells. These SCRAs and did not show mitochondrial damage or changes lysosomal activity or protein synthesis (Koller et al., 2013).

Tomiyama et al. (2014) examined mice forebrain neuronal culture and concluded that the tested SCRAs (including JWH-018) were toxic between 10 and 30 μM. In addition, they concluded that the toxicities of tested SCRA were reversible when the cells were pre-incubated with CB1 receptor blocker but not CB2 receptor

blocker (Tomiyama and Funada, 2014). Interestingly, the N-(3-hydroxypentyl) JWH-018 metabolite was shown to cause a substantial reduction in cell viability at concentrations higher than 25 μM (Couceiro et al., 2016). Moreover, 5F-ADB, MDMB-CHMICA and MMD-CHMICA did not reduce cell viability of mature neuronal cells, suggesting that these SCRA are not overtly toxic at the concentrations tested (0.005-10μM). Recently, a study published regarding cytotoxicity of 5F-ADB and MDMB-CHMICA which utilised a lung carcinoma cell line A549 and buccal carcinoma cell line TR146 only showed toxicity was induced at concentrations higher than 25μM (Grafinger et al., 2019).

So far, there have been few studies published which have investigated the cytotoxic properties of SCRA, probably due to the diversity of the group and the continuous appearance of new compounds (Wojcieszak et al., 2016).

There is limited data available on the bioavailability of SCRA in human blood. Most of the available data are either from post-mortem analysis, emergency department admissions, police forensic samples or pharmacokinetic studies (summarised in Table 4.11). One limitation of these data are the lack of the knowledge of the actual dose being taken by the user and the time between the consumption of SCRA and blood measurement being taken, which varies widely. In addition, the blood concertation usually does not reflect the exact amount introduced into the system as SCRA rapidly disappear from the circulation either due extensive tissue distribution or rapid metabolism (Teske et al., 2010, Toennes et al., 2017). Some of the metabolites may also be more potent than the original parent compound ingested. Moreover, SCRA are highly lipophilic compounds and when they pass through the blood brain barrier, they might accumulate in a concentration higher than blood level.

SCRA	Concentration	Condition	Sample type	Ref.
JWH-018 (n=18)	292.8 pM-582.8 nM	Post-mortem	Blood	(Shanks et al., 2012b)
JWH-018(n=1)	123.0 nM	Case study	Blood	(Hermanns-Clausen et al., 2018)
JWH-018 (n=6)	8.4 nM – 28.9nM	Pharmacokinetic study	Blood	(Toennes et al., 2017)
(JWH-018 (n=2)	23.7 nM-29.2865 nM	Pharmacokinetic study	Serum	(Teske et al., 2010)
JWH-073 (n=8)	305.4 pM–208.5nM	Post-mortem	Blood	(Shanks et al., 2012b)
5F–ADB	503.3 pM	Post-mortem	Blood	(Kusano et al., 2018)
5F-ADB n=4	291.3 pM–5.0 nM	Post-mortem	Blood	(Usui et al., 2017)
5F-ADB	50.33 pM	Case history	Urine	(Minakata et al., 2017)
MDMB-CHMICA	14.5644 nM	Post-mortem	Blood	(Adamowicz, 2016)
MDMB-CHMICA	6.762 nM	Post-mortem	Brain	(Adamowicz, 2016)
MDMB-CHMICA	3.6nM	Post-mortem	Serum	(Westin et al., 2015)
MDMB-CHMICA	8.84-224.70 nM	Case study	blood	(Bäckberg et al., 2017)
5F-APINACA (n=3)	2.3 - 16.9 nM	Driving under influence of drug	Blood	(Karinen et al., 2015)
APINACA (n=3)	656.6 pM- 67.03nM	Driving under influence of drug	Blood	(Karinen et al., 2015)
UR-144 (n=2)	671.7 pM-1.4 nM	Driving under influence of drug	Blood	(Karinen et al., 2015)

Table 4.11 Examples of some SCRA levels found from pharmacokinetics studies, forensics and post-mortem cases.

The available literature suggests that the concentrations of SCRA present in human samples after consumption is lower than the levels examined in toxicity publications. Therefore, the SCRA concentrations range examined in this study were lower to more closely mimic the levels found clinically.

The molecular mechanisms that underlies SCRA cytotoxicity remain to be elucidated. Tomiyama et al. (2014), reported that CB1 cannabimimetic induce apoptosis through a caspase 3 dependant mechanism (Tomiyama and Funada, 2014) and this is the same mechanism suggested for ∆9-THC by increasing cytochrome c release and caspase-3 activity (Campbell, 2001). On the other hand, the cytotoxicity of the CB2 agonist JWH-133 was not mediated by activation of the

CB2 receptor or apoptotic pathways (Wojcieszak et al., 2016). Bologov et al. (2011) indicated that cannabimimetics significantly reduce cell viability when the cells were cultured under stressful conditions (glucose and serum deprived medium), but the same compounds did not affect viability for the same type of cell when cultured under optimal conditions. This raises a possible effect of the environment in potentiating toxicity by mechanisms such as hypoxia (Bologov et al., 2011).

It is worth mentioning that the SCRA used in this study were reference standard quality chemicals and the actual SCRA misused by users are usually in the form of herbal preparations sprayed with chemical that may contain adulterants or contaminants that might also be cytotoxic. Granfinger et al. (2019) examined the cytotoxicity of 5C-AKB 48, ADB-CHMINACA, MDMB-CHMICA, 5F-ADB and NM-2201 in form of reference standards, as smoke condensate and herbal extract preparation. They showed that the cytotoxic effects of 5C-AKB48, 5F-MDMB-PINACA, and MDMB-CHMICA were augmented by plant material (Grafinger et al., 2019). In addition, the cells were continuously exposed to the test compound which is might not reflect the actual exposure, together with the not taking into consideration the effect of blood brain barrier and the metabolic changes that might activate or deactivate the tested chemicals.

The cytotoxicity of tested SCRA was prominent in immature cells but not mature cells. It may be that immature cells are more sensitive to apoptotic effects than mature cells (Downer et al., 2007) and mature cells are more resistant to cell death than immature cells (Kole et al., 2013). In addition, the mechanisms of apoptosis vary between immature and mature neurons (Lesuisse and Martin, 2002).

The natural cannabinoids ∆9-THC was not tested in this study; however, effects of ∆9-THC on viability have been studied previously using the same cell lines and methodology in-house (unpublished data). The results showed that ∆9-THC was not toxic to both mature and immature neuronal cells in a concertation range of up to 20 µM. This would suggest that tested SCRAs are more toxic than ∆9-THC.

To compare the toxicities of SCRA with other NPS, the novel stimulants mephedrone and 25I-NBOMe hallucinogen were investigated for cell viability. The viability experiments showed that mephedrone induced toxicity in a concentration dependant manner for the first seven days. However, the cell viability recovered

after day 14, suggesting that this may be because the concentrations used were stressing the cells rather than killing them. The data in the literature regarding mephedrone induced neurotoxicity is contradictory, for example some studies reported that mephedrone can cause a reductions in dopamine and 5-HT transporters, while others showed no destruction of dopamine nerve endings and no significant changes in monoamine levels in the brain (den Hollander et al., 2014, Martinez-Clemente et al., 2014, López-Arnau et al., 2015, Motbey et al., 2012, Angoa-Pérez et al., 2012). However, due to discrepancies in experimental design, doses and type of cells used, it is difficult to compare the results or draw a final conclusion about mephedrone action (Pantano et al., 2017). 25I-NBOMe showed early neurotoxic effect on immature cells and showed high cytotoxicity after repeated exposure. The high toxicity might because it is a potent 5-HT2A agonist, as suggested by Braden et al 2006 (Braden et al., 2006). Currently, all the data in the literature about 25I-NBOMe relates to case report studies and some pharmacokinetics studies (Hill et al., 2013). No literature has been found about the mechanism of toxicity and this study is the first study *in vitro* to be reported.

To compare the toxicity of SCRA with conventional drug of abuse, the synthetic stimulant MDMA (ecstasy) was examined for cell viability. The viability experiments showed a reduction in cell viability was noticed after 7 days of MDMA exposure at 1, 2 and 5 μM but not 10 μM and the cells retained viability after further exposure. The variability in cell response might be due to that MDMA stressing the cell at the mitochondrial level without killing the cell and later revive from stress (Fulda et al., 2010). Similar to that seen in mephedrone as the cells recovered after 14 days from exposure.

It has been noticed from the NPIS data and published literature that drug abusers usually consume more than one drug of abuse (poly-abuse). This poly-abuse might lead to interactions between different drugs that might lead to different effects when compared to single drug toxicity evaluation. Therefore, a combination of MDMB-CHMICA, 5F-ADB and the conventional drug of abuse MDMA at lower and more clinically relevant concentrations were investigated for effects on cell viability. The combination of MDMB-CHMICA, 5F-ADB and MDMA did not affect viability, the tested concentrations might not affect cell survival, but might induce changes on cellular level that manifest as adverse health effect.

Overall, the viability results suggested that SCRA are toxic to immature cells but not to mature cells. This suggests that the abuse of SCRA by young users or pregnant women may severely affect the young developing brain. Nevertheless, the lack of overt cytotoxic effect in mature cells does not exclude potential changes induced by continued exposure to SCRA. Therefore, additional investigations were done to understand the molecular and cellular changes arising from repeated use. Finally, the different types of NPS can produce different modes of intoxication which require further investigation.

4.5.2 Gene Expression Changes

This is the first investigation using RNA sequencing to assess the impact of SCRA and ∆9-THC using human neuronal stem cell models. In drug discovery and development, RNA sequencing provides a high-throughput tool for simultaneously evaluating the patterns of expression/changes of thousands of genes and identifying relevant functional gene pathways (Khatoon et al., 2014).

To elucidate the molecular changes that occur following repeated exposure to SCRA (MDMB-CHMICA and 5F-ADB) and ∆9-THC, mature neuronal cells were exposed to 10 nM concentrations for 2 weeks. This concentration was chosen in the knowledge that SCRA showed minimal toxicity at 1000 time higher concentrations (10µM), to span the cannabinoids levels reported in the literature (Table 4.11) and to be close in concentration to the EC50 of the selected cannabinoids. Therefore, we selected 10 nM to investigate early changes caused by SCRA rather than nonspecific changes associated with cell death.

At the mRNA level, gene changes detected involved genes that belong to very different functional categories. The number of genes differentially changed in comparison to control after MDMB-CHMICA exposure was 154 genes, while the equivalent for 5F-ADB was only 20 genes. The lower number of genes changed for 5F-ADB is may be due to the variance in response shown by the 4 replicates as shown by the PCA (Figure 4.11). The variation of response might be because of the concertation used (10nM) was not enough to elicit the full response of 5F-ADB, although, the EC50 of 5F-ADB was 0.59 nM. This might suggests that 5F-ADB action is not limited to the CB1 receptor alone. It could also be due to receptor desensitisation that represents a protective mechanism against chronic receptor

overstimulation that limits the response to a full agonist (Ferguson, 2001). Comparing MDMB-CHMICA to ∆9-THC, the magnitude of changes due to the synthetic cannabinoid MDMB-CHMICA was 3 times higher than for ∆9-THC (50 genes significantly changed) suggesting that SCRA might induce more changes in gene expression due to frequent exposure than natural cannabis. Previous studies on the effect of ∆9-THC have been conducted in mice or rats. These transcriptome studies usually target certain aspects like changes in rat cerebellum on chronic use (Colombo et al., 2009) or changes in brain lipidome in mice (Leishman et al., 2018) therefore it is not possible to accurately compare the results to this current study.

The common differential changed genes among the 3 groups were 2 mitochondrial tRNAs, LGI1 and DQX1. It can be noticed that the changes in expression of these genes are in the same direction in the 3 groups, i.e. they are either upregulated or down regulated in all groups. In addition, the fold change was relatively similar. The results showed that two of the genes common to all 3-treatment groups that were upregulated were the mitochondrially encoded tRNA isoleucine (MT-TI) and mitochondrially encoded tRNA glutamine (MT-TQ). They are responsible for transferring the amino acids isoleucine and glutamine, respectively, to the polypeptide chain at the ribosome site of protein synthesis during translation. Mutation of MT-TI leads to many clinical diseases. These include MERRF, which is characterised by, epilepsy, dementia cerebellar ataxia, myoclonus and abnormal cells called ragged red fibers (RRF) (Lorenzoni et al., 2014). Cardiomyopathy and deficiency in complex IV of the mitochondrial respiratory chain have also been shown (Kaido et al., 1995, Taylor et al., 2003). Mutation of MT-TQ is one of the genetic causes of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). It is worth mentioning that the mitochondrial tRNA were the most affected genes. In MDMB-CHMICA treated cells there were 9 MT-tRNA genes significantly changed. The significant genes should have p value less than 0.05 and a fold change more than 1, but it was noticed that there were 8 MT-tRNA genes were significantly changed in p value but did not pass the fold change cut-off value. The same changes in MT-tRNA genes in 5F-ADB treated cells were noticed as 2 TM-tRNA genes were significantly changed in addition to 8 other genes that have been a significant p value but the not the fold change value. In ∆9-THC there were 6 MT-tRNA significantly changed and 6 genes that have been a

significant p value only. These changes in MT-tRNA indicated the adverse effects of cannabinoids in the mitochondria as they are affecting more than 50% MT-tRNA that play an important role in mitochondrial function. The tRNA are non-coding RNAs that helps ribosomes in protein synthesis by decoding mRNA genetic code and translate protein. Recent studies have shown that tRNA play a role in the cellular response to stress (Kirchner and Ignatova, 2015). Cytosolic t-RNA and MT-tRNA bind to cytochrome c; (cytochrome c acts as initiator of apoptosis and caspase activation); this binding will impair the activation of caspase and act as a protective mechanism against cell death (Mei et al., 2010, Huber et al., 2019). In the mitochondria, the MT- tRNA plays important roles in allowing effective, precise, and dynamic protein synthesis (Bohnsack and Sloan, 2018). Mutations in MT-tRNA are responsible for many genetic diseases such as myoclonic epilepsy with ragged-red fiber disease (MERRF) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Goto et al., 1991, Shoffner et al., 1990). The increase in MT-tRNA expression might be a response to the stress induced by MDMB-CHMICA, 5F-ADB and ∆9-THC. The perturbation in MT-tRNA gene expression might indicate changes in mitochondrial protein synthesis, and any disruption or abnormal protein synthesis may affect the components of the mitochondrial respiratory chain or may boost ATP production. In addition, these changes may impact other mitochondrial functions such as involvement in metabolic pathways, apoptosis and reactive oxidative species signalling (Pearce et al., 2017, Bohnsack and Sloan, 2018, Xu et al., 2016).

The consequence of increase or decrease in protein synthesis will lead to a respiratory chain complex defect. This defect will trigger mito-nuclear signalling mechanisms that allow mitochondria to interact with the nucleus and reprogram nuclear gene expression. These signalling mechanisms include mitochondrial proteolytic response, UPR and heat shock response pathways. Therefore, mitochondrial stress in neuronal cells reprogram gene expression and metabolism and consequently regulating neuronal function (Hunt et al., 2019, Khacho et al., 2019). Furthermore, the disruption of mitochondria function, impact the glial cells as well. The induction of UPR in mitochondria and activation of glial stress response pathway have been reported in many neurogenerative diseases (McAvoy and Kawamata, 2019). However, the mechanisms by which Mt-tRNA

affect mitochondrial function are poorly understood. Therefore, prediction of MT-tRNA effect on the neuronal function and clinical outcome extremely difficult; as McFarland et al, reported that “We understand little of the factors determining the pathogenicity of specific MTt-tRNA mutations, making prediction of clinical outcome extremely difficult” (McFarland et al., 2004). Therefore, further investigation about the effect of SCRA on mitochondrial function, neuron mitochondria biogenesis and the proteins and pathways implicated in oxidative-phosphorylation function is required.

The other significantly changed gene was leucine rich glioma inactivated 1 (LGI1). This gene is highly expressed in brain; the exact function in the CNS is still unclear, but evidence shows that it encodes proteins that could regulate the activity of voltage-gated potassium channels and it may be involved in neuronal growth regulation and cell survival. In addition, it regulates synaptic transmission of glutamatergic synapses in the hippocampus (Zhou et al., 2009, National Center for Biotechnology Information). Recent research showed that presence of LGI1 antibodies in encephalitis patients related to structural damage of the hippocampus that causes impairments of verbal and visuospatial memory (Finke et al., 2017). LGI1 has also been linked with faciobrachial dystonic seizures (FBDS), a form of epilepsy which is illustrated by frequent, short-term seizures affecting the arm and face (Plantone et al., 2013).

It can be noticed that the mutations in MT-tRNA and LGI1 are associated with seizures, cardiac side effects and memory loss which are also some of the features of cannabinoid toxicity (Gurney et al., 2014). It can be postulated that the changes in these genes contribute to SCRA adverse effects.

The final commonly differentially changed gene for all 3 groups was DEAQ-box RNA dependent ATPase (DQX1). This gene is related to the RNA-dependent ATPases and plays a role in RNA metabolism. However, the clinical significance and biological role of DQX1 has not been explored.

It can be noticed that there are overlaps in differentially expressed genes among the 3 groups and the direction of change of all of these genes are the same. This might indicates that these changes are mediated through the activation of CB1 receptor. The MDMB-CHMICA induced more changes compared to ∆9-THC might

be explained by MDMB-CHMICA being a more potent and a full agonist on CB1 receptor in comparison to less potent and partial agonist ∆9-THC (Paronis et al., 2012). Therefore, it can be proposed that synthetic cannabinoids are more toxic than cannabis.

The canonical pathway analysis by IPA showed that MDMB-CHMICA, 5F-ADB and ∆9-THC induced a variety of different gene expression changes. MDMB-CHMICA treatment of neuronal cells inhibited both SUMOylation and Sirtuin signalling pathways. Recently, SUMOylation has been identified as a vital posttranslational modification capable of changing stability, regulation of genes, subcellular localization of proteins, and protein-protein interactions. Also, it plays a vital role in cellular processes, for example oncogenesis, cell cycle control, apoptosis, and response to viral infection (Gareau and Lima, 2010). Therefore, any disturbances in the SUMOylation pathway may lead to changes in cellular function (Zhou et al., 2004). The change in SUMOylation pathway may be as a result in changes reactive oxygen species (ROS) production, which has been suggested to be an important regulator of the SUMOylation pathway. Bossis et al reported that SUMO-conjugating enzymes Inactivation may be an early phase in cellular response to oxidative stress and they can be regarded as ROS sensors (Bossis and Melchior, 2006). During oxidative stress, the SIRT1 enzyme (part of the sirtuin signalling pathway) becomes inhibited (Salminen et al., 2013). These findings indicate that MDMB-CHMICA might slightly increase oxidative stress in the cells by increasing the levels of ROS production. The increase in ROS level and loss of antioxidant balance impacts the cell through oxidative damage to cellular proteins, lipids and genetic material. Since the brain is a highly metabolic organ, any disruption to the ROS system will have substantial effects on it (Sies, 1997, Islam, 2017). Any increase in ROS production may be due increase in protein synthesis as shown by increased expression of the MT-tRNA in MDMB-CHMICA treated cells (Han et al., 2013).

The current study showed that MDMB-CHMICA activated the EIF2 signalling pathway, which is an initiator of protein synthesis (Kimball, 1999). The activation of EIF2 signalling might indicate the increase in protein synthesis which is complemented by the activation of mTOR signalling which also indicate increase in protein synthesis. Previous studies showed that continued activation of neuronal

PI3-K/Akt/mTOR signalling can be an early feature of Alzheimer disease due to the accumulation of tau protein (O' Neill, 2013). In addition, TGF-β/ and BMP signalling pathways are involved in many processes at a cellular level and any disruption in their activity lead to many defects. It has been shown that activation of these pathways has a protective effect on the cells (Shan et al., 2018, Guo and Wang, 2009), but cross-talk signalling between these is very complex and data should be interpreted with caution. Taken together, these changes suggest that MDMB-CHMICA activates oxidative stress responses, increases protein synthesis which in turn activates the unfolded protein response. Therefore, further investigation is required to measure the oxidative stress induced by MDMB-CHMICA such as Mito-SOX to measure mitochondria-derived O_2^- and redox prob dichlorodihydro fluorescein (DCFH) to measure intracellular $H_2O_2^-$. In addition to studying changes in mitochondrial transmembrane potential (Δ ψm).

In comparison 5F-ADB showed different canonical pathway changes, with the activation of only 5 pathways and no predictions for inhibition which might attributed to the sample variation mentioned earlier. The activation of p53 induces apoptosis by transactivating pro-apoptotic genes and direct binding to anti-apoptotic mitochondrial proteins, thus successfully provoking apoptosis (Ranjan and Iwakuma, 2016). Whereas, the activation of actin cytoskeleton suggested initiation of apoptosis, as actin cytoskeleton acts as mediator, initiator in apoptotic pathways (Desouza et al., 2012). On the other hand, the activation of NGF and adrenomedullin signalling pathway may reflect a protective response from the neuronal cells. The NGF is a potent pro-survival factor that protects the cells from apoptosis (Mnich et al., 2014), together with the neuroprotective effect of adrenomedullin activation that improves the survival and migration of astrocytes and inhibits apoptosis by eliminating oxidative stress-mediated signals (Xia et al., 2004). It can be noticed that the activated pathways in 5F-ADB treated cells are related to increase apoptosis and consequently cell death. Thus, it is important to understand the mechanisms by which 5F-ADB initiate or trigger apoptosis and hence identify the effects on human brain and how it is translated into clinical manifestations and any long-lasting damages it might cause. This can be done by investigating the effect of 5F-ADB on membrane asymmetry (analysis of annexin V binding), caspase activation markers, and DNA fragmentation.

The gene expression changes induced by ∆9-THC were different and even contradictory to those of MDMB-CHMICA as shown by EIF2 signalling and Wnt/β-catenin signalling being inhibited by ∆9-THC, whereas they were activated by MDMB-CHMICA. As mentioned previously, changes in EFI2 activity arise in response into a broad spectrum of cellular stress and lead to increase in protein synthesis. The reduction in its activity reflects the reduction in protein synthesis (Wek et al., 2006). Furthermore, ∆9-THC inhibited aryl hydrocarbon receptor signalling which plays an important role in the regulation of xenobiotic metabolism, such as the induction of CYP1A1 and CYP1A2. It was also shown to affect the glutathione S-transferase Mu 4 (GSTM4) gene expression. GSTM4 enzyme plays a role in metabolism of various xenobiotic such as drugs, environmental toxins and products of oxidative stress (Denson et al., 2006). The inhibition of phase II metabolism will reduce the detoxification of other drugs and xenobiotic and may enhance their effects, potentially leading to adverse effects and toxicity. In the nervous system, Ephrin signalling is involved in the proliferation of neuronal progenitor cells, the control of axonal growth cones to their synaptic targets and synapse development (Kania and Klein, 2016). In addition, Ephrin-B2 expressed in astrocytes of the hippocampal stimulates EphB4 forward signalling in neural stem cells and, through activation of β-catenin, promotes neuronal differentiation but not proliferation (Ashton et al., 2012). The inhibition of Ephrin-B2 signalling and Wnt/β-catenin signalling in the hippocampus might affect memory and cognition.

Taken together, IPA analysis provides evidence for the potential role of SCRA in cellular stress. The changes induced by MDMB-CHMICA, 5F-ADB and ∆9-THC varied in the targeted pathways, but it can be noticed that affected pathways were related to increase protein synthesis that potentially triggers stress pathways in the cells such as ROS production, activation of unfolded protein response and changes in metabolising enzymes. In addition, there was a difference in responses between the 2 synthetic cannabinoids MDMB-CHMICA and 5F-ADB; as MDMB-CHMICA showed signs of increase cell stress while 5F-ADB triggered apoptotic pathways. This might be attributed to the 10nM concentration used which was similar of MDMB-CHMICA EC50 at CB1 receptor, whereas the EC50 of 5F-ADB at CB1 receptor (0.59nM).The higher 5F-ADB effective concentration and the continuous exposure for 14 days may be responsible for driving different pathways from

MDMB-CHMICA in particular apoptotic pathway. In addition to the inconsistent responses from the quadruplicate samples compromised the IPA analysis and hence different effects on physiological pathways. It should be noted that all these changes are triggered by the relatively low concentration of 10 nM (compared to 10 μM toxic concentration) which was not enough to kill the cells, but enough to induce changes that may help explain some of the neurotoxic effects seen in cannabinoids use.

4.5.3 Proteomic changes due to NPS exposure

The CB1 receptors are the main receptors responsible for the psychotropic effects of cannabinoids. The CB1 receptor expresses 3 isoforms (Bagher et al., 2013), but due to low fluorescence intensity on the western blot only the CB1a isoform was analysable.

Neither MDMB-CHMICA and 5F-ADB or ∆9-THC did not significantly change CB1a protein expression. It is well established that repeated exposure to an agonist can cause down-regulation of receptors expression (Tsao and von Zastrow, 2000). Therefore, down regulation in CB1 receptors might be anticipated after repeated exposure to cannabinoids. Hirvonen et al. (2012) reported that there was down regulation of CB1 receptors selectively in the cortical brain region in chronic cannabis users. This down regulation was reversed after 4 weeks of discontinuing cannabis use (Hirvonen et al., 2012). In addition, the potent CB1 receptor agonist WIN 55,212-3 induced significant down regulation of CB1 receptors in a concentration dependant manner starting from 10 nM (Blair et al., 2009). This down regulation will contribute to development of tolerance to their pharmacological effects (Martin et al., 2004). It can be noticed that MDMB-CHMICA followed the trend in CB1 expression reduction from the previous studies. Interestingly, 5F-ADB increased CB1 receptor protein expression by 19%. Laprairie et al. (2013) reported that endocannabinoids; arachidonyl-2-chloroethylamide, anandamide and methanandamide; can increase CB1 receptor protein expression in a neuronal cell culture model by activation of transcription such as NF-κB. A final conclusion on the effect of the studied cannabinoids on CB1 expression cannot be drawn, because only one isoform was analysed and this represents only a proportion of the CB1 receptor pool. In addition, the CB1 isoform (53 kDa) is the most abundant isoform and was not quantified.

To investigate whether MDMB-CHMICA, 5F-ADB and ∆9-THC induce morphological changes or neuronal injury to mature neuronal cells, β III – tubulin and GFAP were assessed. The tested cannabinoids did not affect neuronal cytoskeletal β III – tubulin, but ∆9-THC significantly increased GFAP expression. GFAP overexpression is characteristic of astrogliosis in which the astrocyte become reactive in response to insults such as trauma, genetic disorder, disease and chemical injury (Eng et al., 2000). However, there are 2 distinctive types of astrocytes, named A1 and A2. The A1 type might be neurotoxic, because they upregulate many genes that suggested to be destructive to the synapses. In contrast to A2 astrocytes, which promote neuronal survival and tissue repair (Liddelow and Barres, 2017); therefore it would be useful to look again with more specific markers to A1 and A2 to see if the response is damaging or protective response.

This result is in agreement with other studies that have showed that ∆9-THC significantly increases GFAP in the rat hippocampus after chronic exposure (Lopez-Rodriguez et al., 2014, López-Gallardo et al., 2012). Furthermore, GFAP expression increased in rat hippocampus during acute and chronic administration of ∆9-THC (Suliman et al., 2018). Another study indicated that twice daily exposure to the SCRA WIN 55,212-2 for 14 days did not induced significant changes in GFAP expression, although there was a significant change in NF-160, NF-200 and MAP-2 neuronal cytoskeletal proteins in rat brain (Tagliaferro et al., 2006). These results highlight that ∆9-THC could induce brain injury which might contribute to the chronic effects of cannabis use.

The overexpression of MT-tRNA suggests an increase in protein synthesis in the mitochondria, including proteins that comprise the OXPHOS system. MT-tRNA is involved in the synthesis of critical subunits of Complexes I, III , IV and two subunits of complex V, depending on the type of amino acid incorporated (Triepels et al., 2001). The Complex I subunits NDUFA9 and NDUFS1 showed non-significant changes in protein expression with 16% reduction of NDUFA9 in ∆9-THC treated cells. Impairment in NDUFA9 expression in mitochondria might lead to an increase in ROS production as it has a proposed role in preventing ROS generation (Abruzzo et al., 2010, Bause and Haigis, 2013). Increased ROS production harms cells by lipid peroxidation and by modification of nucleic acid and protein structures.

In a study by Wolff et al. (2015), showed that ∆9-THC inhibited mitochondrial respiratory chain complexes I, II, and III as well as increasing ROS production in mitochondria extracted from rat brain (Wolff et al., 2015). In the current research, the ∆9-THC effect on NDUFA9 was not statistically significant, but the reduction in expression might lead to increase ROS production and this might induce damage to brain cells during longer term exposure.

MDMB-CHMICA increased Cytochrome c oxidase subunit 1 (MTCO1) expression by 14% and significantly increased Cytochrome c oxidase subunit 5a (CoxVa) by 30%. Cytochrome c oxidase consists of 13 subunits, located in the inner mitochondrial membrane. Cytochrome c oxidase catalyses the last step of the electron transfer chain in mitochondrial oxidative phosphorylation which accounts for 90% of cellular ATP production, in addition to its role as a proton pump together with complex I and III to control ATP synthesis (Little et al., 2018). This suggests that MDMB-CHMICA induces mitochondrial biogenesis, which is a complicated process by which the cell increases the number of mitochondria present to adapt to different physiological and environmental conditions. Therefore, the overexpression indicates an increase in ATP production. The increase Cytochrome c oxidase in MDMB-CHMICA treated cells might be as a compensatory mechanism to a reduction in ATP synthase. Havlíčková Karbanová et al. (2012) reported reduction in inner mitochondrial protein TMEM70 resulting in 30 % reduction in ATP synthase activity and this lead to marked and significant increases in cellular protein content of the subunits of respiratory chain complex III and complex IV (Havlíčková Karbanová et al., 2012). TMEM70 gene expression was significantly inhibited ($p= 0.01$) as shown in the differential gene expression data for MDMB-CHMICA treated cells, but not 5F-ADB or ∆9-THC exposed cells. The 11 % increase VDAC1 expression in MDMB-CHMICA treated cells might further suggests that MDMB-CHMICA increases ROS levels in neuronal cells. It has been suggested that elevated levels in ROS increase cell death observed with VDAC overexpression (McCommis and Baines, 2012). These findings suggests MDMB-CHMICA might lead to increase ROS production to levels that might cause the inhibition of the SUMOylation pathway, Sirtuin pathways and upregulate of mTOR activity (Jang and Sharkis, 2007).

Evidence from the literature proposed that oxidative stress and ROS are correlated to ER stress and represent an essential element of UPR (Bhandary et al., 2013, Zeeshan et al., 2016). UPR generally operates to offset ROS accumulation stimulated by different stresses to protect cells from cell death (Xue et al., 2005). The tested SCRA (MDMB-CHMICA and 5F-ADB) and ∆9-THC did not significantly change PDI and BiP protein expression, but in MDMB-CHMICA treated cells BIP expression increased by 12%. BiP plays a pivotal role as a key ER chaperone with anti-apoptotic properties; it also has the capability to control the initiation of UPR signalling (Lee, 2005).

In addition, phospho Erk1/2 was investigated, given the association between ROS and ER stress (Santos et al., 2009). Phosphorylation of Erk1/2 acts as a cell survivor factor to protect the cells from ER stress- induced apoptosis (Darling et al., 2017). The results showed no significant increase, but there were 14% increase in phospho-Erk1 expression in 5F-ADB. This might indicate that the level of stress induced by 5F-ADB was not enough to activate Erk1 expression. Unfortunately, phospho-Erk2 was not analysed due to low fluorescent signal.

RNA sequencing analysis noted many metabolic enzyme changes, it shows changes in some cytochrome P450 such as CYP17A1 which was 3-4 times significantly upregulated in MDMB-CHMICA and ∆9-THC treated cells. Therefore, both phase I (CYP3A4, CYP2E1 and CYPOR) enzymes and Phase II (UGT1A1, UGT1A6 and GSTP1) enzymes were examined for changes in protein expression. Unfortunately, the CYP's and the UGTs did not produce clear bands on the western blot for analysis. Interestingly, the results showed that phase II enzyme GSTP1 was significantly inhibited in ∆9-THC treated cells whereas the inhibition was non-significant in SCRA treated cells (less than 10%). Glutathione-S-transferase is vital in the metabolism of xenobiotic and endogenous substances and clearance of oxidative stress products (Higgins and Hayes, 2011). There is also recently growing evidence that GSTP can inhibit kinase function and suppress apoptosis through association in the regulation kinases signalling involved in cellular stress, such as Jun N-terminal kinase (JNK), apoptosis and signal-regulating kinase (ASK1) (Tew, 2007, Wu et al., 2006, Elsby et al., 2003). The reduced expression of GSTP1 might modify the capacity to detoxify other chemical and substances

and thus may be causal to other toxicities. This can lead to aggravated intoxication, in particular with poly-drug users.

It is well documented that AHR is involved in xenobiotic metabolism through the induction of cytochrome P450 enzymes. The differential gene expression analysis showed that there are upregulation of some phase I and phase II metabolising enzymes in MDMB-CHMICA and ∆9-THC treated cells such as CYP17A and some GSTs. However, SCRA and ∆9-THC did not directly affect AHR protein expression, but MDMB-CHMICA and ∆9-THC increase AHR expression by 10 % and 6% respectively.

In Summary, the investigated protein changes complement the RNA sequencing data. The increase in Mt RNA might indicate increase in protein synthesis such as the increased expression in MTCO1 and CoxVa in MDMB-CHMICA treated cells. Further proteomic investigation in particular mitochondrial respiratory subunits will be useful to elucidate the effect of SCRA exposure on neuronal cell. In addition, it highlights the effects of cannabinoids on the metabolism enzymes and how these changes can be translated into clinical manifestations.

4.5.4 Neurotransmission

The effect of SCRA on neurotransmission in the brain in particular serotonergic transmission was investigated, because many of the clinical presentations of SCRA users are similar to that of serotonin syndrome. Moreover, numerous behavioural studies on animals reported that cannabinoids might regulate 5-HT neurotransmission (Darmani, 2001, Hill et al., 2006, Franklin and Carrasco, 2013).

In this study, the western blotting analysis of 5-HT_{2A}, showed that there were no significant changes in 5-HT_{2A} expression in the cannabinoid, MDMA and combined exposure groups. In this context, previous studies showed that chronic exposure to the potent cannabimimetic agent HU-210 might upregulate 5-HT_{2A} receptors but causes down regulation of 5-HT_{1A} receptors (Hill et al., 2006). In addition, Franklin and co-authors suggested that repeated exposure to the synthetic cannabinoid CP 55,940 induced upregulation of 5-HT_{2A} at a protein level, but not at mRNA level in the rat hypothalamic paraventricular nucleus (Franklin et al., 2013a). It also

increased 5-HT_{2A} receptor protein levels and 5-HT_{2A} receptor mRNA levels in rat prefrontal cortex and the upregulation of 5-HT_{2A} is mediated through CB2 receptor activation (Franklin and Carrasco, 2012). However, other studies showed that chronic treatment with HU210 or ∆9-THC did not affect 5-HT_{2A} ligand binding and mRNA expression in mouse brain (Dowie et al., 2010). Furthermore, Ibarra-Lecue et al. (2018) reported that chronic ∆9-THC exposure did not induce changes in expression and density of 5-HT_{2A} receptors in mouse brain, but lead to activation of the Akt/mTOR pathway and induced alterations in 5-HT_{2A} receptor functionality (Ibarra-Lecue et al., 2018).

The monoamine oxidase enzymes (MAO) terminate the actions of neurotransmitter, for example 5-HT, dopamine and norepinephrine through metabolism. MAO A has a higher affinity for 5-HT, dopamine and norepinephrine, whereas MAO B has higher affinity for phenylethylamine or benzyl amine (Shih et al., 1999). However, there are few studies of the effects of cannabinoids on MAO activity, Fisar et al. (2010) reported that ∆9-THC, anandamide and WIN 55,212-2 had weak MAO inhibitory activity (Fišar, 2010 522). Therefore, verification of MAO protein expression was performed as their modulation might be involved in neurological dysfunction of many types. The results showed that there was no significant difference in MAO B protein expression in all exposed group, but there was a general increase in protein expression among the treatment groups between 9.5% up to 27% in particular after mixed exposures. This increased expression might result from increases in ROS production as higher concentrations of hydrogen peroxide enhance MAO-B activity (Konradi et al., 1986). DARPP-32, which plays a vital role in controlling the efficacy of dopaminergic neurotransmission in the forebrain, showed no significant changes in protein expression. This is in contrast to mice models using CP55, 940 (Andersson et al., 2005) and ∆9-THC (Borgkvist et al., 2008) both showing an increase in DARPP32 phosphorylation.

4.5.5 Preliminary metabolism experiments

The RNA sequencing analysis showed changes in phase I and phase II metabolising enzymes. In addition, the western blotting analysis of the phase II metabolising enzyme GSTP1 showed that ∆9-THC inhibited its expression. This brought to attention the potential effects of SCRA as inducers or inhibitors of drug

metabolising enzymes. The use of SCRA particularly with other drug of abuse or therapeutic agents adds further risks related to metabolic drug interactions, either by potentiating toxicity or attenuating therapeutic effects.

Studies published in the literature related to the effect of SCRA on metabolising enzyme are very limited. Therefore, the metabolism experiment was conducted to study the effect of cannabinoids on paracetamol metabolism. Paracetamol was chosen as its metabolic pathways, phase I (cytochrome P450) or phase II (conjugation), have been well studied and paracetamol is a readily used painkiller and is available without prescription. The preliminary data suggested that the tested SCRA, (MDMB-CHMICA and 5F-ADB), and ∆9-THC decrease paracetamol concentration in the cell culture media. The results tend to suggest that MDMB-CHMICA, 5F-ADB and ∆9-THC might induce paracetamol metabolism. Previous studies of the effects of cannabis on cytochrome P450 showed that ∆9-THC inhibited CYP2C9 and CYP3A4 function, while CBD inhibited CYP2C19 and CYP3A4 function (Rong et al., 2018). Synthetic cannabinoids MAM-2201 weakly inhibited the activity of CYP1A, in addition MAM-2201 inhibited CYP2C9, UGT1A3 and UGT2B7 (Kong et al., 2018, Alsherbiny and Li, 2019).

Unfortunately, the paracetamol metabolites sulphate, glucuronide and glutathione conjugates in both culture media and in cell lysate were not measured due to technical problem.

Therefore, further investigation is required to elucidate the effects of SCRA on metabolising enzymes activity. Although the results of the metabolism experiments are incomplete and a final conclusion cannot be drawn, it sheds light on the potential indirect toxic effect of SCRA abuse through their interactions with other drugs and chemicals; this may be of particular importance for poly-drug use and clinical treatment of users.

Collectively, the evidence provided in this chapter suggests that the cytotoxicity of NPS varies widely and different SCRA produce different effects on cell viability. It shows that subtle changes in chemical structure might enhance cytotoxicity as illustrated by differences in effects between MDMB-CHMICA and MMB-CHMICA. In addition the study elucidated the effect of repeated exposure of cannabinoids on both protein and gene expression, with induced changes expression of genes

involved in various neural pathways. The results suggest that the SCRAs have more of a profound effect than the natural cannabinoids ∆9-THC. As MDMB-CHMICA significantly changed 154 gene in comparison to 55 in ∆9-THC. Most of these changes are related to oxidative state, protein synthesis and perturbing mitochondrial function; these could potentially lead to subtle but potentially lasting changes in the brain. These changes were reflected in the expression of some of functional proteins such as Cox Va and GSTP1, in addition to the possible drug-drug interactions that might lead to serious consequences. Further studies are required to elucidate more information about the effects of SCRA, taking into consideration the complexity of the pathways involved.

All of these results demonstrate evidence of direct toxicity of NPS on human neuronal cells, especially immature cells, with differences in potency between agents.

Chapter 5 General discussion

Over the last decade, many different NPS, otherwise referred to as “designer drugs” or legal highs”, have appeared in the drug market as alternatives to traditional drugs of abuse. These new substances are usually synthesised with slight modifications in chemical structures to evade legislation but maintain the pharmacological action of the related traditional drug of abuse. More than 730 NPS were reported to the EMCDDA between 2005 and 2018 (EMCDDA, 2019).

The use of NPS is a cause of concern, Firstly, little known about their pharmacology, toxicology and especially their longer-term effects. Secondly, they are perceived as “legal” and thus perhaps “safe” (Corazza et al., 2014) by many users. There is also uncertainty about the type and amounts of NPS sold to users. These substances can cause significant toxic effects that requiring hospitalization and some cases of exposure are fatal. Finally, there is no accurate estimation about the number of users in the population.

The aims of this research were to assess the patterns and incidence with time of NPS toxicity in the UK by analysis of data collected by NPIS, to investigate the pharmacological effects of SCRA on 5-HT transporters and to determine the cytotoxicity and the potential transcriptomic and proteomic changes induced by chronic SCRAs exposure.

NPS are a relatively new phenomena and there is no complete picture of their use. There are challenges in studying NPS prevalence as there is no specific system for collecting data about their use in the UK. There are also large numbers of substances involved and these continue to change in term of chemical types involved and their legal status. NPIS data were selected for studying the prevalence of NPS toxicity because these data are available and collected continuously using consistent methodology, enabling the monitoring of emerging new drugs causing toxicity in UK users (Wood et al., 2014). The findings from analysing the NPIS data between 2009 and 2018 revealed that the most common types of NPS reported were SCRAs (including products) and stimulants. These finding are in line with those from the US (Palamar and Acosta). Most people involved were young and there was a preponderance of males but there was no

obvious geographic localisation of NPS use or evolution within the UK. The types of NPS reported in the UK for the last 10 years varied widely; the first spikes in NPS use were in 2010 with the appearance of mephedrone as a popular stimulant, in particular used by school and university students (Dargan et al., 2010). The number of toxicities and fatalities associated with its use resulted in its legal control in April 2010 (Wood et al., 2010, Maskell et al., 2011). This restriction of mephedrone use lead to replacement with other stimulants such as 'benzo fury' and 'Ivory Wave' and the substances involved in theses preparations were soon also controlled to reduce their harms. The time trend then showed a gradual increase in enquiries related to branded products and SCRA, associated with their easy availability over the internet, appealing marketing and (in some cases) legality. These enquiries started to reduce at the end of 2015.

The increasing and decreasing trends with time can be correlated with the introduction of specific legislation for individual psychoactive substances, culminating with the introduction of the PSA in May 2016. The PSA enactment was considered necessary to break the cycle of replacing controlled drugs with chemically similar uncontrolled alternatives. Since enactment of the PSA, the number of enquires and TOXBASE® access relating to NPS have been significantly reduced. This reduction also mirrors reductions in the numbers of shops and websites selling NPS. As a consequence NPS availability was reduced, prices have increased and street dealers and dark net websites have become the most important sources of NPS (Van Buskirk et al., 2017, Home Office, 2018). Reduction in NPS use are also evidenced by reductions in the total number of admissions for mental and behavioural disorders due to psychoactive substances in England, Wales and in Northern Ireland in 2016-2017 (Home Office, 2018).

Studying the prevalence of NPS use can be challenging, despite there are different data sources, but each source has its limitations (Table 2.1). For this research, the analysis of NPIS data was selected to help to provides an overview of NPS toxicity across the UK and to provide demographic and geographical information about users. It also has important limitations, the most important of which is that there is no analytical confirmation of the substance(s) involved in episodes of toxicity and the rate of reported cases may decline when the substance involved become more familiar. Therefore, toward a comprehensive understanding of NPS use in the UK,

it would be important to combine data related to NPS use from different data sources, giving that each data source has its own limitations. This process is called data triangulation and helps gain multiple perspectives and data validation and decrease the impact of the various limitations of each individual data set (Carter N et al., 2014). For example, in the UK the Identification of New Psychoactive Substances (IONA) study aimed to identify the chemical substances involved in severe toxicity of suspected NPS patients presented to UK hospitals and correlate it with clinical features presented (Thomas et al., 2018). IONA provided information about the changes in NPS use and that SCRA was the most common type being used, in addition to identifications to the changes in NPS types over time which can be linked with the NPIS to confirm the NPS type for users with severe toxicity. Similarly, the STRIDA project in Sweden runs as collaboration between the Swedish Poison information centre (PIC) with Karolinska University Laboratory and provides toxicological analysis for the acute poisoning episodes reported to PIC and emergency departments. The project was able to provide an overview of the drug situation in Sweden and served as warning system to identify new emerging NPS in that country (Helander et al., 2020).

Having defined the incidence of NPS poisoning, there remains limited information about their pharmacological and toxicological effects. In light of that, the next 2 parts of the research focused on understanding some of the basic pharmacological actions and toxicological properties of SCRA, as these were the most commonly used group of NPS identified by the NPIS data. Such understanding is expected to provide an insight into the underling mechanisms of action and potential adverse effects and to inform the development of treatments with the aim of decreasing the adverse effects from use of these potentially hazardous substances.

The adverse effects reported with some SCRA use such as tachycardia, hallucination, vomiting, chest pain, seizures etc. could be consistent with increases in 5-HT activity (Yip and Dart, 2014, Papanti et al., 2014). The direct interaction of SCRA with the serotonergic system is not well studied. The structural similarity between indole core SCRA and 5-HT led to speculation that SCRAs with this core, such as JWH-018, might have a direct inhibitory effect of on 5-HT uptake. The development and optimisation of an ex-vivo rat brain slice 5-HT uptake assay was successfully achieved and validated using the selective serotonin reuptake

inhibitor fluoxetine. Contrary to the original hypothesis, this study did not find a significant effect of either JWH-018 or the non-indole SCRA CP 55,940 on 5-HT uptake. Published studies have tested cannabinoid effects on 5-HT uptake and showed that at high concentration ∆9-THC, anandamide and WIN 55,212-2 inhibited SERT activity leading to increased 5-HT availability (Velenovská and Fišar, 2007, Molina-Holgado et al., 1993, Banerjee et al., 1975). The differences in methodologies, type and concentrations of the tested SCRA with this study might explain the differences. However, the discrepancies between different SCRA in term of chemical structure and potency does not exclude the possibility that other SCRA might have a direct effect on 5-HT uptake. The occurrence of serotonergic effects might also be due direct activation of 5-HT receptors or inhibition of MAO activity (Fišar, 2010) or occur indirectly through upregulation of 5-HT_{2A} receptors (Franklin and Carrasco, 2012, Franklin et al., 2013a). Therefore, 5-HT_{2A} and MAO protein expression were further investigated on mature human neuronal cells using indole core synthetic cannabinoids (MDMB-CHMICA, MMB-CHMICA) and an indazole core SCRA (5F-ADB). There were no significant effects on protein expression but there was a 10 to 14% reduction in 5-HT_{2A} expression and 10 to 17% increase in MAO B protein expression. Collectively, the tested cannabinoids seem not to have indirect effects on the serotonergic system.

The toxicological investigations focused on the neurotoxicity of SCRA in comparison to ∆9-THC and selected stimulants. The choice of SCRA used in this research was based on their frequency of use at the time of research and the dearth of information about their cytotoxicity, particularly in the brain. To model the potential neurotoxicity of NPS, in particular SCRAs, a human neuronal stem cell model was used. This is believed to be the first time a human relevant model has been used and this is important because a species specific model provides a better prediction of human toxicity (Scott et al., 2013). Nevertheless, the use of hNPSC model comes with some limitations. Firstly, the model has not expressed all the cells phenotypes in equivalence to human neuronal cells. Secondly, the 2D *in vitro* model does not mimic all the complexities of tissue or organ toxicity *in vivo*; in term of physiologically relevant conditions such as blood perfusion, drug metabolism, protein binding and passing blood brain barrier. In addition, the cells were exposed continuously to the tested chemical for 14 days which is not a truly representative

of the human exposure although the daily use cannot be excluded. Moreover, the effect of chronic use cannot be withdrawn from 14 days exposure only. Therefore, it would be interesting to investigate the changes after 24 hours,7 days or intermittent SCRA exposure and compare the different time points

The profile of the cytotoxicity of SCRAs was investigated together with other 2 commonly used NPS, mephedrone and 25I-NBOMe. This research revealed diverse effects of these substances on cell viability. The synthetic cannabinoids MDMB-CHMICA, MMB-CHMICA and 5F-ADB; (5-10 μM) and the hallucinogenic stimulant 25I-NBOMe (10 μM) were toxic to immature neuronal cells rather than mature cells with different potencies. 25I-NBOMe showed early and substantial toxicity, whereas MDMB-CHMICA and 5F-ADB showed toxicity after 7-14 days of exposure. In contrast, mephedrone and MDMA were toxic to mature cells with mephedrone showing more marked acute toxicity. The differences in cytotoxicity may be due the differences in mechanisms of action and potencies of the different NPS, as different SCRA may activate or inhibit different pathways.

It is interesting to note that the slight alteration in chemical structure between MDMB-CHMICA and MMB-CHMICA produced a different toxicity profile which could be reflected in important differences in their impact on human health. In addition, comparing toxicities of NPS with the traditionally misused drugs MDMA and ∆9-THC demonstrated that SCRA and other NPS (except mephedrone), were more toxic in immature neuronal cells. Also, they showed earlier toxicity, in particular 25I-NBOMe, and this may put users at greater risk of clinically important neurotoxicity.

To understand the changes induced in hNPSC cell line upon continuous exposure to SCRA, RNA sequencing was used to study potential transcriptomic changes due to SCRA exposure. Understanding the transcriptome is crucial to associate changes in the genome with effects on functional protein expression and on complex biological networks (Ozsolak and Milos, 2011).

The number of genes differentially changed due to cannabinoid exposure varied widely. MDMB-CHMICA induced almost 3 times as many genes changes compared to ∆9-THC (154 genes versus 55 genes). The number of genes differentially changed in 5F-ADB treated cells was only 20, this is believed to be

due to variance in the response from the quadruplicate samples analysed as shown in the PCA.

The MT-tRNA genes were differentially changed among MDMB-CHMICA, 5F-ADB and ∆9-THC treated groups. Taken into consideration that there are only 22 MT tRNA genes expressed in human cells, there were 9 changed in MDMB-CHMICA treated cells, 2 in 5F-ADB treated cells and 6 in ∆9-THC treated cells. Two of the MT-tRNA genes were commonly changed among all 3 exposure groups. The MT-tRNAs play an important role in protein synthesis in the mitochondria. The increase in expression of different MT-tRNA after cannabinoid exposure suggests that the cells are under stress as MT-tRNA can serve as a stress biomarker and are key in initiating a stress response such as increases in the level of ROS, DNA damage, metabolic changes and mitochondrial dysfunction (Huber et al., 2019). These findings could have important implications for cannabinoid users, because the concentrations used in this study were very low in comparison with concentrations used in other studies. 10 nM for each SCRA and ∆9-THC was used to simulate non-toxic, biologically relevant concentration and was intended to trigger their mechanisms of action. This concentration did not significantly change cell viability but was able to disturb their function. This is demonstrated by the results, as the potential increase in ROS, protein synthesis and the perturbation in mitochondrial function triggers protective mechanisms within the cell to relieve the stress and keep the cells viable. The changes disturbances of mitochondrial protein synthesis might suggest the harmful effect of cannabinoids on its function. As mitochondria is the main source of cellular energy and are vital for maintaining the cellular homeostasis through the regulation of many biological processes. LGI1 was commonly down regulated in the 3 groups and a reduction in LGI1 gene expression can lead to structural damage of the hippocampus and short duration seizures (Plantone et al., 2013, Finke et al., 2017). These features are consistent with the reports of poor memory performance in chronic SCRA and cannabis users (Sticht et al., 2015, Cohen et al., 2017).

The functional analysis of the altered genes revealed the involvement of many pathways, most associated with increased cellular stress. The MDMB-CHMICA treated cells showed activation of EIF2 and mTOR, both indicative of increased protein synthesis; this might lead to slight elevation of ROS levels and

consequently inhibit both the SUMOylation and Sirtuin pathways. The activation of the TGF-β, BMP and NOTCH pathways might indicate a mechanism for protection of the cells against apoptosis. In contrast, 5F-ADB stimulated apoptosis through activation of p53 and the actin cytoskeleton pathways. The study suggested that different SCRA activate the apoptotic pathway by different mechanisms, as demonstrated by activation or inhibition of different signalling pathways. The role of apoptosis in induction of cell death by SCRA was suggested previously in other studies (Tomiyama and Funada, 2011, Oztas et al., 2019, Almada et al., 2017). Oztas et al. (2019) suggested that the activation of different death pathways from synthetic cannabinoid exposure is concentration dependant.

The functional changes induced by ∆9-THC were different from those of the SCRA. ∆9-THC did not activate the apoptotic pathway but might affect phase I and phase II metabolism. The IPA analysis showed that ∆9-THC inhibited the aryl hydrocarbon receptor signalling that is involved in xenobiotic metabolism (Nebert, 2017). In addition, ∆9-THC significantly upregulated some cytochrome P450 as shown by the differential gene expression analysis and significantly inhibited the protein expression of the GSTP1 metabolising enzyme. This effect on the metabolic pathways might not only influence the detoxification capacity for other chemicals and xenobiotic but also provoke drug-drug interaction as many cannabis users are also poly-drug users (Hall and Lynskey, 2005, Perkonigg et al., 1999) and this could lead to adverse health effects.

In line with the various effects of ∆9-THC on metabolising enzymes, a preliminary metabolic experiment was conducted to examine the effect of SCRA and ∆9-THC on paracetamol metabolism. The results suggested that ∆9-THC exposure significantly induced paracetamol metabolism. Although the results are incomplete and inconclusive due to technical problems, it indicates the importance of considering drug-drug interaction during clinical assessment.

In addition, the analysis of ∆9-THC induced changes suggested possible adverse effects on learning and memory through the inhibition of Ephrin-B2 and Wnt/β-catenin signalling in the hippocampus. This is consistent with reports that chronic cannabis use is associated with cognitive and learning deficits (Pope Jr et al., 2002, Messinis et al., 2006, Solowij et al., 2002).

To complement the mRNA sequencing analysis of MDMB-CHMICA, 5F-ADB and ∆9-THC, some proteins of interest were investigated by western blotting. An interesting finding from this study was that ∆9-THC induced GFAP expression, while there was no such effect in SCRA exposed cells. Further investigation is required to determine which type of astrocytes involved in relation to ∆9-THC exposure; As A1 reactive astrocytes are more toxic than the A2 reactive astrocytes which proliferate and support neuronal regeneration in models of acute trauma (Liddelow and Barres, 2017).

In addition, MDMB-CHMICA was able to significantly induce mitochondrial complex IV subunit Va which could potentially lead to increased ROS production. In contrast, ∆9-THC slightly inhibited the complex I NDUFA9 subunit which might also lead increased ROS production. The fluctuations in ROS levels play a role in cellular signalling pathways that target proteins, polysaccharides, lipids and DNA (Stadtman and Levine, 2000, Rubbo et al., 1994, Kaur and Halliwell, 1994, Richter et al., 1988). An effect of cannabinoids on mitochondrial respiration is supported by studies that suggested that CB1 receptors are expressed in neuronal mitochondria (mtCB1) and directly control energy production through reduction in cyclic AMP, protein kinase A activity and complex I enzymatic activity (Bénard et al., 2012). In addition to a recently published studies which reported synthetic cannabinoids THJ-2201 and 5F-PB22 promoted the hyperpolarization of mitochondrial membrane and that THJ-2201 disrupted mitochondrial function and triggered apoptotic cell death (Alexandre et al., 2020).

In conclusion, this research started by estimating the incidence of episodes of toxicity associated with different NPS in the UK and established that the substances most commonly implicated in recent years have been SCRA. The NPIS data together with other data sources can be used to monitor the harm caused by recreational drug use and identification of new emerging drugs. This in turn will help policy makers to take steps to reduce the impact on public health, including legal control measures when these are appropriate.

The research then explored the potential pharmacological effects of SCRA on 5-HT uptake as several toxic effects of SCRA might suggest increased serotonergic activity. The research, however, did not demonstrate an effect of indole SCRA on

5-HT reuptake. Toxicological investigations using the hNPSC cell line suggested that synthetic cannabinoids are more potent neurotoxins than ∆9-THC and they are more toxic to immature than mature cells, highlighting their potential dangers to younger users. In addition, this research indicated that SCRA induce different pathways within the cell that might lead to different clinical outcomes. The toxicological investigations indicated that cannabinoids might influence mitochondria, an important finding because efficient mitochondrial function is vital in the CNS These changes in SCRA users could adversely affect human health and might have a long-term effect on brain structure and functions such as memory and cognition. The RNA sequences analysis open the door for further research to fully understand the underlying mechanisms involved in the SCRA effects on human brain and it is not possible to draw any conclusion on their effects and how to link it with the clinical manifestations.

Understanding the prevalence of use and toxicity, pharmacological effects and clinical and cellular toxicology of NPS is important, considering their recent prevalence of use and the lack of information on short and especially longer-term effects.

Further research

The understanding of the condition surrounding NPS use will help in controlling its use. This needs accurate and adequate data collection system to monitor the NPS problem and then to analyse the impact of regulations. This study would suggests developing the NPIS collection data system by including more information about the reported cases such as education and ethnicity. In addition, the NPIS in UK receive enquires from the health professionals only, it would be beneficial if there is specific phone line for taking public enquires. This will enable to include more poisoning cases in particular some drugs users do not prefer seeking medical help. Furthermore, there would be value in co-ordinating other data collecting systems including amalgamating data collected in England, Wales, Scotland and Northern Ireland to get a picture of the whole UK. In addition, collating data about NPS from additional sources, such as data from confirmed analytical samples from hospital admissions as well as post mortem data, together with information reported by the users about side effects from websites and blogs, such as Drugs Forums and surveys will help to develop an overall picture of NPS use in term of pattern of toxicity, demographic use and new emerging substances. This data triangulation will decrease the impact of the limitations of each individual source and will help health professionals and policy makers in managing the problems posed by NPS.
The functional gene analysis showed changes in MT-tRNA, therefore further validation of these results will be needed, for example using Quantitative PCR (qPCR).
Further characterization of the proteins/pathways that are changed by SCRA in neuronal cells is of major interest and will clarify the mechanisms that link SCRA to clinical presentations and permanent changes from repeated use. This could be done using the following methods:

1. Investigation of the morphological changes to neuronal cells using Immunocytochemistry (ICC) techniques.
2. Measuring the oxidative stress that might be triggered by cannabinoid exposure by measuring ROS levels in cells by using the MitoSOX-based assay.
3. Use of mitochondrial function assays such as Mitochondrial Membrane Potential (MMP) Assay and seahorse assay.

4. It would be beneficial to measure not only the increased or decreased in protein expressions, but also the phosphorylated forms. Protein phosphorylation changes the protein function to become activated or deactivated and this plays an important role in modifying protein function such as measuring the phosphorylated form of EIF2 and Erk1/2 proteins. This done by western blotting technique using phosphorylated antibodies.

The brain is just one of many organs that might be affected by SCRA as the adverse effects reported from SCRA was not limited to neurological one only. Further investigations would be needed studying other organ systems such as the heart, respiratory system, kidneys and especially the liver as this is the major site of drug metabolism

Other research that would be of value includes:

1. Further study of the pharmacology and toxicology of prevalent and emerging SCRA
2. Research into the optimum management of acute SCRA intoxication, including evaluation of potential therapies.
3. Development of accurate field tests for SCRA that can adapt to changes in the drug market.
4. Determination of the longer-term health effects of SCRA use, including effects on memory and cognition and on reproductive and foetal health.

Finally, as the existing data about NPS showed that use is largely by younger populations, it would be beneficial to identify the motives for and circumstances of use. This would assist with in planning the most appropriate interventions to reduce use and health impacts, including educational interventions and legislative controls.

Appendix I- The results of RNA sequences

1. List of the differentially expressed genes

1.1 The genes with the most statistically significant changes after MDMB-CHMICA exposure of mature cells.

Ensembl_gene_id	hgnc_symbol	Description	log2FoldChange	pvalue	padj	Chromosome_name
ENSG00000210100	MT-TI	mitochondrially encoded tRNA isoleucine	1.9882	1.080 E-76	2.010E-72	MT
ENSG00000261326	LINC01355	long intergenic non-protein coding RNA 1355	-2.2845	2.290 E-38	1.420E-34	1
ENSG00000105708	ZNF14	zinc finger protein 14	-1.1434	4.218 E-29	1.309E-25	19
ENSG00000252690	SCARNA15	small Cajal body-specific RNA 15	-1.5247	3.900 E-28	1.038E-24	15
ENSG00000120738	EGR1	early growth response 1	1.0825	1.196 E-27	2.784E-24	5
ENSG00000210195	MT-TT	mitochondrially encoded tRNA threonine	1.3404	1.954 E-27	4.043E-24	MT
ENSG00000278864		Novel transcript	-1.0224	7.319 E-24	1.239E-20	17
ENSG00000233937		Uncategorized gene	-1.5595	3.309 E-23	5.136E-20	5
ENSG00000273373		Novel transcript	-1.8770	4.478 E-22	5.957E-19	1
ENSG00000142871	CYR61	cysteine rich angiogenic inducer 61	1.1275	2.113 E-20	2.460E-17	1
ENSG00000272540		Novel transcript	1.8774	6.086 E-20	6.668E-17	6
ENSG00000250251	PKD1P6	polycystin 1, transient receptor potential channel interacting pseudogene 6	1.0007	7.040 E-18	6.243E-15	16
ENSG00000210107	MT-TQ	mitochondrially encoded tRNA glutamine	1.4118	4.274 E-17	3.184E-14	MT
ENSG00000274925	ZKSCAN2-DT	ZKSCAN2 divergent transcript	-1.5762	4.816 E-17	3.450E-14	16
ENSG00000210077	MT-TV	mitochondrially encoded tRNA valine	1.5271	4.607 E-16	2.600E-13	MT
ENSG00000271533		Novel transcript	-1.1121	7.316 E-16	3.785E-13	X
ENSG00000275180		Novel transcript	-1.7873	3.089 E-14	1.338E-11	12
ENSG00000210196	MT-TP	mitochondrially encoded tRNA proline	1.2209	7.749 E-14	3.137E-11	MT
ENSG00000215493		kelch-like 12 (Drosophila) (KLH12) pseudogene	-1.4480	1.427 E-12	4.922E-10	22
ENSG00000210112	MT-TM	mitochondrially encoded tRNA methionine	1.4782	2.827 E-11	7.213E-09	MT
ENSG00000214402	LCNL1	lipocalin like 1	1.0355	1.814 E-10	3.796E-08	9

ENSG00000268403		Novel transcript	-1.3299	3.296 E-10	6.394E-08	11
ENSG00000280214		Uncategorized gene	-1.4552	5.277 E-10	9.545E-08	16
ENSG00000263826		Isoform 2 EIF4A2	1.2778	8.572 E-10	1.451E-07	3
ENSG00000170345	FOS	Fos proto-oncogene, AP-1 transcription factor subunit	1.8559	1.015 E-09	1.672E-07	14
ENSG00000269399		Novel transcript	-1.2372	1.951 E-09	3.002E-07	19
ENSG00000270557		Novel transcript	-1.7006	3.081 E-09	4.590E-07	2
ENSG00000280367		Novel transcript	-3.4385	7.490 E-09	1.000E-06	11
ENSG00000205746	PKD1P4	Polycystin 1, transient receptor potential channel interacting pseudogene 4	1.1436	7.693 E-09	1.023E-06	16
ENSG00000250138		POM121 membrane glycoprotein-like 1 (POM121L1) pseudogene	-2.0564	1.530 E-08	1.940E-06	5
ENSG00000227896		Novel transcript	-1.8051	1.547 E-08	1.946E-06	10
ENSG00000210154	MT-TD	mitochondrially encoded tRNA aspartic acid	1.1378	1.591 E-08	1.989E-06	MT
ENSG00000100181	TPTEP1	transmembrane phosphatase with tensin homology pseudogene 1	-1.3457	1.780 E-08	2.153E-06	22
ENSG00000113739	STC2	stanniocalcin 2 [Source:HGNC Symbol;Acc:HGNC:11374]	1.0262	5.071 E-08	5.523E-06	5
ENSG00000108821	COL1A1	collagen type I alpha 1 chain	-1.7982	1.057 E-07	1.058E-05	17
ENSG00000261136		Novel transcript	-1.3372	1.612 E-07	1.509E-05	15
ENSG00000273723	SUGT1-DT	SUGT1 divergent transcript	-1.6022	2.333 E-07	2.059E-05	13
ENSG00000118972	FGF23	fibroblast growth factor 23	-1.6425	3.045 E-07	2.589E-05	12
ENSG00000245149	RNF139-AS1	RNF139 antisense RNA 1 (head to head)	-1.2990	3.431 E-07	2.873E-05	8
ENSG00000226318	RPS3AP38	ribosomal protein S3a pseudogene 38	-2.1697	3.550 E-07	2.920E-05	10
ENSG00000161381	PLXDC1	plexin domain containing 1	-2.9586	4.000 E-07	3.210E-05	17
ENSG00000210049	MT-TF	mitochondrially encoded tRNA phenylalanine	1.2707	4.138 E-07	3.308E-05	MT
ENSG00000215417	MIR17HG	miR-17-92a-1 cluster host gene	-1.1787	5.922 E-07	4.484E-05	13
ENSG00000276168	RN7SL1	RNA, 7SL, cytoplasmic 1	-1.6897	1.720 E-06	1.089E-04	14
ENSG00000270903	HNRNPA3P9	heterogeneous nuclear ribonucleoprotein A3 pseudogene 9	-2.0692	2.200 E-06	1.320E-04	11

ENSG00000178464	RPL10P16	ribosomal protein L10 pseudogene 16	2.3372	3.370 E-06	1.847E-04	19
ENSG00000255326		Novel transcript	-1.3717	3.615 E-06	1.951E-04	11
ENSG00000213073	CHP1P2	calcium binding protein P22 pseudogene	-1.3723	4.251 E-06	2.224E-04	6
ENSG00000254402	LRRC24	leucine rich repeat containing 24	2.1005	4.770 E-06	2.434E-04	8
ENSG00000254870	ATP6V1G 2-DDX39B	ATP6V1G2-DDX39B readthrough (NMD candidate)	1.6854	4.973 E-06	2.489E-04	6
ENSG00000237491	LINC0140 9	long intergenic non-protein coding RNA 1409	-1.3003	5.772 E-06	2.814E-04	1
ENSG00000084453	SLCO1A2	solute carrier organic anion transporter family member 1A2	1.1476	6.585 E-06	3.052E-04	12
ENSG00000271699	SNX29P2	sorting nexin 29 pseudogene 2	-1.0362	7.112 E-06	3.246E-04	16
ENSG00000279302		Uncategorized gene	-1.5918	7.211 E-06	3.276E-04	8
ENSG00000267519		Novel transcript	-1.0821	7.690 E-06	3.451E-04	19
ENSG00000274012	RN7SL2	RNA, 7SL, cytoplasmic 2	-2.0041	1.200 E-05	5.039E-04	14
ENSG00000269694		Novel transcript	-1.6709	1.773 E-05	6.923E-04	19
ENSG00000279044		Uncategorized gene	-1.6379	1.897 E-05	7.360E-04	19
ENSG00000210156	MT-TK	mitochondrially encoded tRNA lysine	1.0895	2.236 E-05	8.449E-04	MT
ENSG00000224934		Novel transcript	-1.0708	2.441 E-05	9.147E-04	10
ENSG00000259488		Novel transcript	-1.1407	2.477 E-05	9.206E-04	15
ENSG00000260924	LINC0131 1	long intergenic non-protein coding RNA 1311	-1.7542	2.877 E-05	1.046E-03	22
ENSG00000142347	MYO1F	myosin IF	-1.6162	2.925 E-05	1.060E-03	19
ENSG00000140832	MARVELD 3	MARVEL domain containing 3	-1.0521	3.135 E-05	1.114E-03	16
ENSG00000249892		Novel transcript	-1.0055	3.500 E-05	1.214E-03	4
ENSG00000108231	LGI1	leucine rich glioma inactivated 1	-1.3475	3.673 E-05	1.253E-03	10
ENSG00000269929	MIRLET7 A1HG	miRlet-7a-1/let-7f-1/let-7d cluster host gene	-1.1864	4.948 E-05	1.608E-03	9
ENSG00000238186		Novel transcript	1.4305	4.964 E-05	1.611E-03	1
ENSG00000135502	SLC26A10	solute carrier family 26 member 10	1.0097	5.140 E-05	1.654E-03	12
ENSG00000127586	CHTF18	chromosome transmission fidelity factor 18	1.0802	5.961 E-05	1.841E-03	16

ENSG00000260589	STAM-AS1	STAM antisense RNA 1 (head to head)	-1.3850	6.225 E-05	1.895E-03	10
ENSG00000278558	TMEM191B	transmembrane protein 191B	1.8829	7.114 E-05	2.130E-03	22
ENSG00000259984		Novel Pseudogene	-1.7081	7.939 E-05	2.317E-03	1
ENSG00000196826		Novel Zinc Finger Protein	-1.3256	8.527 E-05	2.462E-03	19
ENSG00000268861		Rho/Rac Guanine Nucleotide Exchange Factor	-8.9131	8.670 E-05	2.499E-03	19
ENSG00000174564	IL20RB	interleukin 20 receptor subunit beta	-1.2801	1.042 E-04	2.922E-03	3
ENSG00000196296	ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1	-1.0503	1.062 E-04	2.952E-03	16
ENSG00000274460		Novel Transcript	-1.0889	1.611 E-04	4.172E-03	16
ENSG00000213442	RPL18AP3	ribosomal protein L18a pseudogene 3	1.3249	1.632 E-04	4.216E-03	12
ENSG00000205078	SYCE1L	synaptonemal complex central element protein 1 like	3.1336	1.682 E-04	4.314E-03	16
ENSG00000174951	FUT1	fucosyltransferase 1 (H blood group)	-1.3543	1.686 E-04	4.319E-03	19
ENSG00000149256	TENM4	teneurin transmembrane protein 4	-3.1319	2.046 E-04	4.982E-03	11
ENSG00000226674	TEX41	testis expressed 41 (non-protein coding)	-3.5227	2.199 E-04	5.305E-03	2
ENSG00000226091	LINC00937	long intergenic non-protein coding RNA 937	-1.3762	2.488 E-04	5.844E-03	12
ENSG00000116183	PAPPA2	pappalysin 2	3.1252	2.658 E-04	6.142E-03	1
ENSG00000267053		Novel Transcript	-1.5721	2.732 E-04	6.259E-03	19
ENSG00000273002	ARHGEF2-AS2	ARHGEF2 antisense RNA 2	-1.1658	2.915 E-04	6.565E-03	1
ENSG00000278134		uncategorized gene	-1.0879	2.965 E-04	6.620E-03	9
ENSG00000163694	RBM47	RNA binding motif protein 47	-3.0929	3.008 E-04	6.701E-03	4
ENSG00000158220	ESYT3	extended synaptotagmin 3	-2.0876	3.016 E-04	6.702E-03	3
ENSG00000252464	RN7SKP70	RNA, 7SK small nuclear pseudogene 70	-1.0117	3.298 E-04	7.109E-03	5
ENSG00000233264		peptidylprolyl isomerase (cyclophilin)-like 4 (PPIL4) pseudogene	1.2075	3.428 E-04	7.314E-03	7
ENSG00000268790		Novel protein	1.0557	3.543 E-04	7.464E-03	19
ENSG00000279700		Novel transcript	-1.6674	3.567 E-04	7.497E-03	12
ENSG00000196205	EEF1A1P5	eukaryotic translation elongation factor 1 alpha 1 pseudogene 5	1.1660	3.571 E-04	7.497E-03	9

ENSG00000154734	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1	-2.2652	3.724 E-04	7.733E-03	21
ENSG00000279500		Novel transcript	-1.0169	4.563 E-04	9.002E-03	12
ENSG00000018236	CNTN1	contactin 1	1.5385	5.027 E-04	9.723E-03	12
ENSG00000153902	LGI4	leucine rich repeat LGI family member 4	1.0274	5.530 E-04	1.044E-02	19
ENSG00000127578	WFIKKN1	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1	1.0281	5.895 E-04	1.092E-02	16
ENSG00000177576	C18orf32	chromosome 18 open reading frame 32	-1.1240	6.231 E-04	1.139E-02	18
ENSG00000130600	H19	H19, imprinted maternally expressed transcript (non-protein coding)	-1.0839	6.447 E-04	1.169E-02	11
ENSG00000232725		Novel transcript	1.9045	6.685 E-04	1.204E-02	X
ENSG00000144045	DQX1	DEAQ-box RNA dependent ATPase 1	-3.2767	7.360 E-04	1.285E-02	2
ENSG00000215483	LINC00598	long intergenic non-protein coding RNA 598	-2.2692	8.487 E-04	1.430E-02	13
ENSG00000184343	SRPK3	SRSF protein kinase 3	1.1226	8.739 E-04	1.451E-02	X
ENSG00000206344	HCG27	HLA complex group 27 (non-protein coding)	-1.5323	9.641 E-04	1.563E-02	6
ENSG00000249593		Novel transcript	-2.6103	1.000 E-03	1.599E-02	5
ENSG00000263212		Novel transcript	-2.0290	1.099 E-03	1.715E-02	16
ENSG00000230397	SPTLC1P1	serine palmitoyltransferase long chain base subunit 1 pseudogene 1	-1.3217	1.128 E-03	1.750E-02	10
ENSG00000227081		ribosomal protein S27 (RPS27) pseudogene	-1.6231	1.152 E-03	1.780E-02	12
ENSG00000258302		Novel transcript	-1.0488	1.186 E-03	1.816E-02	12
ENSG00000272931	LRRC8D-DT	LRRC8D divergent transcript	1.2342	1.213 E-03	1.848E-02	1
ENSG00000266955		Novel transcript	-1.4031	1.226 E-03	1.860E-02	18
ENSG00000279595		Uncategorized gene.	-1.7763	1.292 E-03	1.930E-02	15
ENSG00000114841	DNAH1	dynein axonemal heavy chain 1	1.7642	1.325 E-03	1.963E-02	3
ENSG00000271993		Novel transcript,	-1.4313	1.389 E-03	2.036E-02	3
ENSG00000179954	SSC5D	scavenger receptor cysteine rich family member with 5 domains	-1.6710	1.472 E-03	2.125E-02	19

ENSG00000269378	ITGB1P1	Integrin subunit beta 1 pseudogene 1	1.0426	1.479 E-03	2.133E-02	19
ENSG00000205663	FAM239B	family with sequence similarity 239 member B (non-protein coding)	-2.2647	1.580 E-03	2.236E-02	X
ENSG00000267278	MAP3K14-AS1	MAP3K14 antisense RNA 1	1.2478	1.615 E-03	2.268E-02	17
ENSG00000205885	C1RL-AS1	C1RL antisense RNA 1	-1.0011	1.621 E-03	2.275E-02	12
ENSG00000224093	BCAR3-AS1	BCAR3 antisense RNA 1	-4.1488	1.725 E-03	2.393E-02	1
ENSG00000203327		Novel transcript	-1.0648	1.884 E-03	2.542E-02	2
ENSG00000261118		Novel transcript	-1.1651	1.912 E-03	2.569E-02	16
ENSG00000236723		Novel transcript	-1.0153	1.956 E-03	2.611E-02	1
ENSG00000247157	LINC01252	long intergenic non-protein coding RNA 1252	-1.2741	2.290 E-03	2.933E-02	12
ENSG00000226472		Tetraspanin family pseudogene	-1.0202	2.293 E-03	2.933E-02	12
ENSG00000270433	H3P37	H3 histone pseudogene 37	-1.2296	2.298 E-03	2.937E-02	14
ENSG00000167971	CASKIN1	CASK interacting protein 1	1.9154	2.437 E-03	3.055E-02	16
ENSG00000207031	SNORD59A	small nucleolar RNA, C/D box 59A	1.1244	2.488 E-03	3.109E-02	12
ENSG00000272576		Novel transcript	1.2212	2.497 E-03	3.113E-02	4
ENSG00000213057	C1orf220	chromosome 1 open reading frame 220	-1.1096	2.599 E-03	3.208E-02	1
ENSG00000173083	HPSE	heparanase	-1.0606	2.720 E-03	3.307E-02	4
ENSG00000254929		Novel transcript	1.5937	2.734 E-03	3.319E-02	10
ENSG00000183117	CSMD1	CUB and Sushi multiple domains 1	1.2220	2.822 E-03	3.390E-02	8
ENSG00000171931	FBXW10	F-box and WD repeat domain containing 10	1.3914	2.830 E-03	3.398E-02	17
ENSG00000214223	HNRNPA1P10	heterogeneous nuclear ribonucleoprotein A1 pseudogene 10	-1.1906	2.898 E-03	3.461E-02	19
ENSG00000111012	CYP27B1	cytochrome P450 family 27 subfamily B member 1	2.0488	2.972 E-03	3.528E-02	12
ENSG00000163915	IGF2BP2-AS1	IGF2BP2 antisense RNA 1	-5.9053	3.053 E-03	3.602E-02	3
ENSG00000081277	PKP1	plakophilin 1	-1.9184	3.124 E-03	3.662E-02	1
ENSG00000101445	PPP1R16B	protein phosphatase 1 regulatory subunit 16B	2.7226	3.620 E-03	4.099E-02	20
ENSG00000182230	FAM153B	family with sequence similarity 153 member B	-1.2315	3.778 E-03	4.217E-02	5

ENSG00000255142		Novel transcript	-1.0769	3.779 E-03	4.217E-02	11
ENSG00000148795	CYP17A1	cytochrome P450 family 17 subfamily A member 1	3.4324	4.023 E-03	4.392E-02	10
ENSG00000153086	ACMSD	aminocarboxymuconat e semialdehyde decarboxylase	-1.1879	4.073 E-03	4.426E-02	2
ENSG00000165685	TMEM52B	transmembrane protein 52B	2.3586	4.266 E-03	4.577E-02	12
ENSG00000271265		Novel transcript	-1.2092	4.314 E-03	4.607E-02	6
ENSG00000171722	SPATA46	spermatogenesis associated 46	-1.1882	4.379 E-03	4.649E-02	1
ENSG00000246596		SUMO interacting motifs containing 1 pseudogene	-1.0101	4.405 E-03	4.667E-02	5
ENSG00000279104		Uncategorized gene	1.1625	4.700 E-03	4.860E-02	7
ENSG00000138131	LOXL4	lysyl oxidase like 4	1.1227	4.704 E-03	4.861E-02	10
ENSG00000182050	MGAT4C	MGAT4 family member C	1.1565	4.751 E-03	4.894E-02	12
ENSG00000280063		Uncategorized gene	-1.1719	4.836 E-03	4.951E-02	16

1.2 The genes with the most statistically significant changes after 5F-ADB exposure of mature cells.

Ensembl_gene_id	hgnc_symbol	Description	log2Fold Change	pvalue	padj	Chromosome name
ENSG00000210100	MT-TI	Mitochondrially encoded tRNA isoleucine	1.17	4.8E-26	7.8E-22	MT
ENSG00000210107	MT-TQ	Mitochondrially encoded tRNA glutamine	1.06	4.4E-10	1.8E-06	MT
ENSG00000269378	ITGB1P1	integrin subunit beta 1 pseudogene 1	1.50	4.6E-06	2.5E-03	19
ENSG00000196167	COLCA1	Colorectal cancer associated 1	-1.92	7.8E-06	3.2E-03	11
ENSG00000144045	DQX1	DEAQ-box RNA dependent ATPase 1	-4.12	3.2E-05	7.4E-03	2
ENSG00000184343	SRPK3	SRSF protein kinase 3	1.33	7.6E-05	1.4E-02	X
ENSG00000196218	RYR1	ryanodine receptor 1	1.31	1.0E-04	1.6E-02	19

ENSG00000183018	SPNS2	sphingolipid transporter 2	1.05	1.2E-04	1.7E-02	17
ENSG00000171700	RGS19	regulator of G protein signalling 19	2.05	1.3E-04	1.8E-02	20
ENSG00000081479	LRP2	LDL receptor related protein 2	1.13	1.4E-04	1.8E-02	2
ENSG00000108231	LGI1	leucine rich glioma inactivated 1	-1.24	1.3E-04	1.8E-02	10
ENSG00000150667	FSIP1	fibrous sheath interacting protein 1	2.66	2.2E-04	2.4E-02	15
ENSG00000109255	NMU	Neuromedin U	-1.68	3.3E-04	3.3E-02	4
ENSG00000255326		Nnovel transcript	-1.00	4.0E-04	3.7E-02	11
ENSG00000099725	PRKY	Protein kinase, Y-linked, pseudogene	-4.32	4.7E-04	4.0E-02	Y
ENSG00000118972	FGF23	Fibroblast growth factor 23	-1.09	4.8E-04	4.0E-02	12
ENSG00000163694	RBM47	RNA binding motif protein 47	-2.97	4.9E-04	4.0E-02	4
ENSG00000181790	ADGRB1	Adhesion G protein-coupled receptor B1	1.11	5.6E-04	4.4E-02	8
ENSG00000172339	ALG14	ALG14, UDP-N-acetylglucosaminyltransferase subunit	-1.20	5.9E-04	4.5E-02	1
ENSG00000121101	TEX14	Testis expressed 14, intercellular bridge forming factor	1.88	6.5E-04	4.7E-02	17

1.3 The genes with the most statistically significant changes after ∆9- THC exposure of mature cells.

Ensembl_gene_id	hgnc_ symbol	Description	log2F oldCh ange	pvalue	padj	Chrom osome _name
ENSG00000210100	MT-TI	mitochondrially encoded tRNA isoleucine	1.606 2	2.37E-49	3.86E-45	MT
ENSG00000210107	MT-TQ	mitochondrially encoded tRNA glutamine	1.374 5	2.78E-16	9.03E-13	MT
ENSG00000252690	SCAR NA15	small Cajal body-specific RNA 15	-1.069 7	2.28E-16	9.03E-13	15
ENSG00000104435	STMN 2	stathmin 2	-1.440 1	2.33E-15	5.05E-12	8

ENSG00000079102	RUNX1T1	RUNX1 translocation partner 1	-1.0215	1.39E-12	1.62E-09	8
ENSG00000261326	LINC01355	long intergenic non-protein coding RNA 1355	-1.1085	2.75E-12	2.63E-09	1
ENSG00000210112	MT-TM	mitochondrially encoded tRNA methionine	1.4525	6.09E-11	3.96E-08	MT
ENSG00000210077	MT-TV	mitochondrially encoded tRNA valine	1.2058	2.31E-10	1.21E-07	MT
ENSG00000214402	LCNL1	lipocalin like 1	1.0280	2.29E-10	1.21E-07	9
ENSG00000187122	SLIT1	slit guidance ligand 1	-1.3889	5.31E-09	2.01E-06	10
ENSG00000167202	TBC1D2B	TBC1 domain family member 2B	-1.0076	4.95E-08	1.44E-05	15
ENSG00000263826		isoform 2 EIF4A2	1.1230	8.79E-08	2.34E-05	3
ENSG00000151090	THRB	thyroid hormone receptor beta	-1.0202	1.77E-07	3.73E-05	3
ENSG00000272540		Isoform 2 EIF4A2	1.0976	2.20E-07	4.32E-05	6
ENSG00000269399		Novel transcript	-1.0274	2.33E-07	4.51E-05	19
ENSG00000108231	LGI1	leucine rich glioma inactivated 1	-1.6128	7.70E-07	1.23E-04	10
ENSG00000249859	PVT1	Pvt1 oncogene (non-protein coding)	1.3709	7.57E-07	1.23E-04	8
ENSG00000210156	MT-TK	mitochondrially encoded tRNA lysine	1.1918	3.01E-06	3.63E-04	MT
ENSG00000153902	LGI4	leucine rich repeat LGI family member 4	1.3773	3.62E-06	4.19E-04	19
ENSG00000210117	MT-TW	mitochondrially encoded tRNA tryptophan	1.2037	4.31E-06	4.70E-04	MT
ENSG00000254870	ATP6V1G2-DDX39B	ATP6V1G2-DDX39B readthrough (NMD candidate)	1.5816	1.76E-05	1.43E-03	6
ENSG00000261143	ADAMTS7P3	ADAMTS7 pseudogene 3	5.8186	3.12E-05	2.29E-03	15
ENSG00000260589	STAM-AS1	STAM antisense RNA 1	-1.4299	3.26E-05	2.35E-03	10
ENSG00000162572	SCNN1D	sodium channel epithelial 1 delta subunit	1.1313	4.15E-05	2.66E-03	1

ENSG00000196167	COLCA1	colorectal cancer associated 1	-1.7281	5.54E-05	3.44E-03	11
ENSG00000174564	IL20RB	interleukin 20 receptor subunit beta	-1.3267	5.80E-05	3.53E-03	3
ENSG00000148795	CYP17A1	cytochrome P450 family 17 subfamily A member 1	4.7010	7.91E-05	4.48E-03	10
ENSG00000081479	LRP2	LDL receptor related protein 2	1.1653	8.64E-05	4.83E-03	2
ENSG00000236963	LINC01141	long intergenic non-protein coding RNA 1141	1.1807	2.03E-04	8.97E-03	1
ENSG00000253882		Member C (FAM115C) pseudogene	-1.0834	2.05E-04	9.01E-03	7
ENSG00000151651	ADAM8	ADAM metallopeptidase domain 8	1.4878	2.30E-04	9.85E-03	10
ENSG00000269378	ITGB1P1	Integrin subunit beta 1 pseudogene 1	1.1954	2.59E-04	1.08E-02	19
ENSG00000270332	SMC2-AS1	SMC2 antisense RNA 1 (head to head)	-1.4975	2.87E-04	1.14E-02	9
ENSG00000177374	HIC1	HIC ZBTB transcriptional repressor 1	-1.2486	2.88E-04	1.14E-02	17
ENSG00000254402	LRRC24	leucine rich repeat containing 24	1.6708	3.14E-04	1.21E-02	8
ENSG00000196218	RYR1	ryanodine receptor 1	1.2025	3.42E-04	1.27E-02	19
ENSG00000205236		Novel Protein	-6.2693	4.23E-04	1.49E-02	7
ENSG00000196876	SCN8A	sodium voltage-gated channel alpha subunit 8	-1.2012	5.04E-04	1.68E-02	12
ENSG00000181790	ADGRB1	adhesion G protein-coupled receptor B1	1.0942	6.37E-04	1.98E-02	8
ENSG00000227081		Ribosomal protein S27 (RPS27) pseudogene	-1.6836	7.47E-04	2.23E-02	12
ENSG00000161618	ALDH16A1	aldehyde dehydrogenase 16 family member A1	1.0777	8.11E-04	2.35E-02	19
ENSG00000177576	C18orf32	chromosome 18 open reading frame 32	-1.0822	9.85E-04	2.67E-02	18
ENSG00000166840	GLYATL1	glycine-N-acyltransferase like 1	3.6856	1.01E-03	2.72E-02	11
ENSG00000144045	DQX1	DEAQ-box RNA dependent ATPase 1	-3.1991	1.06E-03	2.80E-02	2
ENSG00000122861	PLAU	plasminogen activator, urokinase	1.7646	1.11E-03	2.86E-02	10

ENSG00000013563	DNASE1L1	deoxyribonuclease 1 like 1	1.0646	1.45E-03	3.40E-02	X
ENSG00000215483	LINC00598	long intergenic non-protein coding RNA 598	-2.1137	1.92E-03	4.13E-02	13
ENSG00000236439		ribosomal protein S27 pseudogene	-4.4567	1.97E-03	4.19E-02	1
ENSG00000280852		Adaptor-related protein complex 1	1.0783	1.97E-03	4.20E-02	17
ENSG00000184923	NUTM2A	NUT family member 2A	1.1306	2.30E-03	4.65E-02	10

2. The List of the differentially expressed genes overlapped between MDMB-CHMICA, 5F-ADB and ∆9- THC.

2.1 The most common genes changed between MDMB-CHMICA and 5F-ADB treated cells.

Ensembl_gene_id	hgnc_ symbol	Description	MDMB-CHMICA		5F-ADB	
			log2Fold Change	*padj*	*log2Fold Change*	*padj*
ENSG00000144045	**DQX1**	DEAQ-box RNA dependent ATPase 1	-3.277	0.013	-4.122	7.38E-03
ENSG00000163694	**RBM47**	RNA binding motif protein 47	-3.093	0.007	-2.971	4.00E-02
ENSG00000184343	**SRPK3**	SRSF protein kinase 3	1.123	0.015	1.333	1.35E-02
ENSG00000108231	**LGI1**	Leucine rich glioma inactivated 1	-1.347	0.001	-1.244	1.81E-02
ENSG00000210100	**MT-TI**	Mitochondrially encoded tRNA isoleucine	1.988	2.01E-72	1.169	7.82E-22
ENSG00000118972	**FGF23**	Fibroblast growth factor 23	-1.642	2.59E-05	-1.085	3.98E-02
ENSG00000210107	**MT-TQ**	Mitochondrially encoded tRNA glutamine	1.412	3.18E-14	1.058	1.77E-06
ENSG00000269378	**ITGB1P1**	Integrin subunit beta 1 pseudogene 1	1.0426	0.0213	1.1954	1.08E-02
ENSG00000255326		Novel transcript	-1.3717	0.0002	-1.0016	3.70E-02

2.2 The most common genes changed between MDMB-CHMICA and ∆9-THC treated cells.

Ensembl_gene_id	hgnc_ symbol	Description	MDMB-CHMICA		Δ9-THC	
			log2Fold Change	*padj*	*log2Fold Change*	*padj*
ENSG00000254870	**ATP6V1G2-DDX39B**	ATP6V1G2-DDX39B readthrough (NMD candidate)	1.6854	2.49E-04	1.5816	1.43E-03
ENSG00000177576	**C18orf32**	chromosome 18 open reading frame 32	-1.1240	1.14E-02	-1.0822	2.67E-02
ENSG00000148795	**CYP17A1**	cytochrome P450 family 17 subfamily A member 1	3.4324	4.39E-02	4.7010	4.48E-03
ENSG00000144045	**DQX1**	glycine-N-acyltransferase like 1	-3.2767	1.28E-02	-3.1991	2.80E-02
ENSG00000174564	**IL20RB**	interleukin 20 receptor subunit beta	-1.2801	2.92E-03	-1.3267	3.53E-03
ENSG00000214402	**LCNL1**	lipocalin like 1	1.0355	3.80E-08	1.0280	1.21E-07
ENSG00000108231	**LGI1**	leucine rich glioma inactivated 1	-1.3475	1.25E-03	-1.6128	1.23E-04
ENSG00000153902	**LGI4**	leucine rich repeat LGI family member 4	1.0274	1.04E-02	1.3773	4.19E-04
ENSG00000215483	**LINC00598**	long intergenic non-protein coding RNA 598	-2.2692	1.43E-02	-2.1137	4.13E-02
ENSG00000261326	**LINC01355**	long intergenic non-protein coding RNA 1355	-2.2845	1.42E-34	-1.1085	2.63E-09
ENSG00000254402	**LRRC24**	leucine rich repeat containing 24	2.1005	2.43E-04	1.6708	1.21E-02
ENSG00000210100	**MT-TI**	mitochondrially encoded tRNA isoleucine	1.9882	2.01E-72	1.6062	3.86E-45
ENSG00000210156	**MT-TK**	mitochondrially encoded tRNA lysine	1.0895	8.45E-04	1.1918	3.63E-04

ENSG00000210112	**MT-TM**	mitochondrially encoded tRNA methionine	1.4782	7.21E-09	1.4525	3.96E-08
ENSG00000210107	**MT-TQ**	mitochondrially encoded tRNA glutamine	1.4118	3.18E-14	1.3745	9.03E-13
ENSG00000210077	**MT-TV**	mitochondrially encoded tRNA valine	1.5271	2.60E-13	1.2058	1.21E-07
ENSG00000252690	**SCARNA15**	small Cajal body-specific RNA 15	-1.5247	1.04E-24	-1.0697	9.03E-13
ENSG00000260589	**STAM-AS1**	STAM antisense RNA 1	-1.3850	1.89E-03	-1.4299	2.35E-03
ENSG00000272540		Novel transcript	1.877449	6.67E-17	1.09759	4.32E-05
ENSG00000227081		ribosomal protein S27 pseudogene	-1.62308	1.78E-02	-1.6836	2.23E-02
ENSG00000263826		Isoform 2 EIF4A2	1.277838	1.45E-07	1.12303	2.34E-05
ENSG00000269399		Novel transcript	-1.23716	3.00E-07	-1.0274	4.51E-05
ENSG00000269378	**ITGB1P1**	Integrin subunit beta 1 pseudogene 1	1.042597	2.13E-02	1.19537	1.08E-02

2.3 The most common genes changed between 5F-ADB and ∆9-THC treated cells.

ensembl_gene_id	hgnc_ symbol	Description	5F-ADB		THC	
			log2Fold Change	*padj*	*log2Fold Change*	*padj*
ENSG00000144045	**DQX1**	DEAQ-box RNA dependent ATPase 1	-4.12	7.38E-03	-3.20	2.80E-02
ENSG00000210107	**MT-TQ**	mitochondrially encoded tRNA glutamine	1.06	1.77E-06	1.37	9.03E-13
ENSG00000210100	**MT-TI**	mitochondrially encoded tRNA isoleucine	1.17	7.82E-22	1.61	3.86E-45
ENSG00000108231	**LGI1**	leucine rich glioma inactivated 1	-1.24	1.81E-02	-1.61	1.23E-04
ENSG00000181790	**ADGRB1**	adhesion G protein-coupled receptor B1	1.11	4.44E-02	1.09	1.98E-02
ENSG00000196167	**COLCA1**	colorectal cancer associated 1	-1.92	3.17E-03	-1.73	3.44E-03
ENSG00000196218	**RYR1**	ryanodine receptor 1	1.31	1.60E-02	1.20	1.27E-02
ENSG00000081479	**LRP2**	LDL receptor related protein 2	1.13	1.81E-02	1.17	4.83E-03
ENSG00000269378	**ITGB1P1**	Integrin subunit beta 1 pseudogene 1	1.50	2.45E-03	1.20	1.08E-02

3. The significant canonical pathway analysed by the ingenuity pathway analysis software.

3.1 The canonical pathways that were significantly changed in MDMB-CHMICA exposed mature cells.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Sumoylation Pathway	4.27	0.188	-1.291	GDI1,HDAC2,RCC1,RHOT2,SENP5,PIAS1,SENP1,SP1,PCNA,SUMO3,RHOA,FOS,UBE2I,CTBP2,RHOB,ARHGDIA,RND3,ZNF217
EIF2 Signalling	3.46	0.131	1.698	RPS27L,EIF3G,RPS27,EIF4A2,MT-TM,RPL22L1,DDIT3,RPL29,RPL41,WARS,RPL27A,RPL30,RPS25,SREBF1,VEGFA,RPL21,PIK3R4,RPL22,NOX4,EIF4G2,RPS14,EIF5,EIF2S3,RPL18,RPS17,PIK3R2,HSPA5,MAP2K2,RPL27
Notch Signalling	2.52	0.211	2.236	CNTN1,DLL1,NOTCH1,MAML1,MAML3,HEY1,DLL3,NOTCH3
Estrogen Receptor Signalling	2.32	0.133	#NUM!	NCOR2,MED21,H3F3A/H3F3B,SMARCA4,HNRNPD,MED18,TAF7,GTF2H4,CCNC,GTF2F1,GTF2F2,MED14,CTBP2,MAP2K2,MED23,DDX5,NCOA2
BER pathway	2.21	0.333	#NUM!	OGG1,POLE,APEX1,PCNA
Sirtuin Signalling Pathway	2.18	0.106	-1.512	MT-ND5,MT-ND2,MT-ND1,HIST1H1C,XRCC6,XRCC5,TOMM20,SIRT6,NDUFS8,MT-ND6,H1FX,UCP2,MLYCD,OGG1,SREBF1,ATG2B,SOD1,H3F3A/H3F3B,ATG7,SP1,TRIM28,NDUFS3,LDHA,NDUFA11,LDHD,BAX,DUSP6,PGAM2,APEX1,NDUFB5,LDHB
mTOR Signalling	2.12	0.114	0.535	RPS27L,EIF3G,RHOT2,VEGFA,RPS27,PLD3,PIK3R4,EIF4A2,RPS14,EIF4G2,RHOA,ULK1,GNB1L,NAPEPLD,RPS17,PIK3R2,RHOB,TSC2,PPP2CB,PRKD1,MLST8,RND3,RPS25

BMP signalling pathway	1.95	0.145	1.414	CREB1,MAP2K2,ZNF423,PRKAR1B,MAGED1,TAB1,MAPK11,SMURF1,BMP7,GREM1,SMAD9
TGF-β Signalling	1.92	0.138	1.414	FOS,RNF111,AMH,MAP2K2,ZNF423,TAB1,PMEPA1,MAPK11,SMURF1,BMP7,SMAD9,ACVR2B
1D-myo-inositol Hexakisphosphate Biosynthesis V (from Ins(1,3,4)P3)	1.9	0.667	#NUM!	IPPK,IPMK
Inositol Pyrophosphates Biosynthesis	1.9	0.375	#NUM!	IPPK,IP6K2,IPMK
Superpathway of Inositol Phosphate Compounds	1.85	0.106	1.4	IP6K2,INPPL1,PPP1R16B,STYXL1,FYN,PTPRF,NUDT16,MTMR6,INPP5A,PXYLP1,PALD1,EGFR,PIK3R4,PTPN2,IPMK,PTPN23,PTPN1,PLCD3,PI4KB,IPPK,PTPN12,PIK3R2,PLPP6,RNGTT,PLCG1
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.85	0.111	#NUM!	LOX,EGFR,FGF1,NOTCH1,ID2,PIK3R4,ZEB2,EGR1,FGF23,TCF7L1,DVL1,FZD5,RHOA,PIK3R2,MAP2K2,MAML1,WNT7A,DVL3,SMURF1,NOTCH3,PYGO1
Telomere Extension by Telomerase	1.84	0.267	#NUM!	XRCC6,XRCC5,TNKS2,HNRNPA2B1
Adipogenesis pathway	1.79	0.119	#NUM!	HDAC2,FGF1,TXNIP,SREBF1,HDAC10,ATG7,GTF2H4,KLF3,SMAD9,FZD5,HDAC6,CTBP2,ZNF423,DDIT3,KAT6B,BMP7
Role of BRCA1 in DNA Damage Response	1.79	0.138	0	POU2F1,FANCL,MSH6,ARID2,CDKN1A,BRCC3,SMARCA4,BARD1,E2F3,FANCF,SMARCB1
Thrombin Signaling	1.77	0.108	2.524	CAMK2B,EGFR,GNB3,RHOT2,CREB1,PIK3R4,PTK2,PLCD3,RHOA,GNB1L,F2R,GNAS,PIK3R2,ARHGEF1,RHOB,ARHGEF2,MAP2K2,PLCG1,PRKD1,MAPK11,ARHGEF4,RND3
Mechanisms of Viral Exit from Host Cells	1.76	0.171	#NUM!	SH3GL1,SNF8,CHMP2B,ACTG1,CHMP4B,PRKD1,CHMP6

Breast Cancer Regulation by Stathmin1	1.75	0.107	#NUM!	CAMK2B,GNB3,UHMK1,CALM1 (includes others),TUBB3,PIK3R4,E2F3,TUBB4B,RHOA,GNB1L,GNAS,PIK3R2,ARHGEF1,ARHGEF2,TUBB2B,MAP2K2,CDKN1A,PPP2CB,PRKD1,PRKAR1B,ARHGEF4,ARHGEF18
Heme Biosynthesis II	1.74	0.333	#NUM!	CPOX,UROD,HMBS
Chondroitin Sulfate Degradation (Metazoa)	1.74	0.25	-1	HYAL1,HEXB,GALNS,CEMIP
Wnt/β-catenin Signaling	1.7	0.11	0.471	CSNK1E,CSNK1A1,SOX8,CSNK2B,TCF7L1,DVL1,FZD5,AXIN2,H2BFM,SOX11,WNT7A,PPP2CB,TAB1,FRZB,DVL3,SOX2,SOX4,APC2,ACVR2B
Heme Biosynthesis from Uroporphyrinogen-III I	1.62	0.5	#NUM!	CPOX,UROD
Proline Biosynthesis I	1.62	0.5	#NUM!	PYCR1,PYCR3
Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza	1.51	0.13	0	CSNK1E,CSNK1A1,WNT7A,TCF7L1,DVL3,DVL1,NCOA2,SIAH1,APC2,FZD5
Phospholipase C Signaling	1.49	0.0992	1.342	HDAC2,GNB3,RHOT2,CALM1 (includes others),CREB1,HDAC10,PLD3,PLCD3,RHOA,GNB1L,FYN,HDAC6,GNAS,NAPEPLD,ARHGEF1,RHOB,ARHGEF2,MAP2K2,PLCG1,PRKD1,PLA2G12A,ARHGEF4,RND3,ARHGEF18
1D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)	1.47	0.211	0	IPPK,IPMK,INPPL1,INPP5A
IL-8 Signaling	1.42	0.102	2.065	IRAK1,EGFR,GNB3,RHOT2,VEGFA,PLD3,PIK3R4,NOX4,PTK2,RHOA,FOS,GNB1L,GNAS,NAPEPLD,PIK3R2,RHOB,MAP2K2,PRKD1,BAX,RND3
Serine Biosynthesis	1.42	0.4	#NUM!	PSAT1,PHGDH
2-oxobutanoate Degradation I	1.42	0.4	#NUM!	DLD,PCCB

Protein Kinase A Signaling	1.39	0.0898	1.414	CAMK2B,YWHAQ,CALM1 (includes others),CREB1,TCF7L1,HHAT,HIST1H1C,H2BFM,PRKAR1B,PTPRF,H1FX,PDE12,FICD,PTPN9,GNB3,FLNA,NTN1,PTPN2,H3F3A/H3F3B,PTPN23,PTK2,EYA3,PTPN1,PLCD3,RHOA,GNB1L,GNAS,NAPEPLD,PTPN12,AKAP14,MAP3K1,MAP2K2,PRKD1,PLCG1,DUSP6,APEX1
tRNA Charging	1.38	0.154	0	CARS2,MARS2,PARS2,VARS,WARS,EARS2
Mouse Embryonic Stem Cell Pluripotency	1.32	0.113	1.732	PIK3R2,ID2,PIK3R4,MAP2K2,TAB1,MAPK11,TCF7L1,SOX2,DVL3,DVL1,FZD5,SMAD9
Molecular Mechanisms of Cancer	1.32	0.0888	#NUM!	CAMK2B,BBC3,E2F3,FZD5,FOS,HHAT,H2BFM,FYN,CDKN1A,WNT7A,PRKAR1B,MAPK11,PA2G4,ARHGEF4,RND3,BMP7,ARHGEF18,RHOT2,NOTCH1,PIK3R4,PTK2,DVL1,SMAD9,RHOA,GNAS,PIK3R2,RHOB,ARHGEF2,ARHGEF1,MAP2K2,PRKD1,TAB1,CDK12,BAX,CDK16

3.2 The canonical pathways that were significantly changed in 5F-ADB exposed mature cells.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Molecular Mechanisms of Cancer	2.48	0.0254	#NUM!	CAMK2B,BBC3,PIK3R2,PRKAR1B,BAX,CDK12,CDK17,ARHGEF4,BMP7,RHOA
p53 Signalling	2.46	0.045	1.342	ADGRB1,BBC3,PIK3R2,BAX,TNFRSF10B
Calcium Signaling	1.94	0.0291	#NUM!	CAMK2B,HDAC2,RYR1,PRKAR1B,TPM2,MYH10

Melanocyte Development and Pigmentation Signaling	1.91	0.0408	#NUM!	PIK3R2,PRKAR1B,SH2B2,RPS6KC1
Actin Cytoskeleton Signaling	1.75	0.0264	1.342	PIK3R2,MYH10,FGF23,ARHGEF4,APC2,RHOA
Neuropathic Pain Signaling In Dorsal Horn Neurons	1.68	0.0348	1	CAMK2B,PIK3R2,KCNN3,PRKAR1B
NGF Signaling	1.61	0.0331	1	PIK3R2,BAX,RPS6KC1,RHOA
Superpathway of Cholesterol Biosynthesis	1.58	0.0714	#NUM!	PMVK,LBR
Methylglyoxal Degradation I	1.57	0.333	#NUM!	HAGHL
5-aminoimidazole Ribonucleotide Biosynthesis I	1.57	0.333	#NUM!	PPAT
Hypusine Biosynthesis	1.57	0.333	#NUM!	EIF5A
Cellular Effects of Sildenafil (Viagra)	1.5	0.0305	#NUM!	GUCY1A2,KCNN3,PRKAR1B,MYH10
Adrenomedullin signaling pathway	1.49	0.0258	0.447	GUCY1A2,PIK3R2,KCNN3,PRKAR1B,BAX
Gap Junction Signaling	1.48	0.0256	#NUM!	CSNK1A1,GUCY1A2,SGSM3,PIK3R2,PRKAR1B
Adipogenesis pathway	1.47	0.0299	#NUM!	HDAC2,SREBF1,BMP7,RPS6KC1

Branched-chain α-keto acid Dehydrogenase Complex	1.45	0.25	#NUM!	DLD
Role of BRCA1 in DNA Damage Response	1.44	0.0375	#NUM!	POU2F1,MSH6,SMARCB1
Breast Cancer Regulation by Stathmin1	1.41	0.0244	#NUM!	CAMK2B,PIK3R2,PRKAR1B,ARHGEF4,RHOA
Hereditary Breast Cancer Signaling	1.37	0.0278	#NUM!	HDAC2,PIK3R2,MSH6,SMARCB1
2-ketoglutarate Dehydrogenase Complex	1.35	0.2	#NUM!	DLD
Serine Biosynthesis	1.35	0.2	#NUM!	PHGDH
Myo-inositol Biosynthesis	1.35	0.2	#NUM!	ISYNA1
2-oxobutanoate Degradation I	1.35	0.2	#NUM!	DLD
Notch Signaling	1.33	0.0526	#NUM!	MAML3,DLL3
Role of NFAT in Cardiac Hypertrophy	1.3	0.0228	#NUM!	CAMK2B,CSNK1A1,HDAC2,PIK3R2,PRKAR1B

3.3 The canonical pathways that were significantly changed in ∆9-THC exposed mature cells.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Glutamate Degradation III (via 4-aminobutyrate)	3.5	0.6	#NUM!	GAD2,ABAT,SUCLG2
BER pathway	3.36	0.333	#NUM!	OGG1,LIG1,APEX1,PCNA
Notch Signalling	2.9	0.158	#NUM!	DLL1,PSEN2,MAML1,MAML3,HEY1,DLL3
Adipogenesis pathway	2.87	0.0896	#NUM!	LPIN1,HDAC2,GTF2H2,TXNIP,SREBF1,ZNF423,LPL,RUNX1T1,KAT7,ATG7,BMP7,RPS6KC1
4-aminobutyrate Degradation I	2.52	0.667	#NUM!	ABAT,SUCLG2
Wnt/β-catenin Signalling	2	0.0698	-0.577	AXIN2,CSNK1A1,H2BFM,SOX8,WNT7A,SFRP1,PPP2R5D,DVL3,SOX2,SOX4,APC2,ACVR2B
Rapoport-Luebering Glycolytic Shunt	1.84	0.333	#NUM!	PGAM2,TIGAR
Role of BRCA1 in DNA Damage Response	1.83	0.0875	#NUM!	POU2F1,MSH6,CDKN1A,BRCC3,SMARCA4,BARD1,SMARCB1

EIF2 Signalling	1.56	0.0588	-2	RPS27L,EIF3G,SREBF1,RPS27,EIF4A2,MT-TM,EIF4G2,EIF5,EIF4A3,WARS,RPL14,RPL30,RPS25
Phototransduction Pathway	1.56	0.0943	#NUM!	RGR,GNB3,GUCY1A2,GUCY1A1,OPN3
Ephrin B Signalling	1.52	0.0822	-1.342	EFNB2,GNB3,ITSN2,RGS3,VAV2,RHOA
3-phosphoinositide Biosynthesis	1.52	0.0597	0	DUSP8,EGFR,PI4KB,PPP1R1C,CDIPT,PPP2R5D,PTPRO,NUDT4,SIRPA,PXYLP1,PTPN1,CDC25B
Cell Cycle Control of Chromosomal Replication	1.47	0.0893	#NUM!	CDK11B,MCM2,LIG1,CDK12,PCNA
Aryl Hydrocarbon Receptor Signalling	1.41	0.0638	-2.236	ALDH16A1,NFIB,CDKN1A,GSTM4,SMARCA4,BAX,ALDH1B1,SP1,MGST3
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	1.37	0.0629	-0.333	DUSP8,PPP1R1C,PPP2R5D,PTPRO,NUDT4,SIRPA,PXYLP1,PTPN1,CDC25B
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	1.37	0.0629	-0.333	DUSP8,PPP1R1C,PPP2R5D,PTPRO,NUDT4,SIRPA,PXYLP1,PTPN1,CDC25B
Superpathway of Inositol Phosphate Compounds	1.36	0.0551	0.277	DUSP8,EGFR,PPP1R1C,PTPRO,PTPN1,PI4KB,ITPKB,CDIPT,PPP2R5D,NUDT4,SIRPA,PXYLP1,CDC25B

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