Investigating the Effect of Kappa Opioid Receptor Agonists on Serotonin Transporter Function in HEK-293 Cells

VICTORIA JANE CATHERWOOD

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Abstract

The serotonin (5-HT) transporter (SERT) and the kappa opioid receptor (KOPr) are important brain proteins. Currently, it is known that KOPr modulates 5-HT concentrations but the biochemical mechanisms responsible remain enigmatic. The 5-HT and KOPr relationship is thought to involve SERT because both SERT and KOPr co-localise in the dorsal raphe nucleus (DRN), a brain region involved in drug addiction, affective disorders, and stress homeostasis. Thus, elucidating the KOPr and SERT relationship will clarify biomedical targets for these disorders. To investigate this relationship the effect of KOPr activation on SERT function was examined in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged hSERT. We found the KOPr agonists U50,488H, Salvinorin A (Sal A) and DS-3-240 had no acute (5 min) effect on SERT function as measured by rotating disc electrode voltammetry (RDEV) incubated with 1'-diethyl-2,2'-cyanine iodide (D-22; 1.5 mM) and by confocal microscopy. Interestingly, SERT function significantly decreased with chronic (30 min) exposure to U50,488H (** $p < 0.01$) and Sal A (** $p < 0.01$) as measured with a novel high-throughput assay; this decrease was also attenuated with 5 min pre-treatment of the SERT inhibitor, fluoxetine (** $p < 0.001$). Furthermore, this novel high-throughput assay also replicated our laboratories recent finding that chronic (30 min) exposure to U50,488H (** $p < 0.01$) and Sal A (** $p < 0.01$) significantly increase dopamine transporter (DAT) function in HEK-293 cells co-expressing myc-rKOPr and eYFP-tagged DAT. Collectively, these findings suggest in this cell model that chronic (30 min) KOPr activation by U50,488H and Sal A decreases SERT function.

“If the brain were so simple that we could understand it, we would be so simple that we couldn’t”

Emerson M. Pugh

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Abbreviations

5-HT	serotonin
AC	adenylate cyclase
AFU	arbitrary fluorescent unit
ASP ⁺	4-(4-(dimethylamino)-styryl)-N-methylpyridinium
cAMP	cyclic Adenosine mono-phosphate
CREB	cyclic AMP response element binding protein
D-22	1'-diethyl-2,2'-cyanine iodide; decynium-22
DA	dopamine
DAT	dopamine transporter
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DRN	dorsal raphe nucleus
DYN	dynorphin
eGFP-hSERT	enhanced green fluorescence protein tagged human serotonin transporter
EMT	extraneuronal monoamine transporter
ERK1/2	extracellular-signal regulated kinase 1/2
FCS	fetal calf serum
Fluox	fluoxetine
GABA	γ -Aminobutyric acid
GPCR	G-protein coupled receptor
HEK-293	human embryonic kidney - 293 cells
JNK	c-Jun N-terminal kinase
KOPr	kappa opioid receptor
KREBS	krebs ringer hepes buffer
LB	Luria Broth
myc-rKOPr	myc-tagged rat kappa opioid receptor
MAO-A	monoamine oxidase-A
MAPK	mitogen-activated protein kinase
MEK1/2	mitogen-activated protein kinase kinase 1/2
MPP ⁺	1-Methyl-4-phenylpyridinium
NAc	nucleus accumbens
NET	norepinephrine transporter
OCT	organic cation transporter
PBS	phosphate-buffered saline
PFC	prefrontal cortex
p38-MAPK	p38-Mitogen activated protein kinase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMAT	plasmalemmal monoamine transporter

RDEV	rotating disc electrode voltammetry
ROI	region of interest
Sal A	salvinorin A
SERT	serotonin transporter
SN	substantia nigra
Str	dorsal striatum (striatum)
SSRI	selective serotonin reuptake inhibitor
U50,488H	(Trans-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide methanesulfonate
U69,593	(5 α , 7 α , 8 β)-(+)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide
VTA	ventral tegmental area
eYFP-hDAT	enhanced yellow fluorescent protein tagged human dopamine transport

Chapter 1: Introduction

1.1 General Introduction

The serotonin (5-HT) transporter (SERT) and the kappa opioid receptor (KOPr) are proteins involved in normal brain function and their dysfunction contributes to the etiology of brain diseases. Preclinical evidence suggests the biochemical effects of opioids are mediated, at least in part, by the serotonergic system (Filip *et al.*, 2004; Tao and Auerbach, 2005; Yoshioka *et al.*, 1993). KOPr expresses in numerous brain regions and although the KOPr and 5-HT relationship remains largely undefined it is known that KOPr activation in the dorsal raphe nucleus (DRN) or nucleus accumbens (NAc; ventral striatum) decreases extracellular 5-HT concentrations in the respective region (Tao and Auerbach, 2002; Tao and Auerbach, 2005; Tao *et al.*, 2007; Werling *et al.*, 1989). In addition, KOPr can also modulate 5-HT neurotransmission in the hypothalamus, hippocampus and dorsal striatum (Str) (Yilmaz *et al.*, 2006). Recently, Land *et al.* (2009) illustrated that KOPr and SERT co-localise in the rat DRN and therefore, it is hypothesized that this KOPr and 5-HT relationship is mediated by SERT. The DRN is a brain region that contributes to the etiology of drug addiction, affective disorders, and stress homeostasis. Therefore, elucidating this enigmatic KOPr and SERT relationship will clarify biomedical research targets to contribute overall with understanding our neurobiology.

Classical KOPr agonists attenuate psychostimulant and alcohol drug-taking

and drug-seeking in preclinical models of addiction and is thought to occur by DA signalling (Land *et al.*, 2009; Morani, 2010; 2012; Schenk *et al.*, 2000; Zakarova *et al.*, 2008). Interestingly, chronic cocaine increases KOPr density when the dopamine transporter (DAT) is inhibited with either GBR 12909 or RTI-117 (Schenk and Patridge, 2001), which suggests KOPr modulation occurs through another DA signalling independent mechanism (Collin *et al.*, 2001; 2002). Dual activation of KOPr, other than DAT, highlights SERT as a primary candidate to modulate KOPr. Co-localisation and behavioural studies illustrate and suggest that KOPr modulates SERT *in vitro* (Morani, unpublished; Simonson, unpublished data) and *in vivo* (Morani, 2010; 2012; Zakarova *et al.*, 2008); however, the nature of this KOPr and SERT relationship remains unknown. Collectively, there is rationale to suggest a relationship between KOPr and SERT exists and characterisation of this KOPr and SERT interaction has potential to elucidate key targets for future biomedical research, which may facilitate the development of more effective treatments.

Previously, acute (15 min) incubation of the classic KOPr agonists, (Trans-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide methanesulfonate (U50,488H; 500 nM), and the structurally novel KOPr agonists, Salvinorin A (Sal A; 500 nM) and DS-1-216 (500 nM), decreases SERT function in rat striatal tissue as measured by rotating disc electrode voltammetry (RDEV) (Simonson, unpublished data). In addition, decreases in SERT function were also observed with chronic (30 min) incubation of U50,488H (10 µM) measured with 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP⁺) uptake assay measured by confocal microscopy (Morani, unpublished data). In this study, the interaction

between KOPr and SERT function is investigated by activating KOPr with agonists and measuring SERT function. This is elucidated in HEK-293 cells expressing myc-rKOPr and eGFP-tagged SERT with acute (5 min) incubation with the KOPr agonists U50,488H, Sal A and DS-3-240 measured by confocal microscopy (10 μ M) and RDEV (500 nM), as well as chronic incubation (30 min) with the KOPr agonists U50,488H, Sal A (10 μ M) as measured by a novel high-throughput assay.

1.2 Serotonin

5-HT is synthesised from tryptophan into 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan-5-hydroxylase (Figure 1.1). 5-HTP is then converted into 5-HT by the enzyme 5-hydroxytryptophan decarboxylase and then packaged and stored in vesicles within the presynaptic serotonergic neurons to prevent degradation by enzymes, such as monoamine oxidase (MAO) (Jorgensen, 2007; Nichols and Nichols, 2008). 5-HT is released when an action potential propagates along the presynaptic neuron in the form of an electrical impulse to depolarise the nerve terminal to produce an influx of Ca^{2+} that fuses 5-HT vesicles to the cell membrane and 5-HT is subsequently released into the synaptic cleft. 5-HT diffuses across the synapse to continue the 5-HT signal by binding to postsynaptic receptors. These receptors trigger second messenger molecules that activate effector molecules that amplify the 5-HT signal (Hoffman *et al.*, 1998; Nichols and Nichols, 2008). After binding, 5-HT detaches from the post-synaptic 5-HT receptor and continues to diffuse within the synapse where it can either bind to SERT, MAO-A and B, or glial cells receptors. SERT terminates the signal by re-transporting the 5-HT molecule back

into the presynaptic cell for recycling or degradation, MAO A and B degrade 5-HT into its metabolite 5-hydroxyindoleacetic acid and glial cell receptors can also take up 5-HT to terminate the 5-HT signal.

Fig. 1.1. 5-HT synthesis. Tryptophan is metabolised to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan-5-hydroxylase (also known as L-tryptophan-5-monooxygenase). 5-HTP is then metabolised to 5-HT by the enzyme 5-hydroxytryptophan decarboxylase. Serotonin (5-HT) is then metabolised by monoamine oxidase A and B, which deaminates 5-HT into its metabolite 5-hydroxyindoleacetic acid (5-HIAA). (Diagram modified from Wikipedia, Wikimedia Foundation, n.d. Web, 10 January 2013 http://en.wikipedia.org/wiki/File:Serotonin_biosynthesis.svg)

5-HT is synthesised within the median raphe nuclei and DRN of the brainstem in the midbrain region. The lower raphe nuclei axons terminate at the cerebellum and spinal cord, and higher raphe nuclei axons spread throughout the central and peripheral nervous systems. Neurons that utilise 5-HT are found throughout the brain (Figure 1.2), which facilitates 5-HT to interact with other neurotransmitter systems (Hoyer *et al.*, 2002).

Fig. 1.2. A schematic diagram of the brain with DA and 5-HT reward pathways. The dopaminergic cell bodies originate in the VTA and SNg with the mesolimbic pathway projects from the VTA to the NAc, the mesocortical pathway projects from the VTA to the PFC and the nigrostriatal pathway projects from the SNg to the Str. The serotonergic cell bodies originate in the raphe nuclei and innervate the VTA to feed into the PFC, NAc, Str, amygdala and hippocampus. 5-HT- serotonin, DA- dopamine, NAc- nucleus accumbens (ventral striatum), PFC- prefrontal cortex, SNg- substantia nigra, Str- (dorsal) striatum, VTA- ventral tegmental area. (Diagram adapted from NIDA website <http://www.drugabuse.gov/publications/addiction-science/why-do-people-abuse-drugs/brain-pathways-are-affected-by-drugs-abuse>)

Serotonergic regulation is vital to maintain brain function for optimal mental health and well-being. 5-HT dysfunction is known to be one of the primary factors

contributing to the development and progression of neurological abnormalities. Brain disorders such as major depression (Owens and Nemeroff, 1994), anxiety (Suranyi-Cadotte *et al.*, 1990), bipolar disorder (Bellivier *et al.*, 1997; Velayudhan *et al.*, 1999), suicide (Arango *et al.*, 2002; Purcelle, 2003), impulsive behaviour (Lesch, 2000; Kreek *et al.*, 2005), aggression (Oquendo *et al.*, 2001), obsessive compulsive disorder (Kalueff and LaPorte, 2010), psychostimulant addiction (Baumann and Rothman, 1998; Heinz *et al.*, 2001), stress response, appetite, and sleep (Lotrich *et al.*, 2004) have been linked to 5-HT dysfunction. The co-morbidity of these disorders highlights 5-HT as a superior candidate to investigate further to understand the biological basis of drug addiction, affective disorders and stress homeostasis. In particular, SERT is known to contribute significantly to these disorders because it plays a crucial role in 5-HT reuptake, synthesis, and release. This makes SERT a prime candidate when investigating 5-HT synergy.

1.2.1 Serotonin Signalling

5-HT molecules bind to the 5-HT receptors and 5-HT autoreceptors on the postsynaptic and presynaptic neuronal membrane, respectively. Currently, seven types of 5-HT receptors with at least 15 different subtypes have been identified and cloned and they are known as 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_{3B}, 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆ and 5-HT₇ (Jorgensen, 2007; Nichols and Nichols, 2008).

All 5-HT receptors are G-protein coupled receptors (GPCRs), with the exception of 5-HT_{3A} and 3B, which are ionotropic and found within the gastrointestinal track and link into the enteric nervous system (Hoyer *et al.*, 2002; Jones *et al.*, 2010; O'shea *et al.*, 1998). GPCR's are the largest family of signaling receptors and become activated by a vast array of extracellular ligands. GPCRs are seven-pass transmembrane receptor proteins with a slower, longer-lasting and diverse mode of action to allow specific control of numerous intracellular signalling pathways (Krupnick and Benovic, 1998). 5-HT receptors couple to different types of G-proteins. These G-proteins dissociate from the GPCR and relay the signal to second messenger molecules that interact with effector molecules to amplify and thus, stimulate downstream biochemical signal transduction pathways in the cytoplasm of the cell (Marinissen and Gutkind, 2001). Specifically, activation of stimulatory G-proteins (G_s) leads to the stimulation and activation of an enzyme called adenylate cyclase (AC) which converts adenosine triphosphate (ATP) into the intracellular second messenger, cyclic adenosine monophosphate (cAMP). cAMP subsequently interacts with important target effector signaling proteins, such as Protein Kinase A (PKA). G_s-protein effector molecules regulate Ca²⁺ influx and membrane excitability and PKA phosphorylates specific target proteins that increase gene expression (Nichols and Nichols, 2008). Activation of 5-HT receptors also couples to inhibitory G-proteins (G_i) to inhibit and decrease AC concentrations, which then reduces cAMP levels and thus, decreases PKA activity and downstream gene expression. Activation of G_i produces membrane hyper-polarisation that decreases the rate of neuronal firing but activation of G_s proteins can also cleave

membrane phosphoinositides to form diacylglycerol (DAG) and inositol phosphates, such as inositol triphosphate (IP3) (Nichols and Nichols, 2008). IP3 stimulates intracellular Ca^{2+} membrane channels to additionally increase second messengers, effector molecules and thus, intracellular signal transduction (Nichols and Nichols, 2008).

5-HT homeostasis requires an intricate balance between receptor activation of G_s and G_i . Disruption to this system alters second messenger concentrations that affects the activation of effector molecules by phosphorylation, such as PKC, cGMP, p38MAPK and PKA, which are all involved in SERT modulation (for a comprehensive review see Ramamoorthy, Shippenberg and Jayanthi, 2011).

1.2.2 5-HT in Reward

5-HT is involved in mood and sleep but also plays an important role in reward. Natural reward is a neuroadaptation to ensure survival by reinforcing and motivating behaviours such as eating, sleeping and sexual reproduction (Gawin, 1991; Koob *et al.*, 1998). Reward, reinforcement, and motivation all produce subjective feelings of pleasure and wellbeing. DA predominantly regulates reward through three main DA pathways (Figure 1.2). The DA pathways originate in the brain stem in two main regions, the ventral tegmental area (VTA) and the substantia nigra (SNg). The mesolimbic pathway projects from the VTA to the nucleus accumbens (NAc; ventral striatum), the mesocortical pathway projects from the VTA to the prefrontal cortex (PFC) and the nigrostriatal pathway projects from the SNg to

the Str (Robinson and Berridge, 2000). Each of these brain regions plays a unique role in reward, reinforcement and motivation. The NAc is the reward centre because preclinical and clinical studies illustrate DA release here during motivational behaviour (Koob and Volkow, 2010; Robinson and Berridge, 2000). Interestingly, in the NAc the mesolimbic system significantly modulates structural and functional gamma-aminobutyric acid (GABA) medium spiny neurons (see fig 1.4; Diana, 2011). The PFC is the motivation relay station and is responsible for executive functions such as consequence of actions, goal setting and predicting outcomes (Koob and Volkow, 2010; Robinson and Berridge, 2000). The Str is involved in motor control and creating response to behaviours as a way of learning (Koob and Volkow, 2010; Robinson and Berridge, 2000) and thus, plays a vital role in reinforcement (Everitt *et al.*, 2008). Collectively, these DA pathways control the regulation of reward, reinforcement, attention, motivation, mood and emotions (Kuhar, 2010).

The DA reward pathways also synergistically interacts and overlaps with the serotonergic system. 5-HT synthesis and release occurs in 5-HT cell bodies that are found within the raphe nuclei cluster of neurons in the brain stem. 5-HT neurons project from the DRN and medial raphe nuclei to innervate the VTA and terminate at the PFC, NAc, Str, amygdala and hippocampus. These projections play an important role in reward, reinforcement, attention, motivation, mood, emotions, perception and sensation (Nichols and Nichols, 2008).

1.3 Serotonin Transporter

SERT is an essential protein for brain development, neural plasticity, synaptogenesis, cell proliferation, drug addiction, emotional disorders, and dysfunctional stress response (Kalueff and LaPorte, 2010). Numerous documents of SERT expression in psychopathology cases indicate the importance of SERT expression in brain dysfunction but also in maintaining normal brain function (Murphy *et al.*, 2004). The primary function of SERT is to mediate the recycling of synaptic 5-HT to effectively terminate and regulate overall 5-HT neurotransmission. Where 5-HT neurotransmission is mediated by more than 15 different types of cell-surface receptors (Glennon & Dukat, 1995) it seems that one single gene (Ramamoorthy *et al.*, 1993) encoding for SERT appears to be responsible for majority of the extracellular 5-HT clearance (Blakely *et al.*, 1991; Hoffman *et al.*, 1991; Lesch *et al.*, 1993).

1.3.1 SERT Gene

The human SERT gene is located on chromosome 17q11.2 and consists of 378,000 base pairs spanning 13 exons (Ramamoorthy *et al.*, 1993). SERT exists in homo-multimeric complexes and contains potential phosphorylation sites for several kinases (Ramamoorthy *et al.*, 1993; Schmid *et al.*, 2001). SERT belongs to the SLC6 gene family that also includes DAT, the norepinephrine transporter (NET), GABA and glycine transporters. However, the growing body of SERT literature emphasises the need for more cellular and molecular preclinical models to fully understand the mechanisms and emphasise the clinical importance of SERT (Kalueff and LaPorte,

2010). SERT animal research utilises knockout models, gene regulation and silencing models, as well as epigenetic regulation (Molteni *et al.*, 2010). But there remains a paucity of cell models that investigate SERT function and expression and SERT's relationship with other neurotransmitter systems (Kalueff and LaPorte, 2010). Since the SERT gene was identified many polymorphisms have been discovered as well their corresponding neurobiological and behavioural effects. SERT polymorphisms are associated with a wide range of diseases and disorders such as, attention deficit hyperactivity disorder (ADHD) (Faraone *et al.*, 1998), major depression (Owens and Nemeroff, 1994), anxiety (Suranyi-Cadotte *et al.*, 1990), bipolar disorder (Bellivier *et al.*, 1997; Velayudhan *et al.*, 1999), suicide (Arango *et al.*, 2002; Purcelle, 2003), impulsive behaviour (Lesch, 2000; Kreek *et al.*, 2005), aggression (Oquendo *et al.*, 2001), obsessive compulsive disorder (Kalueff and LaPorte, 2010), psychostimulant addiction (Baumann and Rothman, 1998; Heinz *et al.*, 2001) and substance abuse (Gainetdinov and Caron, 2003).

In particular, the SERT linked promoter region (5-HTTLPR) has been implicated as a potential risk factor for many pathophysiological diseases and disorders but majority of the previous studies remain considerably under powered (Murphy *et al.*, 2013). The 5-HTTLPR is a degenerate repeat polymorphic region on the SLC6A4 gene. This genetic mutation is the most thoroughly researched polymorphism in the SERT literature with well over 300 psychiatric, pharmacogenetic, genetics and medical papers by 2006 (Wendland *et al.*, 2006) and over 100 neuroscience genetics reports published in the first part of 2012 (Murphy *et al.*, 2013). Many of these reports suggest 5-HTTLPR to have two main alleles, the

‘short’ (“S”) and ‘long’ (“L”) (Murphy *et al.*, 2013; Wendland *et al.*, 2006). These two variants have been suggested to result in different SERT mRNA transcription levels; specifically, the “S” variant is suggested to reduce basal transcription levels to decrease SERT mRNA levels (Lesch *et al.*, 1996). A decrease in SERT levels also corresponds to a reported decrease in response to SERT acting compounds, such as selective 5-HT reuptake inhibitors or also known as SSRIs (Purselle and Nemeroff, 2003). SSRIs are commonly used as anti-depressants, and pharmacotherapies, such as fluoxetine (ProzacTM), citalopram (CelexaTM), paroxetine (PaxilTM) and sertraline (ZoloftTM). SSRIs act as SERT antagonists, or blockers, by inhibiting SERT function to prevent 5-HT re-uptake, which increases the 5-HT synaptic concentration. Accordingly, SSRIs were more sensitive to “L” variants because these individuals have more SERT available to inhibit (Purselle and Nemeroff, 2003). However, Purselle and Nemeroff’s (2003) experimental analysis was conducted in post mortem brain tissue (and platelets) of suicide victims and 50% of the previous studies investigating SERT binding in postmortem studies did not identify the same SERT binding and SERT genotype correlation.

The classification of only two alleles does raise serious research methodology issues because less than half (44%) of the 100 neuroscience genetics reports in 2012 investigating this promoter polymorphism 5-HTTLPR (simple long “L” versus short “S” allele classifications) neglect to include other 5-HTTLPR variants and related that have been shown to alter the functional effects of these so called “L” versus “S” variants (Murphy *et al.*, 2013). Thus, interpretation of 5-HTTLPR studies, and SERT

single nucleotide polymorphisms must be interpreted with extreme caution, and all limitations should be stated. Despite discrepancies in SERT polymorphisms and behaviour in the literature, stringent methodology must be performed to reduce bias, chance and confounding in order to reveal a true correlation (Murphy *et al.*, 2013). Nevertheless, it is accepted that SERT is important in behavior, so SERT genetic polymorphisms must continue to be researched so the subsequent biological and behavioural effects can fully recognised. Perhaps Purselle and Nemeroff's (2003) omission of two nearby polymorphisms, rs25531 and rs25532 that substantially alter 5-HTTLPR allele frequencies and functionality or not reporting ethnicity of the subjects genotyped produced bias, chance and confounding (Murphy *et al.*, 2013) that would account for these discrepancies. Until a well-conducted double blind randomized control trial is carried out the definitive answer to this contradicting literature remains the topic of speculation.

Interestingly, other scientific disciplines are contributing to the idea that the 5-HTTLPR "L" vs. "S" alleles account for different behaviours. For example, social sciences are investigating the 5-HTTLPR allele frequency across populations because the genetic differences vary considerably and have been hypothesized to account for cultural differences. Social sciences are attempting to correlate their genetic findings with the cultural patterns to explain differences between cultures. Social sciences illustrate a higher frequency of the "L" allele is found in Europe whereas a lower frequency of "L" is found in Asia (Eisenberg and Hayes, 2011). However, substantial oversights and omissions since 2006 are continuing to be published (Murphy *et al.*, 2013), and thus, research into 5-HTTLPR polymorphism genotypes and related

behaviours should be interpreted with great caution, especially when correlating a few single nucleotide polymorphisms to population wide behaviours.

1.3.2 SERT Protein

The SERT protein contains 630 amino acid residues and hydrophobicity analysis suggests the presence of 12 hydrophobic transmembrane domains with cytoplasmic NH₂ and COOH termini (Ramamoorthy *et al.*, 2011). Transmembranes three and four are separated by a large, hydrophilic loop that contains two canonical sites for N-linked glycosylation (Lesch *et al.*, 1993; Ramamoorthy *et al.*, 1993). SERT is found in the peri and extrasynaptic regions of the presynaptic serotonergic neurons and it regulates synaptic 5-HT concentrations through reuptake into the presynaptic neuron that feeds back the information to the 5-HT system, which modulates overall 5-HT synthesis and release (Blakely *et al.*, 1994; Rudnick, 2006; Zahniser and Doolen, 2001). SERT belongs to the SLC6 sodium (Na⁺) ion and chloride (Cl⁻) dependent plasma membrane transporters family. These transporters exploit the Na⁺ gradient across the neuron membrane to drive secondary active transport of the neurotransmitter from the synaptic cleft back into the presynaptic compartment. This is the most rapid and economical way to remove and recycle synaptic neurotransmitters (Murphy *et al.*, 2004; Sitte and Freissmuth, 2010). SERT requires one Na⁺ ion, one Cl⁻ ion and one 5-HT molecule to facilitate 5-HT transport across SERT (for a review see Torres *et al.*, 2003). Upon 5-HT binding, SERT undergoes conformational changes to form an external barrier that allows SERT to open the 5-HT active site into the cytoplasmic space so 5-HT can detach and

be recycled. The subsequent absence of 5-HT, as well as Na⁺ and Cl⁻, reconfirms SERT back into the original synaptic cleft facing position (for a review see Murphy *et al.*, 2004).

SERT is a phosphoprotein with a multitude of presynaptic receptor-second messenger-kinase and phosphatase linked pathway networks. These networks regulate SERT to alter the protein conformation and dynamically control the transporter expression, activity, trafficking and degradation (Kalueff and LaPorte, 2010; Muller *et al.*, 2007; Rammamoorthy *et al.*, 2011; Torres *et al.*, 2003). Kinase and phosphatase pathways play a pivotal role in the regulation of SERT activity as they are either constitutively operational or activated by extracellular signals (Holley *et al.*, 2013; Ramamoorthy *et al.*, 2011).

SERT function rapidly decreases in response to acute depletion of intracellular Ca²⁺, inhibition of calmodulin, Ca²⁺/calmodulin-dependent protein kinases II (CaMKII), Src-kinase, p38-Mitogen activated protein kinase (p38 MAPK) and activation of PKC (Ramamoorthy *et al.*, 2011). Additionally, data also suggests that cytokines can affect SERT activity through p38 MAPK-dependent mechanisms in some cell lines and mice neurons (Zhu *et al.*, 2006). Conversely, SERT function is increased by an increase in intracellular Ca²⁺ as well as activation of NOS/cGMP and MAPK pathways (PKA). However, the three major protein kinases that are responsible for SERT's phosphorylation state and SERT membrane expression are PKC (Qian *et al.*, 1997) and p38 MAPK (Samuvel *et al.*, 2005) which decrease SERT

function, and PKA (Ramamoorthy *et al.*, 1997) which increases SERT function.

SERT function is regulated by trafficking between the plasma membrane and intracellular compartments through a series of post-translational modifications, however, the exact functional mechanisms and specific residues responsible for the regulation of SERT is not completely understood (Rammamoorthy *et al.*, 2011). Thoroughly understanding the pathways involved in modulating SERT function, particularly decreasing, allow us to better understand the role of transporter phosphorylation and thus, the regulation of 5-HT neurotransmission (Ramamoorthy *et al.*, 2011).

1.3.3 ASP⁺ Uptake as a SERT Uptake Identification Technique

ASP⁺ is a fluorescent analogue of the neurotoxic substrate 1-methyl-4-phenylpyridinium (MPP⁺) (Schwartz *et al.*, 2003). ASP⁺ has previously been used as an identification tool to visualise and quantify monoamine transporter function (Schwartz *et al.*, 2003). Visualising the monoamine transporter function is possible because ASP⁺ fluoresces a bright red colour when it contacts the cell membrane phospholipids that are present in the integral transporter protein. ASP⁺'s red fluorescence provides a timely illustration of monoamine transporter binding, uptake and accumulation within live cultured cells using confocal microscopy (Schwartz *et al.*, 2003). This advanced technique is unique and the first of its kind that allows temporal and visual identification between monoamine transporter binding and uptake. This technique was initially established with NET using cultured cells

expressing eGFP-hNET and it was subsequently demonstrated that ASP⁺ uptake by NET is Na⁺, Cl⁻, cocaine, and temperature dependent (Schwartz *et al.*, 2003). ASP⁺ uptake has further been performed in cultured neurons and in cells transfected with DAT (Bolan *et al.*, 2007; Oz *et al.*, 2010; Zapata *et al.*, 2007) to determine function and regulation, and more recently with SERT (Schwartz *et al.*, 2003). SERT uptake is now known to be sensitive to changes in Na⁺, Cl⁻, temperature, cocaine, fluoxetine and is dependent upon p38 MAPK activation (Oz *et al.*, 2010). Interestingly, the low affinity transporter organic cation transporter 3 (OCT3) also uptakes ASP⁺ (Mayer *et al.*, 2012). However, there remains a paucity of available literature on the low affinity transporters and the uptake of ASP⁺, especially compared with the uptake of the monoamine transporters.

1.3.4 SERT in Drug Addiction

Drug addiction is characterised as a chronic and relapsing disease with reduced pleasure from natural rewards along with the compulsive drug-seeking and drug taking behaviour despite severe health, social and economic implications (Cami and Farre, 2003; Jaffe, 1990; Kalivas *et al.*, 2005; Kelley, 2004; Koob and Le Moal, 2001; Nestler, 1992; Wise, 1996). Genetic polymorphism on 5-HT genes (transporter, receptors or autoreceptors) can create dysfunction within the 5-HT system. It is hypothesised by Kalueff and LaPorte (2010) that individuals with an abnormal 5-HT system also have an abnormal reward system, and with the appropriate environmental conditions this can result in a predisposition to drug-taking and drug-seeking behaviours. Specifically, SERT genetic variations are suggested to

contribute to individual differences in response to drugs of abuse (Ramamoorthy *et al.*, 1993). For example, the association between SERT genetic polymorphism variation have shown a predisposition for alcoholism (Feinn *et al.*, 2005), cigarette smoking (Kremer *et al.*, 2005), psychostimulant (Carroll *et al.*, 1990a; Carroll *et al.*, 1990b) and heroin (Gerra *et al.*, 2004) dependence. This is particularly interesting because DA is largely been believed to be responsible for drug addiction but recent research suggests the involvement of SERT. Suddenly, the synergy of the major neurobiological systems becomes very interesting and more fascinating than previously thought.

Classically, the transition from drug use to abuse is explained by the “dopamine hypothesis” (Prisinzano, 2005) and thus, DA has been the subject of research for pharmacological targets of addiction. However, recent research illustrates the 5-HT system to be heavily involved in reward and related phenotypes (Kalueff and LaPorte, 2010), which highlights SSRI use as a prime pharmacotharapeutic treatment for drug addiction. Cocaine self administration is decreased with the pre-treatment of a SSRI (Carroll *et al.*, 1990b) or by a low L-tryptophan (the precursor of 5-HT) diet (Carroll *et al.*, 1990a). Additionally, alcohol addiction treatment with SSRIs is common because of the established association between alcoholism and affective (mood) disorders (Smith, 2012). Thus, the role of 5-HT in reward, affective disorders, dysfunctional stress responses and the prescribing of SSRIs for alcohol dependence, highlights the role of SERT in drug addiction.

The DA and 5-HT neurotransmitter systems synergistically exist in the brain and it is commonly believed that they interact together in the CNS. Although the specific mechanisms remain to be elucidated, it is known that DA levels are directly modulated by 5-HT through specific 5-HT receptors (5-HT_{1A}, 2A and 2C) that are localised on DA neuronal cells (Barnes and Sharp, 1999; Bubar and Cunningham, 2006; Higgins and Fletcher, 2003; Hoyer *et al.*, 2002). Also, DA can act indirectly on 5-HT through GABA-mediated inhibitory (Eberle-Wang *et al.*, 1998; Stanford and Lacey, 1996), or glutamate-mediated excitatory mechanisms (Di Matteo *et al.*, 2008). In addition, the 5-HT:DA ratio plays a crucial role in determining the abuse liability of psychostimulants (Baumann *et al.*, 2011; Negus *et al.*, 2007; Wee *et al.*, 2005). Table 1.1 compares DA, 5-HT and some psychostimulants to highlight the ability of different molecules triggering both monoamine systems. The lasting physiological reduction of DA and 5-HT after drug dependence has lead to the hypothesis that restoring monoamine activity to pre-drug levels may yield significant clinical improvements in treatment (Diana, 2011). Thus, 5-HT regulates DA and KOPr pathways and other systems, so perhaps a 5-HT dysfunction is enough to create an overall imbalance. If this is true, as suggested by the current literature, then SERT function is particularly relevant in drug addiction (Ramamoorthy *et al.*, 2011).

Drugs of abuse act, directly or indirectly, to increase synaptic DA levels that lead to the increase activation of DA receptors (Koob and Volkow, 2010; Missale *et al.*, 1998). This modulation of DA is hypothesised to be predominantly involved in addiction (Prisinzano, 2005). Pharmacological agents, along with genetic manipulation, facilitates researchers to modulate DA receptors to motivate drug-

taking and drug-seeking in animal models. These methods add to the current knowledge of motivation and reward (Nestler, 2005). Recent studies illustrate that DA neurons in alcohol, opiate, cannabinoid, and other drug-dependent rats have reduced electrophysiological activity, particularly in the NAc (Diana, 2011). Human human imaging studies of cocaine, heroin, and alcohol-dependent subjects show reduced DA receptors and a decrease in the release of endogenous DA into the NAc (Diana, 2011). This offers proof that the brain is “DA-impoverished” in not just animal models after chronic drug abuse too (Diana, 2011). Collectively, these findings illustrate that drug exposure deeply affects DA neurotransmission to reduce natural reinforcement, reward and motivation, which decreases the pleasurable effects of drugs and also effects other biochemical systems.

Table 1.1. The effects of monoamines and psychostimulants on the serotonin (5-HT) and dopamine (DA) transporters

Drug	5-HT release (EC50 nM)	5-HT uptake (Ki nM)	DA release (EC50 nM)	DA uptake (Ki nM)
Dopamine	>10,000	6489	86.9	38.3
Serotonin	44.4	16.7	>10,000	2703
(+)amphetamine	1765	3830	24.78	34
(+)methamphetamine	736	2137	24.5	114
Cocaine	>10,000	304	>10,000	478
(±)MDMA	56.6	238	376	1572

Modified from Rothman and Baumann, 2003.

DAT is an integral membrane protein that transports DA back into the presynaptic DA neurons. DAT expressed in high concentrations in the Str and NAc

where it has been shown to be involved in reward (Boja and Kuhar, 1989). Drugs of abuse, such as cocaine, can target DAT, which prevents DA re-uptake and causes an increase in synaptic DA, that increases synaptic DA to amplify the DA receptor signalling (Mash *et al.*, 2002). Amphetamine acts as a DAT agonist, is taken up into the presynaptic neuron, and also reverses DAT function to release DA into the synapse (Kahlig *et al.*, 2005).

KOPr agonists are known to attenuate drug-seeking behaviour in preclinical and clinical models of psychostimulant addiction and may hold the key to designing better therapeutic compounds to treat addiction. Classical KOPr agonism decreases cocaine conditioned place preference (Shippenberg and Rea, 1997; Zhang *et al.*, 2004), locomotor activity (Heidbreder *et al.*, 1993; Zhang *et al.*, 2004), and attenuates the reinstatement of extinguished drug-taking behaviour and behavioural sensitization (Schenk *et al.*, 1999, 2000, 2001). This is because KOPr increases DAT function and thus, decreases DA neurotransmission (Collins *et al.*, 2001 ; Shippenberg, 2007; Simonson, unpublished data; Svignos *et al.*, 2001; Thompson *et al.*, 2000; Zhang *et al.*, 2004). For example, the KOPr agonists, dynorphin A and R-84760, blocked cocaine-induced increases in striatal DA levels (Zhang *et al.*, 2004). Repeated treatment with the traditional KOPr agonist U69,593 alters D2 receptor density (Izenwasser *et al.*, 1999) and function, (Acri *et al.*, 2001) as well as attenuating the locomotor effects of cocaine in rats (Heidbreder *et al.*, 1998). The oral administration of cyclazocine, a mixed KOPr agonist and mu opioid antagonist, reduced self-administration of cocaine in rats (Glick *et al.*, 1998). Drugs activating the KOPr have also been shown to reduce compulsive drug seeking to alcohol and opiates (Shippenberg *et al.*, 2001;

Chefer *et al.*, 2000; Thompson *et al.*, 2000; Mitchell *et al.*, 2005) and prevent alterations in brain function that occur as a consequence of repeated drug use (Chavkin *et al.*, 2004; Chefer *et al.*, 2005; Mitchell *et al.*, 2005). Collectively, these findings indicate the KOPr opioid receptor / dynorphin system is critically involved in mediating the acute and chronic effects of psychostimulants, and thus offers a novel pharmacological approach to treating drug abuse.

Previous studies have trialed the classical KOPr agonists, such as U50,448H and (5 α , 7 α , 8 β)-(+)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide (U69,593), on cocaine abusing humans and these agonists had little effect on drug-taking behaviour and side effects, such as sedation, depression and dysphoria, limit their therapeutic use (Preston *et al.*, 2004; Walsh *et al.*, 2001). Reasons for the low success in humans are likely to be related to the low doses given in the studies, and that the KOPr agonists selected did not have high enough affinities for the KOPr. The low affinity of classical KOPr agonists for KOPr receptors has support from preclinical studies. Therefore, KOPr agonism has the potential to attenuate drug-taking in humans but the classical KOPr agonists do not show promise as pharmacotherapies.

In preclinical models the classical KOPr agonists, such as U50,448H and U69,593, along with structural derivatives, Sal A and DS-3-240, had anti-addictive effects in rats self-administering cocaine (Morani, 2011). This study highlights that structurally derived KOPr agonist compounds may have clinical pharmacotherapeutic potential, however they just require additional research into

concentration and frequency of dose. For example, although traditional KOPr agonists may not be ideal for pharmacotherapies because of side effects, novel compounds that have reduce side effects may provide an understanding into the mechanisms by which KOPr agonists actively exert their anti-addiction effects. Investigating these anti-addiction effects is important for us to understand the mechanisms of how drugs of abuse up-regulate the KOPr/dynorphin system, and how this effects the additional biochemical systems that are also involved in addiction, reward and motivation. However, it is likely that an increase of KOPr densities after cocaine exposure is not related just to DAT inhibition because selective DAT inhibitors do not increase KOPr's (Collins *et al.*, 2002). Thus, this KOPr agonist increase after drug exposure is thought to occur through a DA independent manner (Collins *et al.*, 2001; Collins *et al.*, 2002). Evidence suggests this DA independent KOPr up-regulation is likely to be modulated by SERT, either directly or indirectly, as shown by *in vitro* (Morani, unpublished; Simonson, unpublished data) and *in vivo* research (Morani, 2010; 2012; Schenk *et al.*, 2000; Zakarova *et al.*, 2008).

1.3.5 SERT in Affective Disorders

Human studies illustrate that reducing 5-HT transporter (SERT) increases the vulnerability for the most common mood disorders, anxiety and depression (Amat *et al.*, 2005; Olivier *et al.*, 2008). Interestingly, the serotonin transporter linked promotor region (5-HTTLPR) variations have been associated with changes in the brain structures associated with executive functioning and emotional conditioning. For

example, a study by Pezawas *et al.* (2005) found less grey matter in the perigenual anterior cingulate cortex and the amygdala for the 'S' variant of the 5-HT linked promoter gene region compared to the 'L' variant of the 5-HT linked promoter gene region variants. However, genetic polymorphisms must be interpreted with caution as a 2008 meta-analysis found no significant overall association between the 5-HTTLPR polymorphisms and reduced grey matter volume in autistic individuals (Huang and Santangelo, 2008). Nevertheless, a landmark study by Caspi *et al.* (2003) hypothesised the gene and environment interaction between the 'S' allele of the 5-HTTLPR and stressful life events and major depression (Caspi *et al.*, 2003; Nierenberg, 2009; Uher and McGuffin, 2008). Unfortunately, methodological errors highlight a flaw in the research and a proposed interaction remains to be conclusively confirmed (Risch *et al.*, 2009). These 5-HTTLPR inconsistencies remain a caveat of SERT research. For example, 14 different allelic variations were found in a population of 200 Japanese and Caucasian subjects and they found no correlation to emotional dysfunction (Nakamura *et al.*, 2000), highlighting that a gene and environment interaction between the 5-HTTLPR variant, stressful life events and major depression may be a case of correlation misinterpreted as causation. Nevertheless, the collective of literature agrees that 5-HT and SERT are both involved in mood disorders (Nestler *et al.*, 2002) but the involvement of the 5-HTTLPR remains to be fully elucidated.

1.3.6 SERT in Stress

Stress is a complex experience with both aversive and motivating properties (Bruchas *et al.*, 2010). Continuous exposure to stressful experiences (chronic stress) is well known to increase the relapse of drug abuse and drug-seeking behaviour, it is also well known to induce despair and thus, escalate the risk of mood disorders such as depression, and stress can also adversely impact health (Bruchas *et al.*, 2010). Specifically, early and/or late life adversity (chronic stress) is suggested to exaggerate vulnerabilities in individuals with a predisposition to these specific brain diseases and disorders (Adamec *et al.*, 2006; Bethea *et al.*, 2005; Caspi *et al.*, 2003; Lee *et al.*, 2005). For example, preclinical studies investigating early life stress in the form of decreased maternal licking and grooming in rat pups and experimenter manipulated stress by pups and dam separation noticed increases the likelihood of alcohol abuse later in life (Kuhar, 2006; 2008; Moffett *et al.*, 2006). In addition, late life adversity in primates in the form of a social hierarchical dominance also increases drug self administration (Kreek *et al.*, 2007). Life stress also reinstates human drug addicts (Hyman *et al.*, 2006; Koob and Volkow, 2010) although the correlation between stress and drug addiction remains to be fully elucidated by clinical studies (Land *et al.*, 2010). There is a growing body of evidence demonstrating that stress is linked with mood (Calabrese *et al.*, 2009; Duman and Monteggia, 2006) For example, stress decreases the expression of brain-derived neurotrophic factor (BDNF) in limbic structures that control mood and that antidepressant treatment (SSRI's) reverses or blocks the effects of stress (Calabrese *et al.*, 2009; Duman and Monteggia, 2006). This indicates a relationship between stress and mood, as well as stress and SERT.

Furthermore, Tjurmina *et al.* (2004) indicate that a lifelong SERT deficiency results in complex alterations in adrenomedullary response to stress. In a landmark study by Caspi *et al.* (2003) a relationship between SERT reuptake, stress and depression was proposed. They suggest that life stressors and certain 5-HTTLPR genetic variants can predispose individuals to depression (Caspi *et al.*, 2003). This finding has also been replicated in other studies (Karg *et al.*, 2011; Kendler *et al.*, 2005) but remains controversial (Risch *et al.*, 2009). Nevertheless, the correlation between a gene and environment interaction between SERT, stressful life events, and brain diseases and disorders remains inconclusive. Bruchas *et al.* (2007) and Land *et al.* (2008) demonstrate that stress (cortisol) and corticotrophin releasing factor (CRF) cause KOPr activation in several key brain structures associated with stress, reward, and pathologies such as addiction and depression. However, the process by which KOPr acts in these structures to modulate affect is a critical question (Bruchas and Chavkin, 2010). KOPr action in the mesolimbic DA circuit is well characterized, however other monoamines and neuropeptide systems are also likely to be involved in KOPr-mediated behaviors. Because both the kappa-opioid system, and thus KOPr's, and SERT are both implicated in drug addiction, mood disorders and stress response a relationship appears appropriate. A relationship between these two proteins (KOPr and SERT) would strengthen the argument that dysfunctional SERT or KOPr proteins are both involved in the predisposition of these diseases and disorders because they are linked.

1.4 The κ -opioid Receptor

KOPr is a pertussis toxin (PTX) sensitive, (Avidor-Reiss *et al.*, 1995) 7-transmembrane GPCR that exerts its effects on the CNS and intestines (Eguchi, 2004). Specifically, within the brain KOPr's are found in high concentration in the NAc, Str, PFC, amygdala, caudate putamen, endoperiform nucleus and in claustrum and low levels found in the VTA and SNg (Mansour *et al.*, 1995; Quirion *et al.*, 1987). Presently, only one KOPr receptor type (KOPr-1) has been cloned, although pharmacological data suggest the existence of three KOPr receptor subtypes (KOPr-1, KOPr-2, KOPr-3) (Horan *et al.*, 1993; Heyliger *et al.*, 1999); yet, a clear demonstration of KOPr heterogeneity remains to be fully elucidated.

A substantial proportion of the literature that investigates the opioid receptor and 5-HT concentration relationship was dedicated to the mu-opioid receptor (Tao *et al.*, 2005). Currently, few studies have investigated the relationship between KOPr and 5-HT (Yilmaz *et al.*, 2006) with even fewer into KOPr and SERT. This is interesting because KOPr activation in the DRN or NAc decreases extracellular 5-HT concentrations in the respective region (Tao and Auerbach, 2002; Tao and Auerbach, 2005; Tao *et al.*, 2007; Werling *et al.*, 1989). In addition, KOPr modulates serotonergic neurotransmission in the hypothalamus, hippocampus and striatum (Yilmaz, 2006). Thus, to date it is believed that mu-Opioids disinhibit and kappa-opioids inhibit serotonin efflux in the DRN (Yilmaz, 2006).

KOPr activation by the endogenous ligand dynorphin, PTX or by synthetic agonists, such as U50,488H and U69,593, leads to a conformational change of the G-protein coupled receptor heterotrimer that comprises of $G_{\alpha i}$, G_{β} and G_{γ} subunits (Connor and Christie, 1999). This activates the intracellular $G_{\alpha i}$ protein trimer to replace GDP with GTP, dissociating the $G_{\alpha i}$ -GTP subunit (Eguchi, 2004). The $G_{\alpha i}$ -GTP subunit subsequently inhibits the enzyme adenylate cyclase (AC), which leads to a decrease in cyclic adenosine monophosphate (cAMP) and subsequently a decrease in protein kinase A (PKA) phosphorylation activity (Avidor-Reiss *et al.*, 1995; Watts and Neve, 2005). Meanwhile, $G_{\beta\gamma}$ binds directly to the potassium ($K_{\text{ir}}3.1$) channel, increasing the efflux of potassium, and binding to the N, L and P/Q type Ca^{2+} channels to decrease the influx of calcium via these voltage gated channels (Rusin *et al.*, 1997). This leads to hyperpolarisation of the KOPr neuron, maintaining its resting state potential and decreasing neuronal excitability (Figure 1.3) (Avidor-Reiss *et al.*, 1995; Bruchas and Chavkin, 2010; Watts and Neve, 2005). Activation of KOPr can trigger any one of the three main mitogen activated protein kinase (MAPK) cell signalling pathways: the c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular-signal regulated kinase 1/2 (ERK1/2) (for a review see Bruchas & Chavkin, 2010).

Fig. 1.3. KOPr signal transduction after acute and chronic activation. The binding of a KOPr ligand leads to a conformational change in the $G\alpha\beta\gamma$ subunits of the receptor which allows the dissociation of α i and $\beta\gamma$ subunits. The $G\alpha$ i protein trimer replaces GDP with GTP that then dissociates as a $G\alpha$ i-GTP subunit. The $G\alpha$ i-GTP subunit inhibits AC leading to a decrease in cAMP, and therefore a decrease in PKA activity. The $G\alpha$ i-GTP subunit also activates ERK1/2 through a PI3K, PKC and Ca^{2+} dependent signaling. The $G\beta\gamma$ subunit increases the efflux of potassium, and decreases the influx of calcium via the voltage gated channels, causing hyperpolarisation, thus decreasing neuronal excitability. Long-term activation of KOPr leads to receptor phosphorylation and kinase activation via GRK3-Arrestin dependent mechanism. AC-adenylyl cyclase; cAMP- cyclic adenosine monophosphate; Ca^{2+} - calcium ion; ERK1/2- extracellular signal-regulated kinase; $G\alpha$ i - G protein alpha subunit; $G\beta\gamma$ - G protein beta-gamma subunit; GDP- Guanosine diphosphate; GRK3- G-protein coupled receptor kinase 3; GTP- Guanosine triphosphate; K^{+} - potassium ion; KOPr- kappa opioid receptor; Kir3- G-protein gated inward rectifying K^{+} channel; P- phosphorylation; p38 - p38 mitogen activated protein kinase; PI3K- phosphatidylinositol-3-kinase; PKA- protein kinase A; PKC- protein kinase C; Src- Src kinase; zif268- zinc finger protein. (Adapted from (Bruchas and Chavkin, 2010)).

1.4.1 Dynorphin

The endogenous KOPr ligand, dynorphin, is synthesised from pro-dynorphin by the enzyme proprotein convertase 2 (Marinova *et al.*, 2005). Pro-dynorphin can be cleaved into several types of active dynorphin: big dynorphin, dynorphin A, B or α/β -neo- dynorphin (Merg *et al.*, 2006). Dynorphin A is the most active form and

preferentially activates KOPr, followed by mu opioid receptor and then delta opioid receptor (Chavkin *et al.*, 1982; Merg *et al.*, 2006). Dynorphin has been found throughout the brain with particularly high levels being detected in the rat forebrain, hypothalamus and SNg, and in low levels in the Str, NAc, amygdala and hippocampus (Goldstein and Ghazarossian, 1980; Weber *et al.*, 1982).

A large body of evidence in animal models indicates that KOPr's (and their endogenous opioid peptide ligands) modulate the effects of central nervous system stimulants (Shippenberg *et al.*, 2001). For, example, administration of cocaine or methamphetamine upregulates the kappa opioid receptor and preprodynorphin mRNA levels (Tzaferis and McGinty, 2001; Collins *et al.*, 2002). Dynorphin and KOPr are located in the nigrostriatal and mesolimbic-mesocortical DA pathways, brain regions involved in reward, reinforcement and motivation (Shippenberg *et al.*, 2007b). Thus, it was suggested that this KOPr/dynorphin system may play an important role in modulating reward to both natural reinforcers and drugs of abuse (Wee and Koob, 2010). KOPr/dynorphin system up-regulation after chronic drug exposure can lead to withdrawal symptoms, stress induced aversion, stress-induced relapse, drug craving and drug-seeking (Shippenberg, 2007). In addition, activation by KOPr agonists can lead to a multitude of effects, including hypersensitive analgesia, immunomodulation, hypothermia, anxiety, dysphoria, anhedonia and water diuresis (Butelman *et al.*, 2012; Liu-Chen, 2004; Shippenberg *et al.*, 2007b). On the other hand, inhibition by KOPr antagonists can lead to anti-depressant and anti-anxiety behaviours (Carlezon *et al.*, 2009).

1.4.2 Dynorphin and KOPr Negative Feedback Loop

KOPr activation directly inhibits DA neurons in the midbrain (Margolis et al., 2003), which modulates DA uptake in the NAc (Thompson et al., 2000). In other words, the KOPr/dynorphin system acts to modulate DA VTA output through a negative feedback loop (Van Bockstaele and Pickel, 1995). This occurs because the medium spiny GABA neurons in the NAc receive a DA input from the DA neurons in the VTA (Figure 1.4). Once DA reaches these stimulatory DA1 receptors AC activity is increased, which increases cAMP and thus, activates CREB resulting in the release of pro-dynorphin and then dynorphin (Nestler, 2001). Dynorphin is released from the NAc and binds to KOPr receptors in the VTA acting to decrease the overall DA tone and thus, the hedonic properties. This activation of KOPr is known as the anti-reward system and thus, KOPr agonists are the subject of anti-addiction pharmacotherapies. Overlap of the endogenous opioid systems with monoamine systems in the brain suggest that other overlapping systems may also interact (Snyder and Pasternak, 2003).

Fig. 1.4. KOPr/dynorphin negative feedback loop. This negative feedback pathway acts to modulate DA release into the NAc. Briefly, the NAc GABA medium spiny neurons receive the DA input through the DA1 receptor. This stimulates AC, which increases cAMP and thus PKA, activating CREB which results in the release of prodynorphin then dynorphin. Dynorphin is then released from the GABA medium spiny neurons in the NAc to bind to KOPr on the DA VTA neurons to decrease DA increase and thus, hedonic properties. NAc- nucleus accumbens; GABA - gamma amino butyric acid medium spiny neuron; VTA - ventral tegmental area; CREB - cAMP response element binding protein; DA- dopamine; D1- DA 1 receptor; Gs- Stimulatory G-protein coupled receptors; KOPr- kappa opioid receptor. (Adapted from Carlezon *et al.*, 2005)

1.4.3 KOPr and DA

It has been known and documented for at nearly 25 years that KOPr has an inhibitory effect on DA neurons in the rat brain (Di Chiara and Imperato, 1988; Werling *et al.*, 1988). These groundbreaking studies illustrates for the first time that KOPr agonists decrease DA concentrations in the NAc and dorsal caudate brain regions of rats, as well as observing decreased motor activity (Kunihara *et al.*, 1993). Since these landmark studies it has been further confirmed that KOPr activation inhibits previously stimulated DA neurons in the nigrostriatal, mesolimbic, tuberunfundibular and tuberohypophysial systems (Manzanares *et al.*, 1991) and

several doses of KOPr activation with U50,488H decreased this effect, suggesting a receptor desensitisation with overstimulation (Schlosser *et al.*, 1995). This inhibitory DA effect induced by KOPr agonists can also be reversed with KOPr antagonists (Manzanares *et al.*, 1991). Co-localisation of KOPr's and DAT was first found in the NAc (Svingos *et al.*, 2001) and KOPr agonists were then found to modulate DA function through an increase of DAT function (in the NAc) (Morani, unpublished data; Simonson, unpublished data; Thompson *et al.*, 2000; Zhang *et al.*, 2004). However, when the biochemical mechanism behind this KOPr activation to increase DAT function was investigated it was found that DAT inhibitors did not prevent the NAc DA increase from baseline. This provided evidence to suggest the rewarding effects of opiates and their receptors may also be occurring through a DA-independent mechanism. Indicating that another similar monoamine transporters, such as 5-HT, may be responsible for this NAc DA increase (Collins *et al.*, 2001). A recent study by Bruchas *et al.* (2011) showed that the activation of p38 MAP-kinase was responsible for kappa-mediated effects on stress in serotonergic neurons, possibly by regulating the function of the SERT (Bruchas *et al.*, 2011). This leads to the possibility that compounds that differentially activate signaling pathways and related monoamine transporters, such as SERT, may have varying effects on addiction, mood, depression, aversion and dysphoria. Therefore, preclinical research illustrates that KOPr modulates DAT, and also suggests KOPr modulates SERT too, thus, providing a new direction for research for future clinical targets.

1.5 Salvinorin A and Derivatives

At present, the Mexican mint *Salvia divinorum* or 'divine sage' is an unregulated hallucinogen that has been traditionally used by Mazatec Indians in shamanistic rituals to induce short, intense hallucinations (Siebert, 1994). The active ingredient in *Salvia divinorum*, salvinorin A (Sal A) (Fig. 1.5), has been shown to be a KOPr agonist both *in vitro* and *in vivo* (Prisinzano, 2005). In fact, Sal A was found to be more similar to dynorphin than the classical KOPr agonists U50,488H (Fig. 1.5) or U69,593 (Chavkin *et al.*, 2004) that have been previously shown to attenuate stress, and drug primed reinstatement. This may be because Sal A has a unique neoclerodane diterpene structure including no nitrogen atoms (Prisinzano and Rothman, 2008). Other novel neoclerodane diterpenes (DS-3-240) are also of interest because they show psychostimulant pharmacotherapy potential, however they differ from Sal A because they have a different C2 side chain (Morani, 2011).

Classic KOPr agonists have anti-addictive properties and also show potential in helping affective disorders, stress dysfunction and pain response studies in animal models but unfortunately, they also lead to negative side effects such as dysphoria and depression, particularly with long-term use (Negus *et al.*, 1997). Sal A is unique in that it has no affinity for the mu opioid, delta opioid, DA, or 5-HT receptors, and unlike all other hallucinogens it had no binding affinity for the 5-HT_{2A} receptor, which is a target for hallucinogens such as lysergic acid diethylamine, dimethyltryptamine, and ibogaine (Munro *et al.*, 2005; Vortherms and Roth, 2006). Yet, few studies have reported behavioural findings with KOPr agonists in non-

primates (Prisinzano, 2005; Shippenberg *et al.*, 2007a; Shippenberg *et al.*, 2007b). It has been found that the classic KOPr agonist U50,488H and Sal A decrease SERT 5-HT uptake in rat striatal tissue after acute (15 min) exposure as shown using RDEV (Simonson, unpublished data) and U50,488H decrease SERT 5-HT uptake in co-transfected HEK-293 cells with chronic (30 min) exposure as shown using confocal microscopy (Morani, unpublished data). Collectively, these studies suggest KOPr activation modulates SERT function *in vitro*.

Fig. 1.5. KOPr agonists. *Sal A is a novel KOPr agonist with a unique structure that has no nitrogen, and was found to be more similar to dynorphin than the classical KOPr agonists U50,488H.*

If these compounds modulate SERT function, they may provide a platform from which pharmacotherapeutics can originate to specifically target KOPr to increase DAT and decrease SERT, allowing the brain to resemble its innate biological state.

1.6 The Serotonin Transporter and Kappa-Opioid Receptor

KOPr expresses in numerous brain regions and KOPr activation in the DRN or NAc decreases extracellular 5-HT concentrations in the respective region (Tao and

Auerbach, 2002; Tao and Auerbach, 2005; Tao *et al.*, 2007; Werling *et al.*, 1989). The 5-HT and KOPr systems are involved in cocaine addiction, affective disorders, and dysfunctional stress responses. In addition, SERT and KOPr co-localise in the DRN, which is a brain region involved in the aetiology of these disorders. To investigate this relationship further preclinical models are extremely important. For example, a study by Ruedi-Bettschen *et al.* (2010) found that fluoxetine, an SSRI, and a KOPr agonist, spiradoline, each inhibited cocaine-induced reinstatement in squirrel monkeys. Interestingly, this effect was reversed for both compounds (fluoxetine and spiradoline) by the 5-HT_{1A} agonist, 8-OH-DPAT, that implies cocaine-induced reinstatement involves 5-HT signalling, which occurs via SERT and KOPr. In addition, KOR activation in the DRN or NAc decreases extracellular 5-HT levels in the respective brain region but whether this effect is because of direct 5-HT release, a direct effect on SERT function or an indirect effect on glutamatergic afferents to the DRN was not determined (Tao and Auerbach, 2002). Further, DRN 5-HT inactivation with the KOPr antagonist norBNI blocked stress-induced cocaine reinstatement in rats and any aversive response observed to U50,488H (Land *et al.*, 2009). Finally, it is known that cocaine exposure up-regulates the KOPr/dynorphin system and this increase is DAT independent because KOPr's did not increase with selective DAT inhibitors (Collins *et al.*, 2001). Thus, it was postulated that SERT may modulate KOPr, either directly or indirectly (Collins *et al.*, 2002) and thus a reciprocal relationship is likely to exist. Collectively, these preclinical models demonstrate sufficient evidence to suggest a relationship between KOPr and SERT.

A potential KOPr and SERT relationship was first documented by Schenk *et al.* (2000) when it was illustrated that cocaine-seeking in rats were attenuated with the traditional KOPr agonist U69,593. This decrease in cocaine-seeking was suggested to occur because kappa-opioids and cocaine interact at common sites on the SERT protein (Schenk *et al.*, 2000). Furthermore, this KOPr and SERT hypothesis was further elaborated by Ramamoorthy *et al.* (2002) who illustrated that SERT activation by the KOPr agonist, U69,593, leads to a decrease in 5-HT re-uptake in rat NAc synaptosomes. Zakharova *et al.* (2008) provided additional evidence to the hypothesis that KOPr modulates SERT, when they illustrated that SERT depletion decreases KOPr activity. Additional recent findings from our laboratory also complement this hypothesis as acute (15 min) KOPr agonist (500 nM; U50,488H and Sal A) exposure decreases SERT function as measured with RDEV on rat striatum tissue (Simonson, unpublished data) as well as chronic (30 min) KOPr agonist (10 μ M; U50,488H) exposure in co-expressing HEK-293 cells using confocal microscopy (Morani, unpublished data). Collectively, an overview of the current literature strongly suggests there is an interaction between KOPr and 5-HT, which is indicated to occur between KOPr and SERT. Therefore, the literature provides rationale to further investigate this relationship by elucidating the effect of KOPr activation on SERT regulation.

1.7 Significance and Hypotheses

SERT and KOPr co-localise in the DRN and NAc which is a brain region vital to the etiology of psychiatric diseases and disorders, however a relationship remains

inconclusive. Elucidating the effect of KOPr activation on SERT function will elucidate key molecular targets to facilitate future psychiatric research and pharmacotherapeutic development for more effective treatment of neurological diseases. It is hypothesised that both acute (5 min) and chronic (30 min) KOPr activation using the classical KOPr (U50,488H) and novel KOPr agonists (Sal A, DS-3-240) will significantly decrease SERT function *in vitro* in HEK-293 cells transiently expressing myc-rKOPr and eGFP-tagged SERT.

1.8 Aims

The aim of the present study is:

- 1) To investigate the cellular effect with acute (5 min) exposure of classical KOPr (U50,488H) and novel KOPr agonists (Sal A, DS-3-240; 10 μ M) on the modulation of SERT function *in vitro* in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT using ASP⁺ uptake assay.
- 2) To investigate the cellular effect with acute (5 min) exposure of classical KOPr (U50,488H) and novel KOPr agonists (Sal A, DS-3-240; 500 nM) on modulation of SERT function *in vitro* in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT using rotating disc electrode voltammetry.
- 3) To develop a high-throughput screening assay to investigate the effect of chronic (30 min) KOPr agonist (U50,488H, Sal A; 10 μ M) exposure on modulation of SERT function *in vitro* using HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT.

Chapter 2: General Methods

2.1 Cell Culture

The cell lines used in this study were grown under PC2 conditions in the School of Biological Sciences, Victoria University of Wellington, New Zealand. All cell culture work was performed in a sterile Email Air Handling Class II Biological safety cabinet (AES Environment Pty LTD, Auburn, Australia) with HEPA air filters. Before the cabinet was used it was sterilized with Ultraviolet (UV) light (1390 mW/m²) for a minimum of 15 min and wiped prior to use with 70% ethanol. The sterile plasticware used for all cell culture work was purchased from BD Biosciences (BD-New Zealand, Auckland, New Zealand) and Greiner Bio One (Raylab, Auckland, New Zealand). Cells were grown in a Heracell incubator (Kendro Laboratory Products, GmbH, Germany) under constant environmental conditions at 37°C with 5% CO₂. In this study human embryonic kidney cells (HEK-293 cells) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were used between passage number 6 and 30.

Human embryonic kidney (HEK-293) cells are an immortalised human embryonic cell line that has been transformed with adenovirus 5. They were cultured in Dulbecco's Modified Eagle's Media (DMEM) high glucose medium (Gibco, Invitrogen, Auckland, NZ) containing a final concentration of 10% fetal calf serum (FCS; IPC biological, Auckland, NZ), 1% Penstrep antibiotic (Penicillin G sodium 5000 units/mL, streptomycin sulphate 5000 units/mL in 0.85% saline, Gibco,

Invitrogen) and 0.1% L-glutamine (Gibco, Invitrogen). Penstrep-free media was used for all transfections and comprised of DMEM containing 10% fetal calf serum and 0.1% L-glutamine. For confocal microscopy and establishment of a stable cell line expressing eGFP-tagged hSERT, HEK-293 cells were plated at a concentration of 5×10^5 cells/mL in a 35 mm glass bottom dishes (Mattec, Loveland, Ohio, USA) and for RDEV HEK-293 cells were plated at a concentration of 5×10^5 cells/mL in 60 mm plastic bottom dishes. The 96-well plate assay and preliminary RDEV experiments used 5×10^5 cells/mL in 100 mm plastic bottom dishes.

2.1.1 Cell growth

Cryovials of cell stocks were stored in liquid nitrogen for experimental use. To defrost cells a cryovial of previously frozen cells was removed from the liquid nitrogen dewer and defrosted at 37°C in a water bath. They were then immediately added to 5 mL of pre-warmed 37°C media in a 15 mL conical centrifuge tube and centrifuged at 300 g for 5 min. The supernatant was aspirated off and cells were re-suspended in 5 mL of media, and transferred into a 75 mL flask fitted with a filter screw cap, placed in the incubator and allowed to grow. The following day the old media was aspirated and fresh media was placed into the flask to feed the cells and encourage growth. Once the cells were 80-90% confluent they were passaged into a 250 mL flask. When this flask became 80-90% confluent the cells were passaged into two new 250 mL flasks, one flask was to continue the cell line for use in experiments, and the other flask was used to replace the removed cell stock.

2.1.2 Cell Passaging and Freezing of Cell Lines

When cells became 80-90% confluent they were ready to be passaged into the sterile plasticware required for experiments or continued growth. The old media was aspirated off and cells were washed with 5 mL of sterile 1x phosphate buffered saline (1x PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4; Appendix 1) and then incubated with 2 mL of TrpLE Express (Gibco, Invitrogen) for 5 min or until cells had detached from the bottom of the flask. Gentle agitation of the flask helped to remove any still adhered cells and when the cells had detached they were diluted with 2 mL of DMEM media, transferred into a sterile 15 mL conical centrifuge tube and centrifuged at 300 g for 5 min. The supernatant was then aspirated off and the cells were re-suspended in 5 mL of media. The concentration of live cells was then determined by using a haemocytometer and 0.4% Trypan blue (Sigma).

Cells were frozen down when in the log growth phase at a concentration of 1×10^6 cells/mL in the corresponding media containing 10% dimethyl sulfoxide (DMSO). Aliquots of 1 mL were frozen in cryovials overnight at -80°C and then transferred to the liquid nitrogen dewar the following day for long term storage.

2.1.3 Cell Transfection

On the first day (Day 1) the cells were passaged into plates at a density for the relevant experiment. The day after plating (Day 2) cells were transfected when 60-80% confluent and in the log growth phase. Cells were transfected using

Lipofectamine 2000 (Gibco, Invitrogen, Auckland, New Zealand) and Optimum I medium (Invitrogen). Transfections were carried out according to manufacturer's instructions. For HEK-293 cells, 3 μ L of Lipofectamine 2000 was made up to 50 μ L with Optimum I in a tube and incubated at room temperature for 5 min. In a separate tube, myc-tagged rat κ -opioid receptor plasmid DNA (myc-rKOPr, kindly donated by L. Devi; Table 2.1) and enhanced green fluorescent protein-tagged human SERT plasmid DNA (eGFP-hSERT, kindly donated by S. Ramamoorthy; Table 2.1) were made up to 50 μ L with Optimum I, to a final concentration of 0.6 μ g/mL and 0.4 μ g/mL, respectively. After a 5 min incubation tube 1 and 2 tube were mixed together and incubated at room temperature for 20 min. Prior to transfection cells were fed with 1 mL of penstrep-free medium for every 100 μ L of the transfection mixture added. Transfected cells were used 48 hours later (Day 4).

Table 2.1. myc-rKOPr and eGFP-hSERT Plasmid Information

Gene	Vector	Resistance	Provided by
myc-rKOPr	pcDNA3	Ampicillin (100 μ g/mL)	L. Devi (Jordan and Devi, 1999)
eGFP-hSERT	peGFP-C1	Kanamycin (50 μ g/mL)	S. Ramamoorthy (Scholze <i>et al.</i> , 2000)

2.2 Plasmid Preparation

2.2.1 Luria Broth (LB) and LB agar

LB broth (10 g/L NaCl, 5 g/L Yeast extract, 10 g/L Bactotryptone) (Appendix 1) and LB agar (10 g/L NaCl, 5 g/L Yeast extract, 10 g/L Yeast agar, 10 g/L Bactotryptone) (Appendix 1) were sterlised by placing in an autoclave at 121°C at 20

lb/in² for 20 min. Solutions were then left to cool to 37°C in a waterbath and then the appropriate amount of the antibiotic was added to each solution (myc-rKOPr:100 µg/mL ampicillin or e-hSERT:50 µg/mL kanamycin). The agar was then gently placed into sterile 100 mm plastic plates and left to set. Agar plates were stored at 4°C until needed but for no longer than 2 weeks.

2.2.2 Transformation of MAX Efficiency DH5αTM Competent cells

A 100 µL aliquot of MAX Efficiency® DH5αTM Competent Cells (Invitrogen) was thawed on wet ice and then divided into two 50 µL aliquots in ice-cold micro centrifuge tubes. 20 ng of plasmid DNA (Table 2.1) was added to one tube, the other tube acted as a negative control. Both aliquots of cells were incubated on ice for 1 h, then heat shocked at 42°C for 2 min in a heat block (Global Science) and then placed on ice for a further 5 min. After cooling, 450 µL of room temperature SOC medium (Invitrogen) was added to each of the tubes, and the tubes were placed in a 37°C Shaker (Bioline, Edwards Instrument Company, Australia) set at 225 rpm for 1 h. Following this, 100 µL of each culture was spread onto a LB agar plate containing the appropriate antibiotic to create a 'lawn' culture. The plates were incubated overnight in a 37°C humid incubator (Bioline).

2.2.3 *Escherichia coli* (E.coli) Cultivation

To cultivate single colonies either: (1) A single colony from a freshly transformed agar plate as previously described above or (2) From a plate streaked with plasmid transformed bacteria from a glycerol stock, was picked off with a

pipette tip and added to 2 mL of LB Broth containing the appropriate concentration of antibiotic. This was only carried out if there was no growth of non-transformed bacteria on the antibiotic containing agar (negative control plate); if this occurred, a new transformation was performed. The bacterial culture was then incubated for 24 h in a shaking, humid 37° C incubator (Bioline). After 24 h, 200 µL of bacteria was removed and added to 200 mL of LB broth containing the appropriate antibiotic, and this culture was incubated for another 24 h in a shaking, humid 37° C incubator. After 24 h the plasmid preparation was carried out. The reason for 2 x 24 h growth phase was because the bacteria was to produce the highest yield of plasmid, and this was shown in the past to be the most effective method.

2.2.4 Plasmid Purification using the Qiagen HiSpeed Maxi Kit

Plasmid DNA was purified from 200 mL bacterial culture following the manufacturer's protocol.

Briefly, the 200 mL bacterial culture was centrifuged at 6,000 g for 25 min at 4°C in a Sorvall Evolution RC centrifuge (Kendro Laboratory Products, Bath, UK) and the supernatant discarded. The bacterial pellet was re-suspended in 10 mL of re-suspension buffer containing RNase A and lysis blue Qiagen reagents (Buffer P1). Following this cells were lysed by transferring the resuspended transformed *E. coli* cells to a sterile 50 mL tube and 10 mL of lysis buffer (Buffer P2) was added and mixed by inverting 4-6 times. The solution was incubated for 5 min at room temperature then 10 mL of chilled neutralisation buffer (Buffer P3) was added to

stop cell lysing and mixed by inverting the tube 4-6 times. This lysate was then transferred into a QIAfilter Cartridge and incubated for 10 min at room temperature. During this time a HiSpeed Maxi Tip was placed over a waste container and equilibrated by the application of 10 mL of Buffer QBT through the filter. The lysate was then filtered through a HiSpeed Maxi Tip into a waste container, trapping the DNA within the tip. The tip was then washed with 60 mL of wash buffer (Buffer QC), and then the DNA was eluted into a sterile 50 mL tube using 15 mL of the elution buffer (Buffer QF). The DNA was then precipitated by incubation with 10.5 mL of room temperature molecular grade isopropanol (Sigma) for 5 min. The isopropanol mixture was transferred into a 30 mL syringe attached to a QIAprecipitator Maxi Module and filtered through the precipitator into a waste container. The DNA remained trapped in the precipitation filter. The precipitator filter was then rapidly washed with 2 mL of room temperature molecular grade 70% ethanol (Ajax Chemicals, Auckland, NZ) through a 5 mL syringe. The precipitation filter was then air dried by quickly pushing air carefully through the syringe through the filter twice then the filter was dried on a paper towel to prevent ethanol carryover. The precipitation filter was then re-attached to the 5 mL syringe and placed over a microcentrifuge tube, 1000 μ L of endotoxin free buffer (Buffer TE) was pipetted into the 5 mL syringe and the DNA was eluted into a 1.5 mL capacity microcentrifuge tube. The eluted DNA was then transferred back into the syringe and filtered a second time through the precipitator into the same 1.5 mL capacity microcentrifuge tube. The yield of DNA was determined using a Nanodrop ND-1000 spectrophotometer (BioLab, Auckland, NZ). The Nanodrop was initialised and then blanked using 1 μ L of double distilled water. A 1 μ L sample of eluted DNA was

placed onto the nanodrop sensor and the amount of DNA present was measured at 230 nM. Yields of above 250 ng/ μ L with an A_{260}/A_{280} ratio of 1.8-1.9 were deemed acceptable and subsequently used for cell transfection protocols.

If yields of 1 mg/mL or above were purified, the 2 mL culture of bacteria was frozen in 500 μ L aliquots in 20% glycerol at -80°C for future use so that transformations were not necessary each time the plasmid was needed. In this case, a scraping from the frozen bacteria stock was streaked onto a LB agar plate containing the appropriate antibiotic to obtain a single colony that could then be cultured in 2 mL of LB broth containing the appropriate antibiotic.

2.3 Reagents

In all experiments Sal A (50 mM stock concentration), DS-3-240 (50 mM stock concentration) (Kind gifts from Dr. Thomas E. Prisinzano, University of Kansas), U50,488H (50 mM stock concentration), fluoxetine-HCl (Tocris, UK) and 1'-diethyl-2,2'-cyanine iodide (decynium-22; D-22) (Sigma Aldrich, St. Louis, MO) were initially suspended in 100% DMSO to make stock concentrations. [4-(dimethylamino)styryl]-1-methylpyridinium (ASP+) (Sigma Aldrich) stock solution was made in sterile, filtered KREBS buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl_2 , 1.2 mM $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 10 mM HEPES, 10 mM D-glucose, pH 7.4) (Appendix 1). All drugs were further diluted into the working concentration using sterile, filtered KREBS (pH 7.4). Serotonin hydrochloride (5-HT HCl) (Tocris, UK)

was dissolved in 1x PBS (pH 7.2) (Appendix 1) to improve stability (Huang and Kissinger, 1996).

Chapter 3: The Effect of Acute (5 min) KOPr Agonist Exposure on Modulating SERT Function in HEK-293 Cells using Confocal Microscopy

3.1 Introduction

Cultured cell lines expressing proteins of interest are an extremely useful tool to understand cellular and molecular processes. Specifically, cell models facilitate the ability to manipulate a wider spectrum than is currently possible in animal models. Previously, SERT function has been measured in tissue and cell models by radioactive 5-HT uptake (Ramamoorthy *et al.*, 1998; Owens *et al.*, 2001), high-speed chronoamperometry (Perez and Andrews, 2005) and fast scan cyclic voltammetry (John and Jones, 2007; Hashemi *et al.*, 2009). However, recent developed temporally-resolved techniques enable a much greater understanding of monoamine transporter function. In this study we utilise confocal microscopy 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP⁺) uptake techniques to investigate the effect of KOPr agonists on SERT function in HEK-293 cells transiently transfected with enhanced green fluorescent protein-tagged human SERT (eGFP-hSERT) and myc-rKOPr. This sensitive ASP⁺ uptake model has multiple benefits over the other available techniques, including visualising eGFP-hSERT to quantify SERT expression levels using single-cell, spatially resolved, real-time fluorescence microscopy analysis (Oz *et al.*, 2010). This advanced technique is unique and the first of its kind to allow separation between ASP⁺ binding and uptake, because rapid binding occurs with

micromolar affinity and then linear uptake occurs for at least 10 min (Figure 3.1), which accumulates intracellularly and binds irreversibly to mitochondria (Schwartz *et al.*, 2003).

Fig. 3.1. Binding and uptake of ASP⁺ in arbitrary fluorescence units (AFU) in a single HEK-293 cell. The initial rapid binding phase is followed by a linear uptake phase for at least 10 min after ASP⁺ addition.

To date it is known that KOPr's co-localise with SERT in a key brain region, the DRN, that is involved in mood, stress, learning and memory (Land *et al.*, 2009). Preliminary data from our laboratory suggests that KOPr's modulate SERT, either directly or indirectly, *in vitro* and *in vivo* (Morani, 2011; Simonson, unpublished data). In addition, Thompson *et al.* (2000) suggest that KOPr's act to modulate DA function, which is confirmed with the discovery that KOPr's co-localise with DAT in the NAc (Svingos *et al.*, 2001). Our laboratory has since illustrated that KOPr agonists increase the function of DAT as a result of change in DAT function in the NAc and in HEK-293 and N2A cells expressing myc-rKOPr and eYFP-hDAT proteins (Simonson,

unpublished data). Because both the SERT and KOPr systems are involved in mood and stress dysfunctions, co-localise within the same brain regions, and have been suggested to interact there is sufficient rational for SERT regulation by KOPr. In this study we utilise a cellular *in vitro* model that measures SERT function pre and post KOPr activation. We use ASP⁺ uptake techniques in a within-cell design to determine if SERT is modulated by KOPr. This will determine if acute (5 min) KOPr agonist exposure modulates SERT function. Overall, this study may aid to develop more effective targets for pharmacotherapy research.

3.1.1 Aim

In this chapter we aim to use confocal microscopy to measure ASP⁺ uptake in transiently transfected (eGFP-hSERT and myc-rKOPr) HEK-293 cells to determine if acute KOPr activation modulates SERT function.

3.2 Methods

3.2.1 Acute ASP⁺ Uptake using Confocal Microscopy

HEK-293 cells were cultured and transiently transfected with myc-rKOPr and eGFP-hSERT plasmids as described in the general methods section (Chapter 2) and used 48 hours after transfection when the cells were 70-90% confluent.

Methods were carried out as previously described (Bolan *et al.*, 2007; Oz *et al.*, 2010a; Schwartz *et al.*, 2003; Zapata *et al.*, 2007). The fluorescent monoamine transporter substrate ASP⁺ (Tocris, UK) was made up into a 10 mM stock solution

using sterile, filtered KREBS buffer (pH 7.4). This stock solution was further diluted to a 10 μ M working solution. Agonists were made into a 50 mM stock concentration in DMSO and then diluted in sterile, filtered KREBS buffer to a working solution concentration of 1.01 mM so that when 10 μ L was added to the dish of cells with 1 mL (10 μ M) ASP⁺ in KREBS buffer the final concentrations of the agonists were 10 μ M. Control experiments were carried out using vehicle (DMSO) matched solutions.

The ASP⁺ and KOPr agonists (U50,488H, Sal A or DS-3-240) were warmed to 37°C along with the solution of KREBS. An Olympus Fluoview FV1000 confocal laser scanning biological microscope (inverted model 1X81) (Olympus, Tokyo, Japan) was used to monitor ASP⁺ uptake and binding of cells, using a 40X water immersion objective (NA=0.9). To maintain the temperature of the cells at 37°C during confocal microscopy, a heat-plated, insulated, double-layer heating chamber (INU-21LCS-F1 model, Tokai Hit Co., Ltd, Japan) mounted on the microscope stage was filled with distilled water and allowed to warm to 37°C. ASP⁺ fluorescence was measured with a 558 nm laser (ASP⁺ filter: 573 nm excitation; 590 nm emission) at settings of 2.0% power, high voltage (HV) 600 V, offset 8%, and gain 1%. eGFP fluorescence was measured with a 473 nm laser (eGFP filter: 472 nm excitation; 595 nm emission) at settings of 0.2% power, HV 650 V, offset 8%, and gain 1%. The microscope pinhole was opened to 500 μ m to allow for more light from a greater z plane to be detected. This decreases confocality and allows ASP⁺ accumulation to be more representative of uptake from the whole cell rather than just a single plane. Images were acquired using Olympus FV10-ASW software.

Acute ASP⁺ experiments were carried out at room temperature in a dark room with limited light so that fluorescence was not affected. First, the culture medium was removed from the 35 mm glass bottom petridish by aspiration and washed once with 1 X PBS then replaced with sterile, filtered KREBS buffer at 37°C. Dishes were mounted on the microscope stage, and the microscope was focused onto an appropriate monolayer collection of cells using differential interference contrast (DIC). Once an appropriate area of cells was located, the KREBS buffer was removed and the time course started (120 scans, every 5 s) and then once the first image was collected using the DIC, eGFP and ASP⁺ filters, 1 mL of 10 µM ASP⁺ at 37°C was added. 300 s after ASP⁺ was added, 10 µL of the KOPr agonist (U50 488H, Sal A and DS-3-240), fluoxetine (1.5 mM), or vehicle control was carefully added to the petri dish. Non-expressing HEK-293 cells served as a negative control to determine non-specific ASP⁺ uptake by non-SERT mediated mechanisms. The amount of ASP⁺ accumulation in cells not expressing GFP-hSERT (non-transfected cells) was compared with those cells expressing GFP-hSERT (transfected cells).

This method allowed the uptake of ASP⁺ before the KOPr agonist addition to be measured, and compared to ASP⁺ uptake after KOPr agonist addition. This allowed SERT function to be determined within single cells. Data is presented as percentage change of ASP⁺ uptake within a single cell. Each condition, along side the negative controls, was measured on at least 2 plates across at least 3 separate transfections.

3.2.2 Data Analysis for Acute ASP⁺ Uptake Studies

Cells expressing eGFP-hSERT were manually outlined using Fluoview FV1000 software, and these regions of interest (ROI) were graphed as integrated pixel density over time to show the changes in ASP⁺ fluorescence. The data from the ASP⁺ uptake graph of each cell that fit the selection criteria was then transferred into a Microsoft Excel spreadsheet, and graphed individually. Cells without linear ASP⁺ uptake were discarded, as this indicated that the cells moved or drifted over the period of the experiment and would not provide a representation of SERT uptake. The initial eGFP fluorescence was also recorded so that cells could be selected based on their eGFP-hSERT expression. Cells expressing less than 200,000 arbitrary fluorescence units (AFU) or more than 900,000 AFU of eGFP-hSERT were not used. Cells with an eGFP-hSERT expression of less than 200,000 were not used because they had low SERT expression as they showed significantly less ASP⁺ uptake at 300 s, did not demonstrate linear uptake and therefore, were classed as non-transfected cells (Figure 3.5) and >900,000 AFU were overexpressing eGFP-hSERT.

To analyse the ASP⁺ uptake data, a linear trend line was generated for a period of 60 s, 1 s before KOPr agonist addition, and compared to a linear trend line created for a period of 60 s after KOPr agonist addition during the time of maximal change in uptake. The change in ASP⁺ uptake was then calculated as a percentage change of the slope difference ((slope after drug at maximal time of effect/slope before drug) x100) and graphed using Graphpad Prism (GraphPad Software Inc, La Jolla, CA, USA) (Appendix 2). Any cells that had a linear uptake trend line of $r^2 < 0.95$

were discarded because ASP⁺ uptake was not linear, and not representative of SERT function.

GraphPad Prism was used to carry out one-way ANOVA followed by Tukey Post test was performed on the average percentage change of ASP⁺ uptake. Significance was defined as $p < 0.05$.

3.3 Results

3.3.1 ASP⁺ Uptake Method Validation

ASP⁺ uptake studies were conducted in transiently transfected HEK-293 cells shown to co-express eGFP-hSERT and myc-rKOPr in HEK-293 cells using a Cy5-myc-rKOPr antibody (Simonson, unpublished data). Preliminary experiments confirmed that ASP⁺ binding was rapid, uptake was linear for 10 min (Figure 3.1) and the correlation with increasing eGFP-hSERT cell expression and ASP⁺ accumulation at 300 s was positive ($r^2 < 0.58$; Figure 3.2). eGFP AFU expression less than 200,000 at 0 s was used as the criteria cut off point for cell selection because there is a significant decrease in ASP⁺ uptake compared to non-transfected cells ($***p < 0.001$; Figure 3.3). This is the same significant decrease found between transfected cells versus non-transfected cells ($***p < 0.001$; Fig. 3.3), justifying the cell selection cut off point. Transfected cells versus fluoxetine pre-treated (5 min) cells also had a decrease in ASP⁺ accumulation ($**p < 0.01$; Figure 3.3). Collectively, these results so far illustrate that ASP⁺ is taken up through SERT. Interestingly, ASP⁺ uptake occurs in non-transfected cells as well as cells with an eGFP AFU at 0 s of less than 200,000 (Figure 3.3). This indicates ASP⁺ uptake through low affinity monoamine transporters, such

as extraneuronal monoamine transporter (EMT), the plasmalemmal monoamine transporter (PMAT), and the organic cation transporters- OCT1, OCT2, and OCT3. For example, ASP⁺ uptake of fluorescent in HEK-293 cells by OCT3 was recently shown in real-time and thus, it is likely that other low affinity transporters also contribute ASP⁺ uptake (Mayer *et al.*, 2012). This idea is further discussed in the final discussion (chapter 6).

Fig. 3.2. ASP⁺ uptake in arbitrary fluorescence units (AFU) at 300 s versus eGFP- hSERT intensity at 0 s have a positive correlation in HEK-293 cells. An increase in eGFP-hSERT expression at 0 s resulted in an increase of ASP⁺ accumulation at 300 s ($r^2=0.5791$, $p<0.001$).

Fig. 3.3. *ASP⁺ accumulation in arbitrary fluorescent units (AFU) at 300 s in HEK-293 cells that were non-transfected, transfected, fluoxetine pre-treated (5 mins), and eGFP less than 200,000 at 0 s. The difference in ASP⁺ accumulation between non-transfected cells and cells with an eGFP AFU at 0 s of less than 200,000 versus transfected cells was significantly different (** $p < 0.001$). Non-transfected cells and cells with an eGFP AFU at 0 s of less than 200,000 versus fluoxetine (1.5 mM) pre-treated (5 min) cells was also significant (** $p < 0.01$). Transfected cells and fluoxetine pre-treated cells were also significantly different (** $p < 0.01$) ($n = 50-200$ cells).*

3.3.2 Effect of Acute KOPr Agonist on SERT Function

ASP⁺ uptake assisted fluorescence investigated the effect of acute KOPr agonists on SERT function. As shown by one way ANOVA with Tukey post test, it was found that there was no statistical significance in the difference of ASP⁺ uptake, as a correlation of SERT function, between any of the KOPr agonists compared to the vehicle matched control (** $p < 0.001$) (Figure 3.6).

Fig. 3.4. Percentage changes of ASP⁺ after acute (5 mins) KOPr agonists exposure and fluoxetine pre-treatment (5 mins). There is no difference in percentage change of ASP⁺ uptake between the DMSO vehicle and any of the KOPr agonists. This was confirmed by inhibition of hSERT by the SSRI, fluoxetine (1.5 mM), for 5 min which significantly decreased the ASP⁺. As shown by a one way ANOVA followed with a Tukey post test (***p*<0.001).

To confirm that no KOPr agonists had a modulatory effect on SERT function we used an SSRI, fluoxetine, as a positive control. Here, it was shown that the hSERT plasmid in this cell model was functional because ASP⁺ accumulation was inhibited significantly with the addition of fluoxetine (***p*<0.001; Figure 3.6). This confirms hSERT is functional, working correctly in this model and again confirming our belief that ASP⁺ uptake occurs through SERT. Based on our knowledge that ASP⁺ uptake is indicative of SERT function, these results suggest acute KOPr activation by the selected agonists have no significant effect on SERT function in this *in vitro* cell

model. Thus, in this transiently transfected HEK-293 cell model, KOPr agonists have no modulatory effect on SERT function as measured by confocal microscopy.

When an appropriate area of cells in the dish was located visualizing with both both the eGFP- hSERT (Fig. 3.4 A and 3.5 A) and DIC image (Fig. 3.4 B and 3.5 B) the KREBS buffer was removed and the time course started (120 scans, every 5 s). The first image was collected without any KREBS containing ASP⁺ solution. This was because ASP⁺ binding is so fast that it was impossible to take an image quick enough with KREBS containing ASP⁺ solution without detecting any ASP⁺ intensity. Immediately after the first image was collected, 1 mL of 10 μ M ASP⁺ at 37°C was added to the cells before the next image was taken. Exactly 300 s after the ASP⁺ had been added to the cells added, 10 μ L of the KOPr agonist (U50,488H, Sal A and DS-3-240), or vehicle control was carefully added to the glass bottom dish containing the visualized cells. Figures 3.4 and 3.5 illustrate this protocol and the time intervals at 0 s (Fig. 3.4 C and 3.5 C), 1 min (Fig. 3.4 D and 3.5 D), 5 min (Fig. 3.4 E and 3.5 E) and 10 min (Fig. 3.4 F and 3.5 F) have been chosen to illustrate the increasing red intensity of ASP⁺.

Fig. 3.5. Time course of ASP⁺ uptake in HEK-293 cells transiently transfected with eGFP-hSERT and myc-rKOPr. Transiently transfected cells were visualised by the confocal microscope to focus on cells expressing eGFP- hSERT (A) cells that consisted of a DIC image (B) of an appropriate monolayer of cells. The ASP⁺ uptake at 0 s (C), 1 min (D), 5 mins (E) and 10 mins (F). The pink arrow indicated a non-transfected cell, and the red arrow indicates a transfected cell.

Fig. 3.6. Time course of ASP⁺ uptake in HEK-293 cells transiently transfected with eGFP-hSERT and myc-rKOPr. Transiently transfected cells were visualised by the confocal microscope to focus on cells expressing eGFP- hSERT (A) cells that consisted of a DIC image (B) of an appropriate monolayer of cells. The ASP⁺ uptake at 0 s (C), 1 min (D), 5 mins (E) and 10 mins (F). The pink arrow indicated a non-transfected cell, and the red arrow indicates a transfected cell

3.4 Discussion

The aim of this chapter was to use confocal microscopy to measure ASP⁺ uptake in HEK-293 cells co-expressing eGFP-hSERT and myc-rKOPr to elucidate if KOPr activation modulates SERT function.

3.4.1 ASP⁺ Uptake Method Validation

Preliminary ASP⁺ uptake experiments were performed to confirm the sensitivity of the ASP⁺ uptake assay. From these preliminary experiments we found ASP⁺ uptake AFU at 300 s versus eGFP-hSERT AFU at 0 s resulted in an overall positive correlation ($r^2=0.58$). This illustrates that as eGFP-hSERT expression increases ASP⁺ uptake also increases, which indicates ASP⁺ uptake occurs through by SERT mediated mechanism. The difference in ASP⁺ accumulation between eGFP-hSERT and myc-rKOPr transiently transfected HEK-293 cells compared to non-transfected HEK-293 cells and also eGFP of less than 200,000 AFU (Figure 3.3) was statistically significant ($***p<0.001$). This suggests that the successful transfection of eGFP-hSERT is required for any ASP⁺ AFU. Again, this illustrates that as eGFP-hSERT expression increases ASP⁺ uptake also increases. Furthermore, co-expressing cells were preincubation for 5 min with fluoxetine (1.5 mM; $*p<0.05$) which significantly decreased SERT function and ASP⁺ AFU. Lastly, there was a significant difference in the fluoxetine pre-treated condition when compared against non-transfected or classified non-transfected HEK-293 cells ($**p<0.01$). Collectively, these findings illustrate the eGFP-tagged hSERT proteins are functional, and this ASP⁺ uptake real-time live cell assay is working appropriately, which means we can continue with the main experiment.

3.4.2 Effect of Acute KOPr Agonist on SERT Function

This confocal microscopy experiment illustrates that the KOPr compounds tested did not modulate SERT function in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT. Thus, acute (5 mins) KOPr agonists (10 μ M; U50,488H, Sal A and DS-3-240) exposure compared to the DMSO vehicle matched control ($p<0.099$) had no effect on SERT function. To confirm the absense of a KOPr agonist effect on SERT function we illustrated that SERT was functional. This was achieved with the inhibition of SERT by the SERT inhibitor, fluoxetine (5 min preincubation of 1.5 mM), which significantly decreased SERT function as analysed by a one way ANOVA followed with a Tukey post test ($***p<0.001$). In conclusion, the KOPr agonists (10 μ M; U50,488H, Sal A and DS-3-240) do not modulate SERT function in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT.

These findings are difficult to compare and contrast with the available research because there is currently no published literature on the effect of KOPr agonists on SERT function in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT by confocal microscopy. However, these results found here with confocal microscopy were replicated with RDEV (see Chapter 3). Interestingly, previous studies from our laboratory by Morani (unpublished data) suggest a difference in SERT function in HEK-293 cell model co-expressing myc-rKOPr and eGFP-tagged SERT (not inhibited by D-22) when incubated with the KOPr agonists U50,488H (10 μ M) but not Sal A (10 μ M) for 30 minutes. Perhaps this discrepancy in

SERT function reflects experimental differences between the two studies. Differences such as incubation time 5 min vs. 30 min.

The current study investigates the KOPr agonist pre-treatment time of 5 min, whereas Morani (unpublished data) investigates a pre-treatment of time of 30 min. If both studies hold true and SERT function is modified with a KOPr agonists 30 min pre-treatment, but not 5 min, then KOPr activation decreases SERT uptake by either, different pathways being activated (one at 5 min that does not modulate SERT and one at 15-30 min which modulates SERT) or a biphasic activation of one pathway (one phase at 5 min that does not modulate SERT and one at 15-30 min which modulates SERT). These hypotheses are discussed in more depth in chapter 6, but briefly it suggests that perhaps different pathways, as hypothesised by Bruchas and Chavkin's (2010; figure 1.3). For example, PKC and ERK1/2 pathways transduce with shortterm KOPr activation, whereas p38 and ERK1/2 pathways transduce with longterm KOPr activation. Or perhaps one single pathway activates a shortterm (acute) and a longterm (chronic) effect where only the latter modulates SERT function. When activated this one pathway has a biphasic signal transduction process where the first signal transduction occurs at 5 min (acute) and the second phase other at 30 min, such as PKC or ERK1/2. It is also entirely possible that both of these explanations are correct if ERK1/2 is involved in decreasing SERT function.

Thus, the absense of an effect between acute (5 min) KOPr activation and SERT function in this experiment does not mean that this interaction does not exist. Rather, it simply implies that the relationship may be more complex than previously thought

and may require additional research. Collectively, these findings suggest either a more complex cell model is required, such as primary cell cultures that has endogenous systems present, or that there simply is no KOPr and SERT relationship in the acute (5 min) model.

From this first experiment a particularly interesting observation was discovered. It was observed that ASP⁺ uptake occurs in non-transfected cells and cells classified as non-transfected (Figure 3.3). This uptake reflects the presence of low affinity monoamine transporters, such as extraneuronal monoamine transporter (EMT), the plasmalemmal monoamine transporter (PMAT), and the organic cation transporters -OCT1, OCT2, and OCT3 in this model. These proteins are known to take up small amounts of monoamine neurotransmitters, yet they remain relatively unstudied (Hagen *et al.*, 2011), although it was recently shown by Mayer *et al.* (2012) that OCT3 is present in HEK-293 cells as shown by real-time uptake of fluorescent ASP⁺. Perhaps our findings that low affinity transporters, perhaps just OCT3, take up ASP⁺ (and thus 5-HT) highlights the overlap of 5-HT and other monoamine uptake into neurons by alternative low affinity transporter proteins. These findings suggest not only that low affinity monoamine transporters are likely to be involved in the same diseases and disorders that other monoamine transporters are implicated in, but also that further experimental analysis in the future should also investigate the effect of these low affinity transporters by using their respective inhibitors to conclusively suggest a new hypothesis. Collectively, these findings highlights how important these less understood low affinity transporters are in modulating the overall signalling of 5-HT and other monoamines.

Chapter 4: The Effect of Acute (5 min) KOPr Agonist Exposure on Modulating SERT Function in HEK-293 Cells using Rotating Disc Electrode Voltammetry

4.1 Introduction

Rotating disc electrode voltammetry (RDEV) is recently developed electrochemical technique to investigate SERT function (Earles and Schenk, 1998). RDEV is an experimental technique that measures the monoamine transporter kinetic parameters, $V_{\max}$ to reflect transporter uptake and K_m to reflect transporter release, in different biological models, such as tissue samples and overexpressing cell cultures. This technique was originally developed to measure the uptake and release of the norepinephrine transporter (NET) in cell and tissue models (Burnette *et al.*, 1996; Chen *et al.*, 1999; Earles and Schenk, 1998). More recently it has been used to measure the real-time uptake and release of 5-HT in tissue samples, such as rat brain synaptosomes (Hagan *et al.*, 2010; Hagan *et al.*, 2011b).

RDEV (Figure 4.1) measures transporter uptake and release through a rotating submerged glassy carbon rotating electrode. This electrode draws the neurotransmitter molecules towards its electrode surface where a constant voltage is maintained and all of the monoamines present in the chamber oxidise and release electrons that produce current, which is measured (Earles and Schenk, 1998; Hagan *et al.*, 2010). The magnitude of this electron current is directly proportional to the

amount of free monoamine molecules in the sample. Therefore, as free monoamine molecules in the solution are transported into cells through a monoamine transporter the current changes accordingly, because there is a decrease in oxidising monoamine molecules in the surrounding sample solution (Earles and Schenk, 1998). The measured current is then calculated to provide the monoamine transporter uptake ($V_{\max}$) and release (K_m) kinetics.

Fig. 4.1. A systematic diagram of RDEV. RDEV consists of a rotating glassy carbon electrode connected to a high-precision rotator set and maintained to a constant 2000 rpm using a digital laser tachometer. The sample of interest sits in a chamber that has an Ag/AgCl reference electrode and a platinum auxiliary electrode submerged into the sample. Voltage is applied to oxidise the monoamine molecules and the resulting current is recorded and used to collect and interpret the transporter kinetics.

The magnitude of current measured at the electrode surface can be defined by the Levich equation:

$$I_L = 0.62nFACD^{2/3}\nu^{-1/6}\omega^{1/2}$$

Where I_L is the limiting current, n is the number of electrons transferred per mole, F is Faraday's constant (96,485 coulombs/equivalent of electrons), A is the area of the electrode in cm^2 , D is the diffusion coefficient of the electroactive species in cm^2/sec , C is the concentration of the electroactive species in the bulk solution in mM, ν is the kinematic viscosity of the solution in cm^2/sec , ω is the angular velocity of rotation in rad/sec , where $\omega = 2\pi N$, and N is the number of rotations per second (Earles and Schenk, 1998). This equation defines the current that is measured at the solid electrode surface.

RDEV is a simple, cost effective technique to establish and provides detailed information about Michaelis-Menten kinetics (Earles and Schenk, 1998), drug effects (Earles and Schenk, 1999; Thompson *et al.*, 2000), ion dependency of transporters (Bjorklund *et al.*, 2007; Chen *et al.*, 1999), and binding information (Chen *et al.*, 1999; Earles and Schenk, 1999). Two methods of RDEV can determine the kinetic parameters of monoamine transporters. The first method is known as the *zero trans* model and involves the single addition of the monoamine neurotransmitter added to a single tissue or cell sample. This is repeated over increasing neurotransmitter concentrations, with each new concentration added to a fresh sample preparation. The second RDEV method is the *low to infinite trans* model, which involves the increasing

neurotransmitter concentrations to be sequentially added, from lowest to the highest, to one sample within the chamber and allowing the nA to reach baseline between each new concentration addition. This method gives comparable results to single addition studies (Povlock and Schenk, 1997), without having to use numerous samples.

Currently, there is insufficient RDEV literature measuring SERT function (Hagan *et al.*, 2010; 2011a; 2012; Holley, unpublished data; Simonson, unpublished data). Hagan *et al.* (2010) were the first to publish peer-reviewed literature showing that RDEV can sensitively measure SERT kinetics in tissue samples. Hagan *et al.* (2010) observed a difference in 5-HT uptake between three different 5-HTTLPR genotypes in mice that was not found with less sensitive 5-HT uptake techniques, such as radioactive uptake (Bengel *et al.*, 1998). Using whole brain synaptosomes Hagen *et al.* (2010) are the first to illustrate that it is possible to measure SERT kinetics using RDEV. They observed that wild type mice have an increased 5-HT uptake when compared to heterozygous or homozygous SERT knockout mice and this increase was attenuated with the SSRI, paroxetine hydrochloride (Hagan *et al.*, 2010). Recently, Hagen *et al.* (2012) further highlight the critical use of the low affinity monoamine transporter inhibitor, D-22 (1.5 mM), when investigating monoamine transporter function because these transporters are endogenously expressed in cells and uptake monoamine transmitters at high concentrations. Thus, experimentation without inhibiting the low affinity transporters could result in confounding (Hagen *et al.*, 2012). At present there is limited peer-reviewed literature investigating SERT function

in cultured cells as measured by RDEV and therefore, much of this protocol is based on a recently published PhD thesis completed in our laboratory (Simonson, unpublished data), past literature (Hagan *et al.*, 2010; 2011a; 2012; Holley, unpublished data) and experimental development in this study within the confines of these previous studies.

4.1.1 Aim

In this chapter we aim to use RDEV to measure transporter uptake ($V_{\max}$) and release (K_m) in HEK-293 cells transiently transfected with eGFP-hSERT and myc-rKOPr to determine if KOPr activation modulates SERT function.

4.2 Methods

4.2.1 Experimental Procedure

RDEV consists of a rotating glassy carbon electrode (Pine Instruments, special order AFMDO3GC, Pennsylvania) that is connected to a high-precision MSR rotator (Pine Instruments) and set to 2000 rpm using a digital laser tachometer. The rate of rotation is maintained and recorded to keep the sample or buffer in the chamber mixing at a custom speed that gives continuous and consistent readings throughout the experiment. The sample is mixed in the chamber with a constant air flow of 5% CO₂/95% with O₂ gently and constantly flowing across the sample solution, while simultaneously being heated to 37°C. The chamber also has two electrodes attached and submerged into the sample. An Ag/AgCl reference electrode is used to apply a +450 mV potential (relative to the electrode) by an eDAQ potentiostat EA161 (eDAQ,

NSW, Australia) and a piece of platinum is used as an auxiliary electrode. Voltage is applied and resulting current was recorded by an eDAQ recorder 2010 (eDAQ) and a PC computer with Chart software (eDAQ) was used to collect and interpret the recordings.

4.2.2 Standard Curve

A standard concentration curve was created at the beginning of each experimental session. 5-HT HCl was dissolved and diluted in 1x PBS (pH 7.2) to decrease oxidation (Chen *et al.*, 1999). A linear regression was calculated using the increase in current (nA) at each 5-HT concentration (0.125, 0.25, 0.5, 0.75, 1.0 and 1.25 μ M). These 5-HT concentrations were chosen because the current literature suggests they are the most reliable to use when measuring SERT function (Hagan *et al.*, 2010; 2011a; 2012; Holley, unpublished data; Simonson, unpublished data). Specifically, Hagan *et al.* (2010) highlight that concentrations above 1.0 μ M cause 5-HT to spontaneously oxidise without the addition of a voltage to oxidise it. In this study, this finding was also seen in RDEV experiments as the linear line (see Fig. A3.1) after 1.0 μ M. This linear line reflects spontaneous oxidation of the 5-HT increase rather than a linear SERT function increase. The purpose of a standard curve of increasing 5-HT concentration allows data collected from the experimental cell samples to be transformed into μ M, based on the measured nA values and their corresponding μ M value from the standard curve as established by other protocols (Chefer and Shippenberg, 2002; Chen *et al.*, 1999). Each 5-HT HCl solution was made fresh for each

experimental session and used within 2 hours of preparation to prevent oxidation confounding effects (Meiergerd and Schenk, 1995).

4.2.3 Cell Preparation

HEK-293 cells were cultured and transiently transfected with myc-rKOPr and eGFP-hSERT plasmids (as described in Chapter 2) and used 48 hours after transfection when the cells were 70-90% confluent. In RDEV optimisation experiments 100 mm plastic bottom dishes with 3.5×10^6 cells in 7 mL were used but the increase in cell incubation time with D-22 in the chamber meant that fewer cells were required at any one time. So to minimise consumables 60 mm plastic bottom dishes were used with 1.5×10^6 cells in 3 mL instead. Additional cells were seeded in parallel for each transfection onto a glass bottom plate to allow the percentage of transfection to be determined by eGFP-hSERT expression using confocal microscopy.

For RDEV cells were rinsed twice with 37°C saline (138 mM NaCl, 4.1 mM KCl, 5.0 mM KH_2PO_4 , 2% w/v D-glucose, pH 7.3) (Appendix 1) and then harvested by mechanical agitation with 37°C KREBS buffer saturated with 5% CO_2 /95% O_2 . Cell suspensions were then centrifuged at 300 g for 5 min, re-suspended in 1 mL of 37°C KREBS buffer saturated with 5% CO_2 /95% O_2 , and stored in an incubator at 37°C until needed but for no longer than 30 min.

For 5-HT cell uptake assays, 294 μL of the cell suspension was added to the RDEV chamber, the rotator lowered and turned on, and the cells were incubated until

a constant baseline was reached. The low affinity monoamine transporter inhibitor D-22 (1.5 mM) was then added for a total of 9 min before any 5-HT addition. In the KOPr agonist pre-treatment condition KOPr agonists (500 nM) were incubated for 5 min before 5-HT addition. Control experiments were carried out as above instead either a vehicle (DMSO) matched solution or fluoxetine (1.5 mM) pre-treatment was added 5 min before 5-HT addition. After each experiment the a live count was determined 30 sec 5-HT addition, with the addition of 0.4% trypan blue and manually counted on a microscope using a haemocytometer.

4.2.4 Data Analysis

To measure the initial SERT uptake of the cells 30 seconds of uptake data was collected after the addition of the 5-HT concentration. Using Microsoft Office Excel linear regression was calculated from this data. Any linear regression with $r^2 < 0.95$ was not used because this suggested SERT function was not representative of linear uptake and therefore, not comparable. The concentration curve plotted before each experiment was used to convert the measured charge in nA into $\mu\text{M/s}$, and this value was adjusted or normalised to the number of alive cells counted. The final measurement of SERT function was expressed as $\text{pmol/s}/1 \times 10^6$ cells. For this *zero trans* model the values were then entered into GraphPad 5 software, and a Michaelis-Menten curve was fitted. The Michaelis-Menten curve was used because the Lineweaver-Burk (LWB) plot was widely used to determine important terms in enzyme kinetics, such as V_{max} and K_m , before the wide availability of powerful computers and non-linear regression software. Unfortunately, the double reciprocal

plot (LWB) distorts the error structure of the data, and it is therefore unreliable for the determination of enzyme kinetic parameters (Serap *et al.*, 2006). Although it is still used for representation of kinetic data (Serap *et al.*, 2006) because the y-intercept is equivalent to the inverse of $V_{\max}$, and the x-intercept of the graph represents $-1/K_m$. This also gives a quick, visual impression of the different forms of enzyme inhibition, such as competitive or non-competitive (Lineweaver and Burk, 1934) but is of little use when simply measuring precise kinetic parameters of one protein with no enzymatic activity. However, non-linear regression or alternative linear forms of the Michaelis-Menten equation are generally used for the calculation of precise parameters, such as $V_{\max}$ and K_m (Greco and Hakala, 1979). The $V_{\max}$ and K_m of each individual sample was calculated and the average and SEM of these was computed. Student's *t*-tests were performed for each treatment group on $V_{\max}$ and K_m values to determine whether significant differences were attained. Significance was defined as $p < 0.05$

4.3 Results

RDEV experiments were conducted using transiently transfected HEK-293 cells that co-express eGFP-hSERT and myc-rKOPr in HEK-293 cells shown with a Cy5-myc-rKOPr antibody (Simonson, unpublished data).

4.3.1 Preliminary Experimental Procedure

Preliminary experiments highlight the need for the low affinity 5-HT transporter, D-22, for experimental samples to reach kinetic saturation (Figure 4.2 and Table 4.1). In addition, D-22 incubation time increased from 5 mins as suggested by

(Hagan *et al.*, 2010) to 9 min because this is when saturation was seen (Figure 4.3 and Table 4.2).

Fig. 4.2. The effect of acute (5 min) KOPr agonists on 5-HT uptake by SERT in transiently transfected HEK-293 cells in the absence of D-22 preincubation. Transiently transfected cells were pre-treated with the classic KOPr agonists U50 488H, Sal A, the novel KOPr agonist DS-3-240, the vehicle (DMSO) for 5 min, in the absence of D-22. A total of 9 min D-22 preincubation was also used as a comparison. Data points shown are average $\pm$ SEM ($n = 8-12$).

Table 4.1. D-22 inhibition.

Treatment	Vmax (pmol/s/10 ⁶ cells)	Vmax SEM	Km	Km SEM
D-22	10.80	5.20	0.71	0.53
U50	1.49 x 10 ²³ ***	1.32 x 10 ¹³ ***	2.10 x 10 ²² ***	1.86 x 10 ¹² ***
Sal A	2.21 x 10 ²³ ***	1.86 x 10 ¹² ***	3.33 x 10 ²² ***	3.49 x 10 ¹² ***
DS-3-240	1.81 x 10 ²⁴ ***	2.92 x 10 ¹³ ***	2.18 x 10 ²² ***	3.52 x 10 ¹² ***
Fluoxetine	1.87 x 10 ²⁶ ***	5.50 x 10 ¹⁶ ***	4.01 x 10 ²⁵ ***	1.18 x 10 ¹⁶ ***
DMSO	1.13 x 10 ²⁵ ***	4.55 x 10 ¹⁴ ***	2.21 x 10 ²⁴ ***	8.89 x 10 ¹³ ***

*** $p < 0.001$ compared to control $V_{\max}$ and K_m

Fig. 4.3. The effect of different D-22 incubation periods on 5-HT uptake by SERT in transiently transfected HEK-293 cells. Transiently transfected cells were pre-treated D-22 for 5 min, 7 min and 9 min. Data points shown are average $\pm$ SEM ($n = 4-6$).

Table 4.2. Uptake kinetics of SERT in transiently transfected HEK-293 cells with with different D-22 preincubation increments.

Treatment	V _{max} (pmol/s/10 ⁶ cells)	V _{max} SEM	K _m	K _m SEM
D-22 (5 min)	4.9 x 10 ²⁴ ***	2.29 x 10 ¹⁴ ***	1.2 x 10 ²⁴ ***	5.56 x 10 ¹³ ***
D-22 (7 min)	524.3	108.2	108.8	21.45
D-22 (9 min)	14.58	0.77	2.48	0.27

*** $p < 0.001$ compared to control V_{max} and K_m

4.3.2 Effect of Acute KOPr Agonist on SERT Function

To investigate the effect of KOPr agonists on modulating SERT it was necessary to incubate cell suspensions with the low affinity transporter inhibitor, D-22 (1.5 mM), for 4 min to block any low affinity transporters endogenously expressed in HEK-293 cells. The cell suspension was then incubated with a test KOPr agonist compound (500 nM) for 5 min before 5-HT addition at the appropriate concentration. Results showed (Figure 4.4 and Table 4.3) that none of the KOPr agonists investigated

had an acute (5 min) effect on SERT function when compared with the DMSO vehicle matched control. These results were statistically analysed using a Student *t*-test and the $V_{\max}$ values were U50,488H ($p < 0.4665$), Sal A ($p < 0.3054$), and DS-3-240 ($p < 0.1242$) and the K_m values were U50,488H ($p < 0.8525$), Sal A ($p < 0.7467$), and DS-3-240 ($p < 0.1346$). The selective 5-HT reuptake inhibitor, fluoxetine, significantly decreased SERT uptake, $V_{\max}$, ($***p < 0.001$) and binding affinity, K_m , ($***p < 0.001$). This finding was confirmation that SERT is functional. Thus, we can conclusively say that SERT is functional and the absence of an observed effect by KOPr agonists on SERT function cannot be because of a dysfunctional SERT protein.

Fig. 4.4. The effect of acute (5 min) KOPr agonists on SERT function in transiently transfected HEK-293 cells in with 9 min D-22 preincubation. Transiently transfected cells were preincubated with D-22 (9 min) and then pre-treated with the classic KOPr agonists U50,488H, Sal A, the novel KOPr agonist DS-3-240, or the vehicle (DMSO) for 4 min, as well as the fluoxetine pre-treatment (5 min). All cells were pre-incubated with D-22 for a total of 9 min. Data points shown are average $\pm$ SEM ($n = 8-12$).

Table 4.3. Uptake kinetics of SERT in transiently transfected HEK-293 cells with acute (5 min) exposure to KOPr agonists and a total of 9 min D-22 preincubation.

Treatment	V _{max} (pmol/s/10 ⁶ cells)	V _{max} SEM	K _m	K _m SEM
D-22	14.47	1.89	0.81	0.21
D-22 + U50	24.14	12.33	2.068	1.526
D-22 + Sal A	27.12	17.14	2.318	2.051
D-22 + DS-3-240	18.71	7.285	1.131	0.7504
D-22 + DMSO	15.85	2.875	0.8143	0.2942
D-22 + Fluoxetine	5.45 × 10 ³⁵ ***	1.09 × 10 ²⁴ ***	8.52 × 10 ³⁴ ***	1.71 × 10 ²³ ***

*** $p < 0.001$ compared to control V_{max} and K_m

The control samples, D-22 and the DMSO vehicle matched control, show a significant difference between the two (Figure 4.5 and Table 4.4), V_{max} (** $p < 0.001$) and K_m (** $p < 0.001$). This confirms that D-22 has the ability to block other 5-HT uptake transporters in these HEK-293 cells. The inhibition of low affinity 5-HT transporters means only 5-HT is taken up by SERT at a rate that fits the Michaelis-Menten enzyme kinetics, up to 1.25 μ M where saturation is observed (Figure 4.5). The 5-HT uptake saturation provides a V_{max} and K_m. The linear line of the DMSO vehicle control reflects the increasing concentration of 5-HT increments that are oxidising and creating a current.

Fig. 4.5 The effect of 9 min D-22 preincubation versus DMSO matched controls on SERT function in HEK-293 cells. Transiently transfected cells were either pre-incubated with D-22 or the vehicle (DMSO) for 9 min. Data points shown are average $\pm$ SEM ($n = 8-12$).

Table 4.4. Uptake kinetics of SERT in HEK-293 cells in 9 min D-22 preincubation versus DMSO matched controls.

Treatment	V _{max} (pmol/s/10 ⁶ cells)	V _{max} SEM	K _m	K _m SEM
D-22	14.47	1.89	0.81	0.21
DMSO	3.91 x 10 ³² ***	7.76 x 10 ²³ ***	4.32 x 10 ³¹ ***	8.57 x 10 ²² ***

*** $p < 0.001$ compared to control V_{max} and K_m

Simultaneous inhibition of both SERT with the SSRI, fluoxetine, and the low affinity SERT transporters, by D-22, resulted in a much higher V_{max} (** $p < 0.001$) and K_m (** $p < 0.001$) than with just D-22 or fluoxetine alone (Figure 4.6 and Table 4.5). Again, when the D-22 alone or D-22 + DMSO condition values are compared with DMSO, DMSO (D-22) + DMSO (Fluox), D-22 + Fluox, or Fluox condition values the V_{max} (** $p < 0.001$) and K_m (** $p < 0.001$) are significantly different. Again, this highlights the

importance of D-22 when using Michaelis-Menten kinetics to measure monoamine transporter function.

Fig. 4.6. The effect of matched controls on SERT function in transiently transfected HEK-293 cells. Transiently transfected cells were pre-incubated with either D-22 (9 min), the matched vehicle (DMSO) control or fluoxetine for 5 min. All cells were treated with their control for the respective time and cells from all the groups were in the chamber for a total of 9 min before 5-HT was added. Data points shown are average $\pm$ SEM ($n = 8-12$).

Table 4.5. Uptake kinetics of SERT in transiently transfected HEK-293 cells in D-22 (9 min) versus fluoxetine with matched controls.

Treatment	Vmax (pmol/s/10 ⁶ cells)	Vmax SEM	Km	Km SEM
D-22	14.47	1.89	0.81	0.21
DMSO	3.91×10^{32} ***	7.76×10^{23} ***	4.32×10^{31} ***	8.57×10^{22} **
D-22 + Fluoxetine	5.45×10^{35} ***	1.09×10^{24} ***	8.52×10^{24} ***	1.71×10^{22} ***
D-22 + DMSO	15.85	2.875	0.8143	0.2942
DMSO (D-22) + DMSO (Fluox)	3.36×10^{26} ***	2.93×10^{16} ***	3.76×10^{25} ***	3.28×10^{15} ***
Fluoxetine	2.44×10^{29} ***	1.09×10^{24} ***	1.09×10^{28} ***	4.56×10^{23} ***

*** $p < 0.001$ compared to control $V_{\max}$ and K_m

4.4 Discussion

The aim of this chapter was to use RDEV to measure 5-HT transporter uptake ($V_{\max}$) and binding affinity (K_m) in HEK-293 cells co-expressing eGFP-hSERT and myc-rKOPr to determine if KOPr activation modulates SERT function.

4.4.1 Preliminary Experimental Procedure

The initial experimental results illustrate that it is impossible to obtain SERT transporter saturation without the presence of the low affinity transporter inhibitor, D-22 (1.5 mM; Figure 4.2 and Table 4.1). Also, it was found that in this overexpressing cell model the incubation time of D-22 needs to be increased from 5 min, as suggested by Hagan *et al.* (2010; 2011), to 9 min because this is when SERT saturation occurs and thus, this is when the low affinity transporters are completely inhibited (Figure 4.3 and Table 4.2). This increase in D-22 incubation time suggests that HEK-293 cells have more low affinity transporters present than in the rat synaptosome model (Hagan *et al.*, 2010). The D-22 concentration (1.5 mM) was not adjusted because it was desired to have comparable results with Hagan *et al.* (2011) because no other RDEV model has used D-22, let alone a cell model, and adjusting the concentration would add more variables to compare in the results.

4.4.2 Effect of KOPr Agonists on SERT Function

In this HEK-293 cell model co-expressing myc-rKOPr and eGFP-tagged SERT, we found none of the KOPr compounds tested had a modulatory effect on SERT function as measured with RDEV. SERT function as indicated by the kinetic parameters, $V_{\max}$ (transporter uptake) and K_m (transporter binding affinity), was not

modulated with acute (5 min) KOPr agonist exposure when compared to the DMSO matched control, $V_{\max}$ (U50,488H ($p<0.4665$), Sal A ($p<0.3054$), and DS-3-240 ($p<0.1242$)) and K_m (U50,488H ($p<0.8525$), Sal A ($p<0.7467$), and DS-3-240 ($p<0.1346$)) were non-significant. The absence of a KOPr agonist modulatory effect on SERT function was reassured to be correct by further experiments to identify that SERT was functioning correctly. SERT function was investigated with the preincubation of transiently transfected HEK-293 cells with the SSRI, fluoxetine (1.5 mM), for 5 min. Results show a significant decrease in SERT $V_{\max}$ (** $p<0.001$) and K_m (** $p<0.001$) with a fluoxetine (1.5 mM) pre-treatment of 5 min when compared to the vehicle matched control DMSO. These RDEV findings suggest that in this HEK-293 cell model co-expressing myc-rKOPr and eGFP-tagged SERT we found no presence of an acute (5 min) KOPr activation effect by the KOPr agonists (U50,488H, Sal A, and DS-3-240) on SERT function as indicated by the RDEV kinetic parameters, $V_{\max}$ and K_m .

These findings are difficult to compare and contrast with the current literature because research into the effect of KOPr agonists on SERT function in HEK-293 cells co-expressing myc-rKOPr and eGFP-tagged SERT inhibited with D-22 by RDEV has never been published. In addition, an incubation time of 5 min has never been investigated by our laboratory either. However, the results found here with RDEV was replicated by confocal microscopy (see Chapter 3). Interestingly, previous studies from our laboratory by Simonson (unpublished data) suggest a difference in SERT function in HEK-293 cell model co-expressing myc-rKOPr and eGFP-tagged SERT (not inhibited by D-22) when incubated with the KOPr agonists U50,488H (500 nM), Sal A (500 nM) (and DS-1-216, 500 nM) for 15 minutes. Perhaps the discrepancy in

SERT function reflects experimental differences between the two studies. Differences such as incubation time 5 min vs. 15 min, D-22 present vs. no D-22, HEK-293 co-expressing cells vs. rat striatal tissue, or maximum 5-HT concentrations for enzyme kinetics, 1.25 μ M vs 3 μ M.

The current study investigates the KOPr agonist pre-treatment time of 5 min, whereas Simonson (unpublished data) investigated a pre-treatment of time of 15 min. Interestingly, Simonson also did not find a SERT decrease by the KOPr agonists U50,488H (500 nM), Sal A (500 nM) and DS-1-216 (500 nM) at the time intervals of 4 or 10 min, but there was a decrease at 15 min. If both studies are factual (irrespective of if Simonson had used D-22) and SERT function is modified by KOPr agonists somewhere between 5 min and 15 min exposure time it is possible this is due to either different pathways being activated, or a biphasic activation of one pathway. These hypotheses are discussed in more depth in chapter 6, but briefly it suggests that Bruchas and Chavkin's (2010; figure 1.3) hypothesis of different KOPr pathways are activated depending on time, and thus intensity. This means that one pathway is activated at 5 min (acute), which involves ERK1/2 and PKC, the other at 15 min (chronic), which involves ERK1/2 and p38. Another explanation for the difference in results found between 5 min and 15 min pre-treatment could be because the main pathway responsible is biphasic, such as is the case with PKC. It is also possible that both of these explanations are correct because both p38 and PKC are known to decrease SERT function (Ramamoorthy *et al.*, 2011).

Another interesting variable between the two studies investigating KOPr function on SERT function measured with RDEV is that Simonson (unpublished data) did not use the low affinity transporter inhibitor, D-22. This is important to note because in the current study it was impossible to obtain realistic (if any) SERT transporter function enzyme kinetic data without D-22 preincubation. Hagen *et al.* (2010; 2011) also state the importance of using a low affinity transporter inhibitor when measuring SERT transporter function. Simonson (unpublished) also failed to show any decreases of SERT function by KOPr activation at 4 min and 10 min and it is possible that these results may also complement the current study. However, because D-22 was not used in the comparison study, any real differences between the two studies should be made with caution.

Different preclinical models also offer another explanation for discrepancies between the current study and Simonson's (unpublished) study. In the current study we used cultured cells and Simonson (unpublished) used 4-5 rat striatal tissue samples. Or perhaps a possible reason for the difference is simply that the cell model lacks the additional endogenous biochemical pathways that are necessary for KOPr activation to modulate SERT function.

Lastly, it has been suggested that the use of 5-HT concentrations above 1 μM are too high for use in RDEV to measure SERT function (Hagan *et al.*, 2010). Hagen *et al.* (2010) state that 5-HT is unstable at concentrations above 1 μM because it oxidises in solution so rapidly, which makes measuring free 5-HT difficult and inconsistent. This makes 5-HT oxidation difficult to differentiate between an modulation in SERT

function or just the oxidation of 5-HT in solution because of high 5-HT concentrations. In this study, it was found that a maximum 5-HT concentration of 1.25 μM was necessary to saturate SERT because 1 μM did not show kinetic saturation as measured by the kinetic parameters V_{max} and K_m . A 5-HT concentration higher than 1.25 μM , in the optimisation experiments, resulted in the SERT function saturation increasing again and produce the same linear increase of 5-HT uptake that is illustrated by Hagan *et al.* (2010; Appendix 3). This was suggested by Hagan *et al.*, (2010) to be indicative of 5-HT oxidation, rather than uptake and this explanation is accepted here as a possible reason into why Simonson's data (unpublished data) differed from our finding. Collectively, It would have been interesting to have investigated the effect of a KOPr agonist preincubation time of 15 min to definitively justify these explanations for result differences.

However, these findings and possible suggestions should be interpreted with caution because this experiment is one cell model of many possible preclinical models. Thus, it would be interesting to further investigate additional cellular models, such as primary cell cultures, which endogenously express other neurotransmitter systems that may act in a synergistic manner to facilitate the KOPr and SERT relationship. Animal models and different brain regions would also be very interesting to add to this literature. Finally, additional studies by RDEV will also help to further elucidate the interaction between KOPr and SERT.

Chapter 5: Developing a Potential High-Throughput Screening Protocol to Elucidate the Effect of Chronic (30 min) KOPr Agonist Exposure on Modifying SERT Function

5.1 Introduction

Neurotransmitter transporters, such as SERT, DAT and NET, represent established pharmaceutical targets because they play an essential role in regulating monoamines that play a role in many neurological diseases and disorders. Specifically, SERT has been implicated in the role of brain development, neural plasticity, synaptogenesis, cell proliferation, and emotional disorders such as depression (Owens and Nemeroff, 1994), anxiety (Suranyi-Cadotte *et al.*, 1990), bipolar disorder (Bellivier *et al.*, 1997) and addiction to psychostimulants (Baumann and Rothman, 1998; Heinz *et al.*, 2001). Thus, the ability to monitor neurotransmitter uptake is key to a better understanding of these diseases.

Currently, researchers remain relatively limited with transporter assays that provide cost effective, rapid and reliable data. An assay with these qualities would allow for high-throughput screening for potential SERT modifying compounds, particularly those that modulate mood, stress, learning and memory. Recent introduction of novel transporter assay kits provide researchers with the tools to

screen live-cell kinetic uptake for key neurotransmitters in a homogeneous, fluorescence-based, and high-throughput format. This means that temporally limited techniques, such as radioactive labeled compounds, are being replaced with kinetic assays suitable for mechanistic studies, or as a high throughput screening with readable endpoints. This is possible because these neurotransmitter transporter assay kits use fluorescent substrates that mimic the biogenic amine neurotransmitters and when taken into the cell through the transporter it results in an increase of intracellular fluorescence intensity that can be monitored in real-time using a bottom-reading microplate reader. In this convenient procedure, cells can be incubated with a compound of interest and transferred into the plate reader for evaluation with a fluorescent reagent.

A high-throughput screening assay to identify SERT modifying compounds, such as this, requires a uniform cell population for consistent replication across relatively similar samples. The development of stable cell lines can be a difficult procedure to perform given that there are so many potential variables. This process requires transfection with the transporter plasmid of interest and then growth in media substituted with an antibiotic to render any cell with the plasmid immune from the appropriate antibiotic (Schwartz *et al.*, 2005). Immune cells are assumed to contain the plasmid of interest, then they are isolated to a single cell in the hope that this cell will divide to produce an identical population. This constant population is the sample population to screen the potential SERT modifying compounds, as is presumed that each cell has similar SERT cell surface expression. The development of a high-throughput assay to monitor SERT function is required for the prompt development

of SERT modifying pharmacotherapeutics.

5.1.1 Aims

The aim of this chapter is to develop a high-throughput screening assay to investigate the effects of chronic (30 min) KOPr agonist exposure on SERT function in HEK-293 cells transiently expressing myc-rKOPr and eGFP-tagged hSERT.

5.2 Methods

5.2.1 Cell Preparation

To investigate the chronic (30 min) effect of KOPr agonists on SERT function, HEK-293 cells were cultured and transiently transfected with myc-rKOPr and eGFP-hSERT plasmids as described in the general methods section (Chapter 2) and used 48 hours after transfection when the cells were 70-90% confluent. Preliminary experiments required cells to be plated onto 100 mm plastic bottom dishes with 3.5×10^6 cells in 7 mL, but for the final experimental procedure, 60 mm plastic bottom dishes were used instead with 1.5×10^6 cells in 3 mL.

5.2.2 Experimental Procedure

Cells were rinsed twice with 37°C saline (138 mM NaCl, 4.1 mM KCl, 5.0 mM KH_2PO_4 , 2% w/v D-glucose, pH 7.3) (Appendix 1) and then harvested by mechanical agitation with 37°C KREBS buffer saturated with 5% CO_2 /95% O_2 . The cell suspension cell count was then determined with the addition of 0.4% trypan blue and manually counted using a haemocytometer. Cells were subsequently diluted to the required concentration, 2×10^5 cells/mL, with 37°C 5% CO_2 /95% O_2 saturated KREBS buffer. 200

μL of this cell solution was placed into each well of a 96-well plate (Invitrogen) using a multipipettor with 10 μM of the KOPr agonist pre-treatment and loaded into the plate reader at 37°C. After 30 min, ASP⁺ (3 μM) was added and re-loaded into the plate reader and fluorescence was read after 5 min. Control experiments were carried out using a vehicle (DMSO) matched solution, fluoxetine (1.5 mM) pre-treatment and cells transiently transfected with myc-rKOPr and eYFP-hDAT plasmids. Non-expressing HEK-293 cells served as a negative control to determine non-specific ASP⁺ uptake by non-SERT mediated mechanisms. Each condition, along side the negative controls, was measured on at least 2 plates across at least 3 separate transfections.

Table 5.1. eYFP-hDAT Plasmid information

Gene	Vector	Resistance	Provided by
eYFP-hDAT	pciHyg	Ampicillin (100 μg/mL)	J. Javitch (Scholze <i>et al.</i> , 2000)

5.2.3 Establishing a Stable Cell Line Expressing eGFP-tagged hSERT

HEK-293 cells were cultured and transfected as described in the general methods section (Chapter 2), however these cells were transfected with 1.0 μl/mL eGFP- hSERT plasmid and then grown in PS free DMEM media substituted with Geneticin (G418) antibiotic as in (Schwartz *et al.*, 2005). Transfected cells were selected at 400 μg/mL G418 and maintained at 300 μg/mL so only transfected cells were grown. The reason a stable cell line was produced but not used in any of the previous experiments is because this stable cell line was developed at the same time as the

experiments were performed in the previous chapters. More time would be necessary for a stable transiently transfected cell line to be used in the earlier experiments.

Those that survived the selection process were subcultured in a maintenance process for eight weeks. Individual cell lines were cloned by limiting the dilution of cells. Briefly, selected and maintained cells were diluted to a concentration of 2.5 cells/mL and then plated out in a 96-well plate. Immediately following cell seeding each well was visualised under a fluorescent microscope to find each well with a single fluorescent cell. When these cells became 70-80% confluent they were mechanically removed and placed into a 24-well plate and the growth process was continued. This process was repeated into a 12-well plate and then into a 6-well plate. Cells were then grown in a small 30 cm² flask and then a 75cm² flask. At each passage step cells were visualised for eGFP expression using the confocal microscope to ensure they still contained the eGFP- hSERT plasmid. Cell lines that had even cell surface expression in 90% or more of the cells were deemed acceptable and allowed to continue growth. When cells became 70-90% confluent in the 75cm² flask they were passaged and frozen as in the general methods section for future use.

5.2.4 Data Analysis for Chronic ASP⁺ Uptake Studies

Data was exported from the plate reader to analyse. Values were entered onto an Excel spreadsheet and the average AFU was calculated for each condition. The average for each condition was then normalised to the average of the vehicle condition (ASP⁺ + Cells + Vehicle) and expressed as a percentage of change. This

percentage of change was then entered into GraphPad Prism and a repeated measures ANOVA followed by a Tukeys post test was performed.

5.3 Results

5.3.1 Fluorescence-Based SERT Activity Measurement

Fluorescence is when light is absorbed at one wavelength and emitted at a different, and longer, wavelength and thus, it was necessary to use fluorescence spectroscopy to optimise the excitation and emission wavelengths of ASP⁺ to ensure that only ASP⁺ uptake was being measured by the the 96-well plate reader (EnSpire® Multimode Plate Readerm 2300-001M, PerkinElmer, USA). It was found that ASP⁺ was excited at 447 nm and emitted at 600 nm by plate reader. In order to validate the novel fluorescence-based SERT assay a series of preliminary experiments using the plate reader were performed. The plate reader can read a 96-well plate simultaneously across a time period. This allows for fluorescence over time to assess the uptake of ASP⁺. Figure 5.1 represents the change in fluorescence for eGFP-hSERT transfected HEK-293 cells versus non-transfected HEK-293 cells for a total of 30 min at 60 s intervals as indicated by Δ RFU (relative fluorescent units). From this graph is can be observed that eGFP-hSERT transfected HEK-293 cells uptake ASP⁺ at a rate that is significantly different (** $p < 0.01$) from the control matched conditions of no cells, no ASP⁺, and fluoxetine (1.5 mM) pretreated. This is indicative that eGFP-hSERT is working as it should in this experiment and that the ASP⁺ fluorescent wavelengths are correct as only ASP⁺ uptake is being measured. Although parental HEK-293 cells display some ASP⁺ uptake the transfected cells have a significantly higher accumulation of ASP⁺, demonstrating that ASP⁺ uptake is highly specific over time

compared to non-transfected cells, yet, ASP⁺ is taken up by non-SERT mediated mechanisms. Also, the substrate uptake appears to be at a linear rate constant for at least 30 min.

Fig. 5.1. Time dependent uptake of ASP⁺ fluorescence in transiently transfected HEK-293 cells versus controls. eGFP-hSERT and myc-rKOPr co-expressing cells uptake ASP⁺ at a linear rate for a total of 30 min at 60 s intervals that is significantly different (** $p < 0.001$) from the controls. Monoamine transporter activity (i.e uptake of the fluorescent substrate) is depicted as relative fluorescence unit changes (Δ RFU). Data points are presented as mean $\pm$ SEM.

5.3.2 Uptake Inhibition by Re-uptake Inhibitors

To further investigate the use of ASP⁺ as a fluorescent substrate in this high-throughput assay, preliminary experiments were performed to ensure SERT function was being measured. To determine this uptake was inhibited by the re-uptake inhibitor, fluoxetine. As seen in Figure 5.2 it was illustrated that fluoxetine significantly decreased ASP⁺ uptake when compared to the vehicle matched DMSO control (** $p < 0.001$).

5.3.3 Effect of Chronic (30 min) KOPr Agonist on SERT Function

In addition to other preliminary experiments, a transiently transfected positive control was also performed. Chronic (30 min) KOPr agonist experiments were conducted in transiently transfected HEK-293 cells that have previously shown to co-express eGFP-hSERT and myc-rKOPr in HEK-293 cells using a Cy5-myc-rKOPr antibody (Simonson, unpublished data).

Fig. 5.2. Percentage changes of ASP⁺ uptake by eGFP-hSERT after chronic (30 mins) KOPr agonist exposure (10 µM), DMSO and fluoxetine pre-treatment (5 min). There is a significant difference in percentage change of ASP⁺ uptake between the DMSO and U50,488H (***p*<0.001) but not between DMSO and Sal A (***p*<0.001). Fluoxetine was statistically different from vehicle (***p*<0.001) and from U50,488H (***p*<0.01) but not from Sal A (*p*>0.743). As shown by a repeated measured ANOVA followed with a Tukey post test.

Fig. 5.3. *Percentage changes of ASP⁺ uptake by eYFP-hDAT after chronic (30 mins) KOPr agonist exposure (3 μM), and DMSO. There is a significant difference in percentage change of ASP⁺ uptake (3 μM) between the DMSO and U50,488H (***) as well as between DMSO and Sal A (***) as shown by a repeated measured ANOVA followed with a Tukey post test.*

5.3.4 Establishing a Stable Cell Line Expressing eGFP-tagged hSERT

The procedure was repeated twice, in the first attempt no cells had eGFP-hSERT expression. In the second attempt two subcultures expressed eGFP-hSERT. These were visualised under the confocal microscope and are referred to as ‘high eGFP-hSERT concentration’ (Figure 5.4) and ‘low eGFP-hSERT concentration’ (Figure 5.5). This cell line was frozen, unfrozen and then grown to assess the integrity each week for a total of 5 weeks and illustrated acceptable consistent integrity (Figure 5.6) because there was no obvious expulsion of the plasmid by the cell, which would

produce an image that had significantly less eGFP expression than the previous inspection.

Fig. 5.4. Stable SERT HEK-293 cell line with high eGFP-hSERT expression viewed using confocal microscopy. Different views after selection and 8 weeks of maintenance in G418 substituted media as visualised under the confocal microscope at 40X water immersion.

Fig. 5.5. Stable SERT HEK-293 cell line with low eGFP-hSERT expression viewed using confocal microscopy. Different views after selection and 8 weeks of maintenance in G418 substituted media as visualised under the confocal microscope at 40X water immersion.

Fig. 5.6. 5 weeks of growth of the stable SERT HEK-293 cell line with high eGFP-hSERT expression viewed using confocal microscopy. After freezing, unfreezing and growing for a total of 6 weeks. (A) at week 1, (B) at week 2, (C) at week 3, (D) at week 4, and (E) at week 5. As visualised under the confocal microscope at 40 x water immersion.

5.4 Discussion

The aim in this chapter was to develop a high-throughput screening assay to investigate the effects of chronic (30 min) KOPr agonist exposure on modulating SERT function in HEK-293 cells transiently expressing myc-rKOPr and eGFP-tagged hSERT. It was found that chronic (30 min) KOPr activation can directly modulate SERT function using this cell model and novel technique. This novel protocol provided a readily available screening tool for potential SERT modifying compounds. A stable SERT cell line was also developed to facilitate further screening of SERT modifying compounds using a uniform cell population.

5.4.1 Effect of Chronic (30 min) KOPr Agonist on SERT Function

Preliminary experiments provided excitation and emission wavelengths to provide data only indicative of ASP⁺ uptake as measured by arbitrary fluorescent units (AFU). This ASP⁺ fluorescence reflects SERT uptake, so any changes in ASP⁺ uptake (Figure 5.2) suggest SERT function differences. These findings reveal chronic (30 min) exposure to the KOPr agonists, U50,488H (10 μ M) and Sal A (10 μ M), significantly decrease SERT function (** p <0.01) as measured by ASP⁺ uptake assay using a 96-well plate reader. SERT functional integrity was confirmed with the 5 min exposure to the SSRI, fluoxetine (** p <0.01) to produce a significant reduction in SERT function. In addition, it was found in HEK-293 cells transiently expressing myc-rKOPr and eYFP-tagged hDAT with chronic (30 min) exposure of U50,488H (10 μ M) and Sal A (10 μ M) significantly increased DAT function (** p <0.01). DAT function increases have been illustrated before by our laboratory (Simonson, unpublished data) when it

was illustrated that DAT function increases by 5% for U50,488H (1 μ M), 3% for Sal A (1 μ M), 12% for U50,488H (5 μ M), and 20% for Sal A (5 μ M) as well as 20% for U50,488H (10 μ M) and 45% for Sal A (10 μ M). Therefore, based on Simonson's (unpublished) findings, the findings in the current study that DAT function increases by 9% for both U50,488H (3 μ M) and Sal A (3 μ M) appears to be in line with other research. Our laboratory also illustrated that chronic (30 min) exposure of U50,488H (10 μ M) decreased SERT function by about 7% using confocal microscopy (Morani, unpublished data). Because the DAT findings are in line with past findings within our laboratory and because SERT is performing in line with past findings in our laboratory, it can be strongly suggested that SERT function decreases significantly, by about 8%, with chronic (30 min) exposure to the KOPr agonists U50,488H (10 μ M) and Sal A (10 μ M) in HEK-293 cells transiently expressing myc-rKOPr and eGFP-tagged hSERT as measured with a 96-well plate reader.

Our findings are very interesting because SERT function was not modulated by acute (5 min) KOPr agonist exposure but it was significantly decreased with chronic (30 min) KOPr agonist exposure. Unfortunately, at the time of publishing there is no published literature, other than from our laboratory, investigating the effects of chronic (30 min) KOPr agonist exposure on modulating SERT function in immortalised cells. Therefore, these findings are difficult to compare and contrast with the current literature.

Interestingly, previous studies from our laboratory by Morani (unpublished data) suggest a difference in SERT function in HEK-293 cell model co-expressing myc-

rKOPr and eGFP-tagged SERT (not inhibited by D-22) when incubated for 30 min with the KOPr agonist U50,488H (10 μ M) but not Sal A (10 μ M) measured by confocal microscopy. This discrepancy in SERT function results may reflect experimental differences between the two studies or the difference may exist because of experimenter error. Measuring SERT function after KOPr agonist exposure in repeated experiments might help to clarify if experimenter error is the reason.

Another interesting variable between the two studies investigating KOPr function on SERT function measured with RDEV is that Morani (unpublished data) did not use the low affinity transporter inhibitor, D-22. This is important to note because studies using other biological techniques (Hagen *et al.*, 2010; 2011) insist on the importance of using a low affinity transporter inhibitor when measuring SERT transporter function. Because D-22 was not used in the comparison study, any real differences between the two studies should be made with caution.

Additionally, the discrepancy between acute (5 min) KOPr exposure and chronic (30 min) exposure on SERT function may reflect the signal transduction pathways that are being activated. The potential pathways are discussed further in the final discussion (chapter 6). Further experiments need to investigate different time points to look at to see if the KOPr agonist concentration increases can act to decrease SERT function and if the time points have an effect on SERT function. This could help to explain signal pathways activation over time. It would also be interesting to look at different KOPr agonists such as DS-3-240 and U69,593 to elucidate if SERT functional changes discriminate against different KOPr agonists. Collectively, the above findings

reveal that chronic (30 min) exposure to the KOPr agonists, U50,488H (10 μ M) and Sal A (10 μ M), significantly decrease SERT function (** p <0.01) as measured by ASP⁺ uptake assay using a 96-well plate reader.

5.4.2 Establishment of a Stable Cell Line Expressing eGFP-tagged hSERT

Here, two stable cell lines expressing eGFP-tagged hSERT, the high expression, (Figure 5.4) and the low expression (Figure 5.5) were developed. The high expression cell line was frozen, unfrozen and grown for 6 weeks (Figure 5.6) to investigate the integrity of this cell line after being frozen for future experimental use. The cells appeared to grow after being thawed without compromising their integrity. These cells were intended to be used in the above experiment with transfected myc-rKOPr, however, due to time constraints co-transfection of myc-rKOPr in the stable SERT cell line was not performed. In addition, it was also anticipated that the findings above could be confirmed using confocal microscopy, however time constraints also prevented this from occurring. Together this chapter illustrates that SERT is functioning correctly, and that this novel highthrough put assay has the ability to show 5-HT transporter changes with pharmacological agents.

The finding that chronic (30 min) KOPr activation modifies SERT would be of particular interest as a pharmaceutical target given its intrinsic role in addiction, mood and stress. Any future research in our laboratory investigating the SERT and KOPr relationship would be far more economically feasible now because this SERT stable cell line could be transfected with just the myc-rKOPr plasmid, minimizing

expensive consumables. Initially, this would require co-localisation of KOPr but using a more uniform SERT expressing population would produce more consistent results across different experimental procedures. This SERT function (uptake) assay provides a fast, simple, and reliable fluorescence-based assay for the detection of SERT activity in a stable expressing cell line.

Chapter 6: General Discussion

SERT is an important protein essential for normal brain function and SERT dysfunction is heavily involved in the etiology of brain disorders. Specifically, SERT dysfunction occurs because of environmental stressors, such as drugs of abuse or life adversity. Environmental stressors can modify the SERT gene, such as the 5-HTTLPR, and the SERT protein through epigenetic modifications. These modifications contribute to the aetiology of SERT related brain diseases and disorders such as drug addiction, affective disorders, stress homeostasis and pain. Many of these SERT related diseases and disorders are also correlated with KOPr dysfunction. This overlap of SERT and KOPr diseases and disorders suggests that SERT and KOPr pathways are similar and/or they overlap, which indicates some sort of direct or indirect relationship. Recently, the co-localisation of both KOPr and SERT in the rat DRN (Land *et al.*, 2009) highlights that there is a relationship between these two proteins.

The KOPr and SERT relationship was first proposed by Schenk *et al.* (2000) when it was observed that the DAT inhibitor, RTI-55, produces cocaine-seeking in rats, which was subsequently attenuated with the traditional KOPr agonist U69,593. In other words, when the DAT inhibitor, RTI-55, increases DA levels cocaine-seeking increases in rats the subsequent administration of U69,593 shortly after reduced this cocaine-seeking behaviour. Schenk *et al.* (2000) suggest this inhibition of cocaine-seeking by U69,593 occurs because cocaine and κ -opioids interact at a common site on the SERT protein. Furthermore, Ramamoorthy *et al.* (2002) consolidate this hypothesis

by showing that U69,593 decreases 5-HT re-uptake by SERT in rat striatal synaptosomes. Zakharova *et al.* (2008) also indicate KOPr modulates SERT function by illustrating a decrease in rat KOPr activity when SERT levels are reduced. In line with this proposed KOPr and SERT relationship, our laboratory show KOPr activation decreases SERT function with acute (15 min) exposure to U50,488H (500 nM), and Sal A (500 nM) as measured with RDEV on rat striatum tissue (Simonson, unpublished data) and with chronic (30 min) exposure to U50,488H (10 μ M) in co-expressing myc-rKOPr and eGFP-hSERT HEK-293 cells using confocal microscopy (Morani, unpublished data). In the current study we also found chronic (30 min) but not acute (5 min) KOPr activation by U50,488H (10 μ M), and Sal A (10 μ M) decreases SERT function as measured with a novel high throughput assay.

The aim of this current study is to investigate the effect of KOPr activation on SERT function in HEK-293 cells co-expressing myc-rKOPr and eGFP-hSERT. We found that acute (5 min) exposure by the KOPr agonist U50,488H (500 nM) and the novel KOPr agonists, Sal A (500 nM), and DS-3-240 (500 nM) had no effect on SERT function in transiently transfected (myc-rKOPr and eGFP-hSERT) HEK-293 cells as measured with RDEV as well as measured by confocal microscopy (U50,488H, 10 μ M; Sal A, 10 μ M; & DS-3-240, 10 μ M). However, it was found that chronic (30 min) exposure to U50,488H (10 μ M), and Sal A (10 μ M), significantly decreases SERT function in transiently transfected (myc-rKOPr and eGFP-hSERT) HEK-293 cells measured with a novel high-throughput assay.

	5 min RDEV	5 min confocal	15 min RDEV	30 min	30 min
KOPr agonist(s)	U50/ Sal A / DS-3-240	U50/ Sal A / DS-3-240	U50/ Sal A / DS-3- 240	U50	Sal A
Simonson	✗ 500nM	-	✓ 500nM	-	-
Morani	-	-	-	✓ confocal microscopy 10µM	✗ confocal microscopy 10µM
Current study	✗ 500nM	✗ 10µM	-	✓ novel assay 10µM	✓ novel assay 10µM

Table 6.1: Summary of KOPr agonist effects on SERT function. Acute (5 min) exposure to the KOPr agonists U50,488H (500 nM) and the novel KOPr agonists, Sal A (500 nM), and DS-3-240 (500 nM) had no effect on SERT function in transiently transfected (myc-rKOPr and eGFP-hSERT) HEK-293 cells as measured with RDEV as well as measured by confocal microscopy (U50,488H, 10 µM; Sal A, 10 µM; & DS-3-240, 10 µM). However, KOPr activation decreases SERT function with acute (15 min) exposure to U50,488H (500 nM), and Sal A (500 nM), as measured with RDEV on rat striatum tissue (Simonson, unpublished data), as well as chronic (30 min) exposure to U50,488H (10 µM) in co-expressing HEK-293 cells using confocal microscopy (Morani, unpublished data). In this study chronic (30 min) exposure to U50,488H (10 µM), and Sal A (10 µM) significantly decreases SERT function in transiently transfected (myc-rKOPr and eGFP-hSERT) HEK-293 cells measured with a novel high-throughput assay.

Interpretation of these results should be made with caution because this cell model, along with any preclinical model, it has its limitations. This co-expressing cell model is an oversimplified preclinical model that serves the purpose to investigate a direct relationship between two proteins by overexpressing them both. Thus, our observation of no effect by acute (5 min) KOPr activation on SERT function suggests that there is no KOPr activation effect on SERT function, but this may only be present in this cell model. Perhaps, acute (5 min) KOPr activation of SERT function may involve additional molecular systems, as has been suggested by Collins *et al.* (2001; 2002), that are not present in this experimental cell model.

Further experiments to clarify if an acute (5 min) KOPr and SERT relationship exists is necessary. For example, a preclinical model that better resembles the neuron, such as a primary cell culture, may provide more insight into the acute KOPr and SERT relationship. Primary neuronal cultures have endogenously expressing monoamine transporter systems that may be required for an acute (5 min) KOPr and SERT relationship. Overexpressing KOPr and SERT in a primary cell culture and measuring the acute (5 min) effect of KOPr agonists on SERT function would provide a mechanism to further clarify if an acute KOPr and SERT relationship requires monoamine transporter synergy. However, like all preclinical models primary cell cultures have their limitations too. For example, protein expression can change over time in these cultures and the absence of supporting glial cells, that may interact with primary neurons in a whole brain, presents additional confounding variables. Nevertheless, utilisation of additional monoamine neurotransmitter systems in other preclinical models will elucidate if other systems are synergistically involved in an acute (5 min) KOPr activation to modulate SERT function or if an acute (5 min) KOPr and SERT relationship exists at all.

It was observed in this current study that SERT function significantly decreases after chronic (30 min) exposure to the KOPr agonists U50,488H (10 μ M) and Sal A (10 μ M). This finding suggests a direct relationship between KOPr and SERT. Further additional experiments to test these KOPr agonists in confocal microscopy, with the low affinity monoamine transporter inhibitor D-22, would conclusively confirm that chronic (30 min) KOPr activation decreases SERT function.

To summarise, the KOPr and SERT relationship appears to be absent at 5 min because no SERT function differences were measured in transiently transfected (myc-rKOPr and eGFP-hSERT) HEK-293 cells with confocal microscopy and RDEV (current study). However, it appears a long term (chronic) KOPr and SERT relationship is present that is being activated somewhere between 5 min and 30 min. A KOPr and SERT relationship is present at 30 min because KOPr activation decreased SERT function as measured in transiently transfected (myc-rKOPr and eGFP-hSERT) HEK-293 cells with confocal microscopy (Morani, unpublished) and a novel high-throughput assay (current study). But it is likely that this is occurring at 15 min because KOPr activation for 15 min decreased SERT function as measured in rat striatum tissue with RDEV (Simonson, unpublished).

The molecular pathway responsible for this decrease in SERT function between 5 and 15-30 min KOPr activation remains to be fully elucidated. However, SERT function is known to rapidly decrease in response to acute depletion of intracellular Ca^{2+} , inhibition of calmodulin, Ca^{2+} /calmodulin-dependent protein kinases II (CaMKII), Src-kinase, p38-Mitogen activated protein kinase (p38 MAPK) and activation of PKC (Ramamoorthy *et al.*, 2011). Additionally, data also suggests that cytokines can affect SERT activity through p38 MAPK-dependent mechanisms in some cell lines and mice neurons (Zhu *et al.*, 2006). Conversely, SERT function is increased by an increase in intracellular Ca^{2+} as well as activation of NOS/cGMP and MAPK pathways (PKA). However, the three major protein kinases that are responsible for SERT's phosphorylation state and SERT membrane expression are

PKC (Qian *et al.*, 1997) and p38 MAPK (Samuvel *et al.*, 2005) which decrease SERT function, and PKA (Ramamoorthy *et al.*, 1997) which increases SERT function. Currently, there are two major protein kinases responsible for decreasing SERT function, the inhibition of p38 MAPK (Samuvel *et al.*, 2005) and also the presence of PKC (Qian *et al.*, 1997), and each pathway is discussed below. In addition, to the known pathways that modulate SERT function, known KOPr pathways are also discussed as proposed by Bruchas and Chavkin (2011). Suggested KOPr pathways (Bruchas and Chavkin, 2011) for acute (short term) KOPr activation involve PKC and PKC dependent ERK1/2 and chronic (long term) KOPr activation involves p38 and independent ERK1/2 (see Fig. 1.3) KOPr signal transduction. Therefore, given the collective pathways above the p38, ERK1/2 and PKC pathways are all possibilities for the observed KOPr activation and subsequent SERT function decrease.

The mitogen-activated protein kinases (MAPK's) activate SERT in midbrain synaptosomes and heterologous cell models, whereas inhibition of p38 MAPK inhibits SERT activity either pharmacologically or by specific siRNAs-mediated knockdown (Ramamoorthy *et al.*, 2011). Furthermore, activation of p38 MAPK stimulates SERT activity either by overexpressing a constitutively active form of MAP kinase kinase 3b, an upstream kinase activator of p38 MAPK, exposure to anisomycin, an activator of p38 MAPK, or adenosine receptor (AR₃) activation (Samuvel *et al.*, 2005; Zhu *et al.*, 2005; Zhu, Hewlett, Feoktistov *et al.*, 2004; Zhu *et al.*, 2007). The extent that p38 MAPK regulates SERT surface expression remains controversial and requires further research with evidence supporting both trafficking dependent and independent mechanisms

(Ramamoorthy *et al.*, 2011). Interestingly, p38 signalling is activated by KOPr chronic (long term) exposure Bruchas and Chavkin (2010); however, Ramamoorthy *et al.* (2011) suggest an increase of p38 signalling increases SERT function. Ramamoorthy *et al.*'s (2011) hypothesis explains how stress can induce affective disorders. For example, stress increases p38 MAPK to increase SERT function, which decreases 5-HT signalling and increases anxiety and depression. However, the observation that KOPr chronic signaling increases p38, and *increases* SERT function too does not fit together with our data, which observed a *decrease* in SERT. Nevertheless, the brain is a complex and extremely difficult organ to understand and it is possible that perhaps p38 upregulates SERT in some brain cell regions and down regulates in other brain regions. If p38 increases (KOPr chronic activation) can downregulate SERT function in some cells too, this pathway might explain the findings observed in the HEK-293 cells from this study. Further investigation into this chronic (30 min KOPr agonist treatment) cell model is necessary to further investigate p38 as the pathway responsible for KOPr activation decreasing SERT function.

The biochemical mechanism between KOPr activation and SERT function decrease remains unknown. Another possible pathway to explain the chronic (30 min) relationship between KOPr and SERT is ERK1/2. The ERK1/2 pathway is the most commonly studied MAPK pathway activated by KOPr; Yet, although ERK1 and ERK2 are two separate proteins, they are largely investigated as the same pathway known as ERK1/2 (Assenio *et al.*, 2006). This signalling pathway is possible because ERK1/2 is phosphorylated and activated by MEK1/2 in biphasic stages with the first stage occurring after 5 minutes of exposure, and this biphasic stage is reflected in our data

with no acute (5 min) effect but an effect is seen after 5 min. Early phase ERK1/2 phosphorylation is seen after 5-15 min and is PI3K, PKC and Ca²⁺ dependent and β -arrestin independent. Late phase ERK1/2 phosphorylation is seen after 2 h and is GRK3 and β -arrestin dependent (Belcheva *et al.*, 2005; McLennan *et al.*, 2006). Based on the current ERK1/2 phosphorylation literature it appears that the SERT modulation Simonson (unpublished data) observed with 15 min KOPr activation was the early phase ERK1/2 activation, and this was also observed in the chronic (30 min) SERT modulation seen in the current study. This could explain why we did not see any acute (5 min) effects, because the pathway between KOPr and SERT takes 5-15 minutes to occur, which is why Simonson (unpublished) and the current study observes SERT function decreases at 15 and 30 min. If the ERK1/2 early phase pathway is involved in SERT modulation then it would be interesting to investigate further to see if inhibiting or modifying this pathway can attenuate the KOPr decrease on SERT function. This would confirm if ERK1/2 is responsible in this cell model for the chronic (30 min) KOPr activation, by U50,488H or Sal A, and subsequent decrease of SERT function. However, because this pathway is abundant within the cell this could prove difficult to achieve. Prasad *et al.* (2005) showed that p38 MAPK activation modulates function of the SERT, but because KOPr activation of ERK1/2 activation is in line with the observed SERT modulation at 15 and 30 min, ERK1/2 appears more likely to be the pathway responsible. Future experiments are necessary to investigate the role of ERK1/2 in KOPr agonist induced SERT function decreases. Co-expressing HEK-293 cells and performing a Western blot with a pERK1/2 antibody is required to investigate ERK1/2 activation at 5 and 15 min time points. If ERK1/2 appears to be responsible, then differentiating ERK1 from ERK2 would be required to see which

protein was specifically responsible. If ERK1/2 concentrations do not show any changes at the 5, 15 and 30 min time points then it would make sense to look further into p38 or PKC.

In contrast to p38 and ERK1/2, PKC is also suggested to be a major protein kinase responsible for decreasing SERT function (Qian *et al.*, 1997). Because inhibiting extracellular signal-regulated kinase (ERK1/2) or c-Jun N-terminal Kinase 1 (JNK) is without effect on SERT (Oz *et al.*, 2010; Samuvel *et al.*, 2005), PKC may be a better candidate to explain the relationship between KOPr activation and a decrease in SERT function. Recently, time course studies observed a decrease in SERT surface expression by SERT phosphorylation from the PKC-dependent pathway, which triggers SERT internalization and reduces 5-HT uptake to result in a synaptic increase of 5-HT (Ramamoorthy *et al.*, 2011). Bruchas and Chavkin (2011) suggest PKC and PKC dependent ERK1/2 to also be involved in acute (short term) KOPr activation. PKC regulates SERT in a biphasic manner, where the initial phase (1 – 5 min) of 5-HT uptake inhibition occurs independently of trafficking but the later phase (5 – 60 min) is associated with enhanced endocytosis (Jayanthi *et al.*, 2005). The biphasic inhibition of SERT is accompanied with sequential phosphorylation of plasma membrane SERT because it was shown that phosphorylation of the serine (Ser) residues precedes that of the threonine (Thr) residue(s) (Jayanthi *et al.*, 2005). It has also been suggested that the initial phosphorylation on the Ser residues are responsible for changes in the intrinsic properties and/or silencing of SERT, and that Thr phosphorylation may trigger internalization of phosphorylated SERT (Ramamoorthy *et al.*, 2011). If PKC is responsible for KOPr activation decreasing SERT function it would be interesting to

investigate the Ser and Thr phosphorylation patterns (Jayanthi *et al.*, 2005) at 5, 15 and 30 min time points to see if they correlate with SERT cell function and expression.

The recent finding that PKC activation increases SERT internalization and phosphorylation, whereas inhibition of p38 MAPK reduces basal SERT phosphorylation and plasma membrane insertion suggests that p38 MAPK and PKC must act at different phosphorylation sites (Ramamoorthy *et al.*, 2011). And if direct phosphorylation is involved in both processes, then these kinases are likely to target SERT at different locations (Ramamoorthy *et al.*, 2011). Future efforts directed towards the identification of the specific sites of PKC and p38 MAPK mediated phosphorylation will illuminate the cellular and molecular basis of SERT regulation by these kinases (Ramamoorthy *et al.*, 2011), and perhaps that of KOPr activation on SERT.

An interesting finding in the current research worth mentioning is that ASP⁺ uptake occurs in HEK-293 cells. Non-transfected HEK-293 cells and cells classified as 'non-transfected' (Figure 3.3), as measured by confocal microscopy, as well as transfected HEK-293 cells with SERT inhibited by fluoxetine, but not D-22, can uptake 5-HT (Figure 4.3). These findings reflect the abundance and significance of low affinity monoamine transporters in 5-HT uptake, even though SERT is thought to play a largest role in 5-HT uptake. Low affinity monoamine transporters, extraneuronal monoamine transporter (EMT), the plasmalemmal monoamine transporter (PMAT), and the organic cation transporters-OCT1, OCT2, and OCT3, are known to take up small amounts of monoamine neurotransmitters but they remain relatively unstudied

(Hagen *et al.*, 2011). Recently, Baganz *et al.* (2008) illustrated that OCT3 regulates extracellular 5-HT in SERT-deficient mice. Furthermore, Mayer *et al.* (2012) proved that OCT3 is present in HEK-293 cells as shown by real-time uptake of fluorescent ASP⁺. Collectively, these findings consolidate the data found in the current study that these low affinity transporters clearly contribute to 5-HT clearance. This highlights the overlap between 5-HT uptake and other monoamine uptake by these alternative integral transporter proteins. Now it is apparent that other monoamine transporters also contribute synergistically to monoamine signalling. The low affinity 5-HT transporters are also sensitive to corticosteroids, which increases their sensitivity to stressors and 5-HT uptake (Daws, 2009). Thus, it is likely that these low affinity monoamine transporters are involved in drug addiction, affective disorders and dysfunctional stress homeostasis. These findings highlight the importance of more research into these enigmatic low affinity transporters and their overall potential role in monoamine signalling. Controlling for confounding variables, such as these low affinity transporters, in clinically relevant scenarios is important for future neurobehavioural research (Kalueff and LaPorte, 2010).

6.2 Significance

In the current study we found chronic (30 min) but not acute (5 min) KOPr activation decreases SERT function. This has enormous clinical applications because of SERT's intrinsic role in addiction, mood, stress and pain. Currently, KOPr agonists and antagonists are known to attenuate different stages of the addiction cycle and SSRIs are used to treat depressive, anxious and stressed states, as well as alcohol addiction (ondansetron-SSRI). Given the proposed relationship between KOPr and

SERT, the overlap in targeting different systems in therapeutic compounds for similar disorders is one step closer to being understood. Novel KOPr agonists compounds also have the potential to treat pain, cough, diarrhoea, stimulant dependence, and mood disorders; unfortunately, Sal A contains undesirable side effects, such as dysphoria and anxiety (Prisinzano, 2005). Thus, it has limited for therapeutic use. Based on this information the relationship between KOPr and SERT is of particular interest for therapeutic targets. Medicinal chemistry efforts continue to explore the structure and activity relationship of Sal A. To date these have mainly focused on Sal A's high affinity and selectivity for the KOPr with few investigators looking at interactions with transporters or new analogues for pharmacotherapeutics with fewer side effects (Prisinzano and Rothman, 2008). Several models suggest that the affinity and selectivity of Sal A for KOPr's is the result of interactions with unique residues compared with other KOPr ligands (Prisinzano and Rothman, 2008). Further experiments are required with other KOPr ligands to further investigate interactions with unique residues compared with other KOPr ligands. And these findings may unveil the KOPr residue responsible for the decrease in SERT function. Further elucidating the interaction between these two important proteins has the potential to unveil key targets, which will facilitate future therapeutic research and treatment.

6.2 Future Directions

To define the KOPr and SERT relationship further, investigations with a multitude of different variables are necessary. Firstly, it is necessary to define the effect of different KOPr agonist preincubation times on SERT function using real-time confocal microscopy to definitively conclude when there is a KOPr and SERT

relationship. The next step is to investigate varying KOPr agonist concentrations (1 μ M to 10 μ M) to provide insight into the strength of KOPr agonists necessary to induce these SERT function changes. Also measuring the effects of different KOPr agonists, such as DS-3-240 and U69,593, at these time points and concentrations would clarify any KOPr agonist differences in modulating SERT function. This would enlighten us to specific KOPr residues that the KOPr agonists transduce the signal to modulate SERT. Then the signal transduction pathways responsible for each residue effect will need to be identified. Additional variables to investigate in future experiments also include different preclinical models to elucidate if the same biochemical trends occur in different clinical models. Additionally, protein co-immunoprecipitation of rat brain samples, and perhaps even human brain samples, also need to be further illustrated to identify the specific locations of the co-localisation of KOPr and SERT in the DRN in rats. Ultimately, PET scans may highlight the specific locations where KOPr and SERT co-localise in the primate brain. This will facilitate extrapolation of this information to form hypotheses on human brain and behaviour based on the neurobiology revealed.

Future experiments are necessary to elucidate this potential and exciting interaction between KOPr and SERT. These experiments allow us to be much closer to understanding the molecular mechanisms between KOPr and SERT and thus, the underlying pathways involved in drug addiction, affective disorders, and abnormal stress homeostasis so that they can be treated more effectively.

Appendix 1

Chapter 2 Solutions

Krebs Ringer Hepes buffer (KREBS)

	Concentration	g/L
NaCl	130 mM	7.6
KCl	1.3 mM	0.10
CaCl ₂	2.2mM	0.24
MgSO ₄ .6H ₂ O	1.2mM	0.27
KH ₂ PO ₄	1.2mM	0.16
HEPES	10mM	2.6
D-Glucose	10mM	1.8
	pH 7.4	

10 x Phosphate Buffered Saline (PBS)

	Concentration	g/L
NaCl	1.4 M	80
KCl	26.8 mM	2.0
Na ₂ HPO ₄	81.0 mM	26.8
KH ₂ PO ₄	14.7 mM	2.4
	pH 7.4	

1x PBS for cell culture

	Concentration	g/L
NaCl	137 mM	8
KCl	2.7 mM	0.2
Na ₂ HPO ₄	8.1 mM	2.68
KH ₂ PO ₄	1.5 mM	0.24
	pH 7.4	

1x PBS RDEV 5-HT solute

	Concentration	g/L
NaCl	137 mM	8
KCl	2.7 mM	0.2
Na ₂ HPO ₄	8.1 mM	2.68
KH ₂ PO ₄	1.5 mM	0.24
	pH 7.2	

LB Agar

	g/L
NaCl	10
Yeast Extract	5.0
Bactotryptone	10
Yeast Agar	10

LB Broth

	g/L
NaCl	10
Yeast Extract	5.0
Bactotryptone	10

Appendix 2

Chapter 3: Calculation of Maximal Effect on Cell by Confocal Microscopy

ASP⁺ uptake after acute KOPr agonist pre-treatment

Fig A.3.1. ASP⁺ uptake. Initial ASP⁺ studies were conducted to investigate when the KOPr agonists had their maximal uptake effect within 5 min. To calculate this ASP⁺ uptake 60 seconds before the addition of the KOPr agonist was compared to ASP⁺ uptake + 60 seconds after the KOPr agonist was added. It was found that U50,488H (A) showed its maximal effect at 210-270 s, Sal A (B) showed its maximal effect at 240-300 s, and DS-3-240 (C) showed its maximal effect at 120-180 s. $n = 43-57$ cells, across a minimum of 2 dishes in each transfection, over a total of 3 different transfection days. These time points were used to adjust all of the following studies, in case the time of action looked as if it was trending to occur after the 5 min mark. There was no statistically significant difference between the vehicle and any of the KOPr agonists and ASP⁺ uptake, so this data was not required. However, this was performed regardless to assist any future researchers into SERT and KOPr agonists.

Appendix 3

Chapter 4: RDEV in Transiently Transfected HEK-293 Cells with Increasing Increments of 5-HT Concentration

Fig. A3.1. SERT function in transiently transfected HEK-293 cells with increasing increments of 5-HT concentration. 5-HT is unstable at concentrations above 1 μM because it rapidly oxidises making measuring free 5-HT difficult because it becomes impossible to differentiate whether the electron current measured is an increase in oxidation or change in SERT function at higher 5-HT concentrations.

Table. A3.1. SERT function in transiently transfected HEK-293 cells with increasing increments of 5-HT concentration

Treatment	Vmax (pmol/s/10 ⁶ cells)	Vmax SEM	Km	Km SEM
U50	1.43 x 10 ³⁷	8.97 x 10 ²⁶	1.22 x 10 ³⁶	7.67x 10 ²⁵
Sal A	2.21 x 10 ³⁶	7.72 x 10 ³⁰	3.33 x 10 ³⁵	9.22 x 10 ²⁹
DS-3-240	1.81 x 10 ³⁷	2.64 x 10 ³²	2.18 x 10 ³⁶	2.09x 10 ³¹
DMSO	9.26 x 10 ²³	1.46 x 10 ¹³	9.96 x 10 ²²	1.57 x 10 ¹²
Fluoxitene	1.65x 10 ²³	1.41x 10 ¹³	2.02 x 10 ²²	1.73 x 10 ¹²
HEK-293	1.26 x 10 ²⁵	2.98 x 10 ¹⁸	1.36 x 10 ²⁸	3.23x 10 ¹⁷

In this study, initial optimisation experiments justify the use of a 5-HT concentration up to 1.25 μM because 1 μM did not show kinetic saturation and

concentrations higher than 1.25 μM resulted in the same linear increase of 5-HT uptake that is illustrated in Hagan *et al.*, (2010) which is suggested to be indicative of 5-HT oxidation, rather than uptake. Therefore, the last 5-HT concentration before this linear increase was deemed to be where 5-HT was saturating SERT.

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