

COMPARISON OF MEMBRANE PERMEABILITIES OF TRACE AMINES AND CORRESPONDING NEUROTRANSMITTERS

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By

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ABSTRACT

Trace amines (2-phenylethylamine, *p*-tyramine, *p*-octopamine and tryptamine) are endogenous compounds structurally similar to the monoamine neurotransmitters and distributed throughout the nervous systems of vertebrates. However, they are not thought to be stored in synaptic vesicles, nor released in an activity-dependent manner. Their synthesis, however, is regulated with the enzyme aromatic L-amino acid decarboxylase being a rate limiting factor. Distinct post-synaptic effects of trace amines have been demonstrated and a family of G-protein-coupled Trace Amine-Associated Receptors (TAAR) has been identified. The TAAR protein, though, is poorly translocated to the cell membrane and remains intracellular. Hence, in order to bind to post-synaptic TAAR, trace amines have to cross cell membranes. This was previously thought to occur by simple diffusion. Recent computer simulations have, however, predicted a high-energy barrier associated with this process.

Here the membrane passage of trace amines in the absence of transporters has been measured directly for the first time using the Fluorosome system. The trace amines tyramine ($p < 0.01$), and tryptamine ($p < 0.001$), had significantly greater membrane permeability than the comparable monoamine neurotransmitters, with trace amine permeability half-lives under 15 seconds. The effect of membrane transporters on the permeability of a representative trace amine (tyramine) and neurotransmitter (dopamine) was examined in Caco-2 and synaptosome studies. Tyramine accumulation ($\approx 7-8\%$ of administered concentration) was approximately twice that of dopamine (3-4%) in Caco-2 cells. Equilibration of both tyramine and dopamine occurred in less than 10 minutes. In

synaptosomes both tyramine and dopamine uptake equilibrated within 1 minute. Tyramine release from synaptosomes was significantly faster ($p < 0.001$) than that of dopamine. Dopamine release in depolarized membranes was significantly faster ($p < 0.01$, $F = 6.95$) while tyramine release was significantly slower ($p < 0.05$, $F = 5.86$) than in non-depolarized membranes. Release from synaptosomes was significantly slower than Fluorosome membrane passage for both tyramine ($p < 0.0002$, $F = 13.63$) and dopamine ($p < 0.0001$, $F = 56.77$) indicating the involvement of processes other than simple diffusion. In conclusion, the trace amines are more permeable than the corresponding neurotransmitters both in the absence and presence of transporters.

Table of Contents

PERMISSION TO USE	i
ACKNOWLEDGEMENT	ii
ABSTRACT.....	iii
Table of Contents	v
List of Figures.....	viii
List of Tables	x
List of Abbreviations	xi
1 INTRODUCTION.....	1
2 LITERATURE REVIEW	4
2.1 SYNTHESIS OF TRACE AMINES	4
2.1.1 RELATIONSHIP BETWEEN TRACE AMINE LEVELS, AADC ACTIVITY AND NEUROTRANSMITTERS	4
2.2 DEGRADATION OF TRACE AMINES	6
2.3 FUNCTIONAL EFFECTS OF TRACE AMINES.....	7
2.3.1 Indirect Sympathomimetic Effects.	7
2.3.2 Physiological Effects	8
2.3.3 Trace Amine Associated Receptor Mediated Effects	11
2.3.3.1 TAAR1	13
2.3.4. Toxicologic and Pathophysiological Effects	17
2.3.4.1 Toxicologic	17
2.3.4.1.1 Drugs of Abuse	17
2.3.4.1.2 Cheese Effect	18
2.3.4.1.3 Migraine.....	18
2.3.4.2 Pathophysiological.....	19

2.3.4.2.1 Schizophrenia.....	19
2.3.4.2.2 Depression.....	20
2.3.4.2.3 Parkinson’s Disease	21
2.4 MEMBRANE PERMEABILITY OF NEURONAL MONOAMINES	22
2.4.1 Release mechanisms	23
2.4.2 Uptake processes	24
2.4.3 Methods for studying membrane passage.....	27
2.4.3.1 Polarity-based predictions.....	28
2.4.3.2 Artificial membrane based systems	28
2.4.3.3 Cell culture.....	30
2.4.3.4 Synaptosomes	31
2.5 AIMS AND HYPOTHESIS	31
2.5.1 Hypothesis	32
3 MATERIAL AND METHODS	33
3.1 FLUOROSOMES	33
3.2 PARTITION COEFFICIENT, TOPOLOGICAL POLAR SURFACE AREA AND DISTRIBUTION COEFFICIENTS.....	34
3.2.1 Data Analysis.....	35
3.3 Caco-2 CELL CULTURE.....	36
3.3.1 Materials	36
3.3.2 Cell Culture.....	36
3.3.2.1 Subculture of Cells.....	37
3.3.2.2 Culture into Transwell® Plates.....	37
3.3.2.3 Trans-epithelial Electrical Resistance.....	38
3.3.2.4 Lucifer yellow (LY).....	38
3.3.2.5 Permeability Experiment	39

3.4 SYNAPTOSOMES.....	41
3.4.1 Preparation of Synaptosomes.....	41
3.4.2 Uptake Assay	41
3.4.3 Release Assay	42
3.4.4 Release in Depolarized Synaptosomes Assay.....	42
3.4.5 Protein Assay	43
3.4.6. Data Analysis.....	43
4 RESULTS	44
4.1 FLUOROSOMES	44
4.2 PARTITION COEFFICIENT, TOPOLOGICAL POLAR SURFACE AREA AND DISTRIBUTION COEFFICIENTS.....	55
4.3 CELL CULTURE	64
4.4 SYNAPTOSOMES.....	69
5 DISCUSSION.....	80
6 SUMMARY AND CONCLUSION	88
7 FUTURE DIRECTIONS.....	90
REFERENCES.....	91

List of Figures

Figure 1: Molecular structure of selected neurotransmitters and trace amines	2
Figure 2: Endogenous routes of synthesis for trace amines and monoamine neurotransmitters.	5
Figure 3 : Effect of 9.4 mM TRYP on free fluorosome probe fluorescence.	45
Figure 4 : Effect of 6.25 mM tyramine on free fluorosome probe fluorescence.	46
Figure 5: Effect of 85 mM PE on free fluorosome probe fluorescence.	47
Figure 6: Effect of amiloride on fluorosome fluorescence.	48
Figure 7: Quenching of Fluorosome fluorescence following addition of 93.8 mM dopamine and 46.9 mM tyramine.	49
Figure 8: Quenching of Fluorosome fluorescence following addition of 93.8 mM NA and 46.9 mM OCT.	50
Figure 9: Quenching of Fluorosome fluorescence following addition of 2.3 mM 5-HT and 9.4 mM TRYP.	51
Figure 10: Comparison of pooled neurotransmitter and trace amine membrane permeability measures.	54
Figure 11: Relationship of predicted TPSA and log P scores to Fluorosome membrane permeability coefficients.	57
Figure 12: Representative standard curve of dopamine in PBS.	59
Figure 13: Representative standard curve of dopamine in octanol.	60
Figure 14: Representative standard curve of tyramine in PBS.	61
Figure 15: Representative standard curve of tyramine in octanol	62
Figure 16: Relationship of log $D_{7.4}$ to Fluorosome membrane permeabilities.	63
Figure 17: Permeability of [^3H]-tyramine in Caco-2 cell culture upon apical and basal additions.	65
Figure 18: Permeability of [^3H]-dopamine in Caco-2 cells following apical and basal addition.	66
Figure 19: Standard curve for [^3H]-tyramine application.	67
Figure 20: Standard curve for [^3H]-dopamine application	68
Figure 21: Standard curve for protein assay.	70
Figure 22: Uptake of [^3H]-tyramine in rat brain synaptosomes.	71
Figure 23: Uptake of [^3H]-dopamine in rat brain synaptosomes.	72
Figure 24: Release of [^3H]-tyramine under basal and high K^+ -induced depolarization conditions.	74
Figure 25: [^3H]-Dopamine release from synaptosomes under basal and high K^+ -induced depolarization conditions.	75
Figure 26: Comparison of the release of [^3H]-tyramine and [^3H]-dopamine from synaptosomes under basal conditions.	76

Figure 27: Comparison of the release of [³H]-tyramine and [³H]-dopamine from synaptosomes following high K⁺-induced depolarization..... 77

List of Tables

Table 1: Interactions of Neurotransmitters and Trace amines	9
Table 2: Species variation in TAAR showing number of functional and pseudogenes in different species.....	12
Table 3: Biogenic amine permeability coefficient and diffusion half-lives across Fluorosome lipid bilayer membranes.	52
Table 5: Rate constants and half-lives for [³ H]-tyramine and [³ H]-dopamine release under various conditions.....	78
Table 6: Comparison of the rate constants and half-lives for tyramine and dopamine in synaptosome and Fluorosome assay systems.	79

List of Abbreviations

3-MT - 3-Methoxytyramine

4-MT - 4-Methoxytyramine

5-HT - 5-Hydroxytryptamine

AADC - Aromatic L-amino acid decarboxylase

Ach - Acetylcholine

ATP - Adenosine-5'-triphosphate

COMT - Catechol-O-methyl transferase

CNS - Central nervous system

D2 - Dopamine receptor 2

DA- Dopamine

DAT - Dopamine transporter

DBH - Dopamine beta hydroxylase

GABA - Gamma amino butyric acid

GPCR(s) - G-protein-coupled receptor(s)

HBSS - Hank's balanced salt solution

HEPES - Hydroxyethyl piperazineethanesulfonic acid

KO- Knock-out

Log D - Distribution coefficient

Log P - Partition coefficient

LY – Lucifer yellow

mL - Milliliter

MAO - Monoamine oxidase

NA - Noradrenaline

NET - Norepinephrine / noradrenaline transporter

OCT - Octopamine

PE - 2-Phenylethylamine

PBS - Phosphate buffered saline

PNMT - Phenylethanolamine N-methyl transferase

PH - Phenylalanine hydroxylase

SEM - Standard error of the mean

SLC - Solute carrier

SERT - Serotonin transporter

SYN - Synephrine

T1AM - 3-iodothyronamine

TAAR- Trace amine-associated receptor

TEER - Trans-epithelial electrical resistance

TH - Tyramine hydroxylase

TPSA - Topological polar surface area

TRYP - Tryptamine

μL - Microliter

μM - Micromolar

VAT - Vesicular amine transporter

VACHT - Vesicular acetylcholine transporter

VMAT - Vesicular monoamine transporter

1 INTRODUCTION

Trace amines are a group of endogenous amines found in the central nervous system of all species studied so far (Philips et al., 1978; D'Andrea et al., 2003; Berry, 2004; Jacob and Presti, 2005). These include 2-phenylethylamine (PE), *para*-tyramine, tryptamine, *para*-octopamine and *para*-synephrine. They have structural similarities with neurotransmitters such as DA, noradrenaline and serotonin (5-HT) (Figure 1). However, in vertebrates the trace amines have very low concentrations, usually in the range of 0.1-10 nM, which is less than 1% of neurotransmitter concentrations (Durden et al., 1973; Durden and Philips, 1980; Durden and Davis, 1993), hence, the name trace amines.

In invertebrates the trace amines are found in higher concentrations and, because of this, octopamine and tyramine function as primarily neurotransmitters (Axelrod and Saavedra, 1977; David and Coulon, 1985; Evans and Robb, 1993; Roeder, 1999; Saraswati et al., 2004). In vertebrates the trace amines are not thought to be neurotransmitters mainly due to the fact that they are not stored in synaptic vesicles (Juorio et al., 1988), are not released in an activity-dependent manner (Dyck, 1989) and moreover do not affect neuronal excitability at physiological levels (Jones, 1981; Harris et al., 1988; Berry et al., 1994). However, the synthesis of trace amines is regulated (Paterson et al., 1990) and they alter the neuronal responses of neurotransmitters (Henwood et al., 1979; Jones and Boulton, 1980; Lundberg et al., 1985; Paterson, 1993).

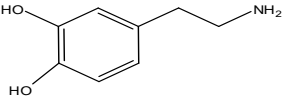
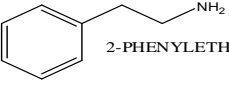
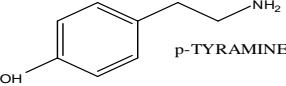
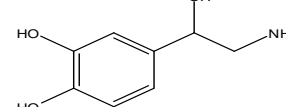
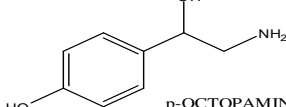
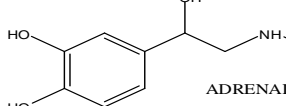
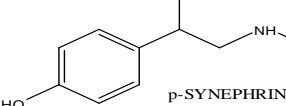
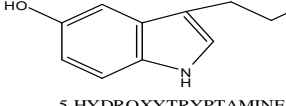
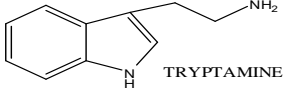
NEUROTRANSMITTERS	TRACE AMINES
 <p data-bbox="529 386 630 407">DOPAMINE</p>	 <p data-bbox="951 302 1166 323">2-PHENYLETHYLAMINE</p>  <p data-bbox="967 407 1084 428">p-TYRAMINE</p>
 <p data-bbox="610 596 769 617">NORADRENALINE</p>	 <p data-bbox="967 583 1110 604">p-OCTOPAMINE</p>
 <p data-bbox="651 722 769 743">ADRENALINE</p>	 <p data-bbox="984 722 1110 743">p-SYNEPHRINE</p>
 <p data-bbox="505 907 737 928">5-HYDROXYTRYPTAMINE</p>	 <p data-bbox="984 877 1101 898">TRYPTAMINE</p>

Figure 1: Molecular structure of selected neurotransmitters and trace amines

This suggests that trace amines have physiological significance, an assertion supported by the discovery of Trace Amine-Associated Receptors (TAAR), which are intracellular G-protein-coupled receptors (GPCRs) selectively activated by trace amines (Borowsky et al., 2001; Bunzow et al., 2001). Post-synaptic effects of trace amines are presumed to be mediated through TAAR (Berry, 2004; Sotnikova et al., 2004; Lindeman et al., 2008) and for these to occur, the trace amines would have to cross the cell membranes. This membrane passage was assumed to occur by simple diffusion (Paterson et al., 1990; Tchercansky et al., 1994; Berry, 2004), but computer simulation studies suggest a high-energy barrier associated with diffusion across a lipid bilayer (Berry et al., 2011). Trace amines have also been implicated in a number of neurological and psychiatric disorders (Berry, 2007) and TAAR are potently activated by a number of drugs of abuse (Bunzow et al., 2001; Reese et al., 2007). Determination of the mechanisms by which trace amines interact with their receptors is of importance for a better understanding of trace amine functioning under physiological and pathological conditions. This will be addressed in this thesis by determining the ability of trace amines to cross lipid bilayer membranes both in the absence and presence of endogenous transporter proteins. Furthermore, the membrane passage will be compared to that of the more widely studied monoamine neurotransmitters.

2 LITERATURE REVIEW

2.1 SYNTHESIS OF TRACE AMINES

The trace amines are synthesized by initial decarboxylation of the aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan by aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28) in a single enzymatic step (Boulton, 1982) (Figure 2). Although AADC is not the rate-limiting factor in the synthesis of neurotransmitters, it is in the synthesis of trace amines as it is the only enzyme involved in their synthesis (Saavedra et al., 1974; Dyck et al., 1983; Berry et al., 1996).

2.1.1 RELATIONSHIP BETWEEN TRACE AMINE LEVELS, AADC ACTIVITY AND NEUROTRANSMITTERS

Agonists at DA and noradrenaline receptors cause a decrease in AADC activity and subsequent decrease in the trace amine levels (Rossetti et al., 1990; Hadjiconstantinou et al., 1993; Zhu et al., 1993), while antagonists cause an increase in AADC activity and the levels of trace amines (Zhu et al., 1992; Cho et al., 1997; Neff et al., 2006). These changes in AADC activity however, were insufficient to cause alterations in the levels of neurotransmitters. Further, treatments that increase the synaptic levels of neurotransmitters also decrease AADC activity and trace amine levels (Juorio et al., 1991).

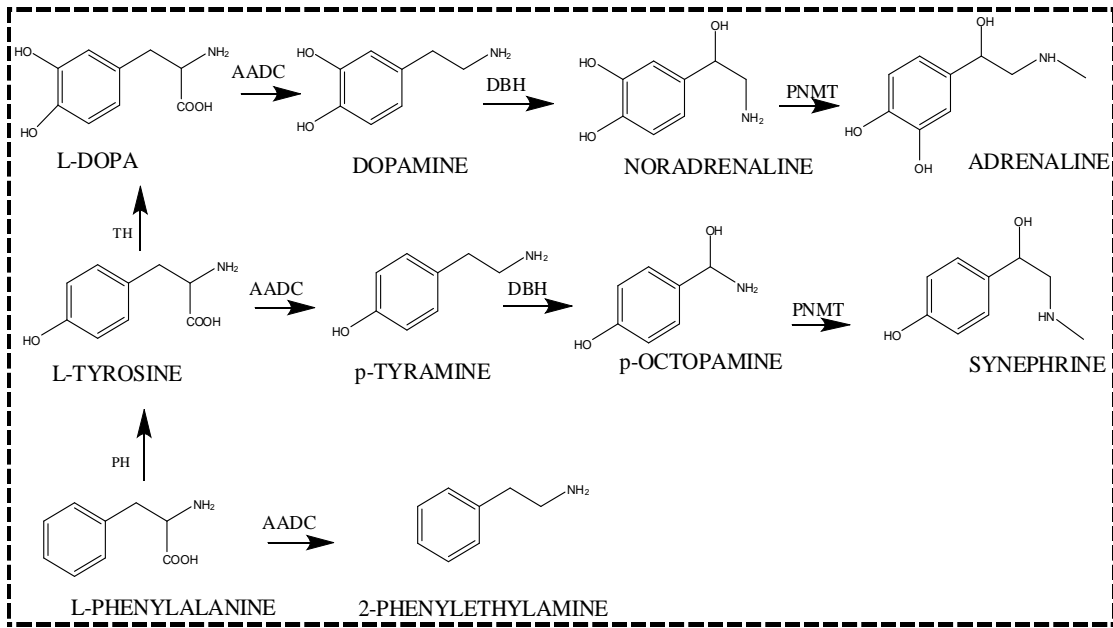


Figure 2: Endogenous routes of synthesis for trace amines and monoamine neurotransmitters.

AADC: Aromatic L-amino acid decarboxylase;

DBH: Dopamine β -hydroxylase;

PNMT: Phenylethanolamine N-methyl transferase;

TH: Tyrosine hydroxylase;

PH: Phenylalanine hydroxylase

For example, amphetamine increases extracellular DA levels (Carboni et al., 2001), causes a decrease in AADC activity (Buckland et al., 1996) and decreases accumulation of the trace amine PE (Boulton, 1982). Also reserpine, which causes depletion of neurotransmitters (Wessel and Joh, 1992), increases AADC activity and the accumulation of PE (Juorio, 1988). Thus, drugs that increase stimulation of neurotransmitter receptors cause a decrease in the AADC activity and a subsequent decrease in trace amine levels. Treatments that decrease stimulation of neurotransmitter receptors enhance AADC activity and thereby increase trace amine levels. Hence, the trace amine levels appear to be modulated by regulation of AADC activity in response to synaptic neurotransmitter levels.

2.2 DEGRADATION OF TRACE AMINES

The trace amines are inactivated primarily by oxidation of the amino group by the enzyme monoamine oxidase (MAO; EC 1.4.3.4) (Philips and Boulton, 1979). There are two isoforms of MAO: MAO-A and MAO-B (Shih and Chen, 2004). Each of these isoforms displays characteristic preference for particular trace amines. For example, 2-phenylethylamine is primarily oxidized by MAO-B (Yang and Neff, 1973) whereas other trace amines are acted upon by both MAO-A and MAO-B (Philips and Boulton, 1979; Durden and Philips, 1980). The rate of metabolism is very high, giving trace amines a high turnover rate, with an endogenous half-life of approximately 30 seconds (Durden and Philips, 1980). This is also consistent with the apparent lack of storage of trace amines (Juorio et al., 1988).

2.3 FUNCTIONAL EFFECTS OF TRACE AMINES

Both pharmacological and physiological effects of trace amines have been described. In addition trace amines have been implicated as playing a causative role in a number of toxicological and pathophysiological conditions.

2.3.1 Indirect Sympathomimetic Effects.

The effects of trace amines have been described as indirect sympathomimetic or amphetamine-like (Parker and Cubeddu, 1988; Paterson, 1993; Barroso and Rodriguez, 1996). These effects require much higher levels of trace amines than normal, which is micromolar levels compared to the usual nanomolar levels (Reynolds, 1979; Baud et al., 1985; Parker and Cubeddu, 1988; Paterson, 1993; Barroso and Rodriguez, 1996). Here they increase the synaptic transmitter concentrations by blocking re-uptake processes and displacing vesicular stores of neurotransmitters (Burchett and Hicks, 2006). These effects result in a number of pharmacological responses and were previously thought to be the mechanism of amphetamine action (Burchett and Hicks, 2006). For example, in CNS the trace amines produce responses similar to amphetamine showing increased alertness, euphoria, decreased appetite, irritability, insomnia and tremor (Zhu and Juorio, 1995). In the cardiovascular system effects such as tachycardia and vasomotor effects leading to both hypertension and hypotension are seen (Horn and Snyder, 1972). While these effects may have pharmacologic importance, the high concentrations of trace amines required for their induction, make them unlikely to be of physiological relevance.

2.3.2 Physiological Effects

Several electrophysiological iontophoretic studies have been done on rat brain to determine the effects of trace amines on neurotransmission (Henwood et al., 1979; Jones and Boulton, 1980; Lundberg et al., 1985; Paterson, 1993). Trace amines have been shown to modify the *in vivo* responses of neurotransmitters at doses that induce no change in electrophysiological properties alone. Table 1 summarizes the interaction of various trace amines with different neurotransmitters. PE potentiates the response of dopamine (DA) and its agonists (Harris et al., 1988; Paterson et al., 1990; Berry et al., 1994) and noradrenaline (NA) (Jones, 1982b; Paterson, 1988; Paterson, 1993). No effects are seen with 5-HT (Jones, 1982b), acetylcholine (Jones, 1982b; Paterson, 1993), gamma-amino butyric acid (GABA) (Jones, 1982b; Paterson, 1988) or glutamate (Jones, 1982b). However, one group has reported PE reduced GABA receptor mediated pre-synaptic inhibition (Brancucci et al., 2004; Berretta et al., 2005). Similarly *p*-tyramine potentiates responses of DA (Jones and Boulton, 1980; Harris et al., 1988), and NA (Harris et al., 1988) with no effects on 5-HT (Jones and Boulton, 1980), glutamate (Jones and Boulton, 1980) or GABA (Harris et al., 1988) responses. Interactions with acetylcholine (ACh) have not been investigated. Very little is known about the interactions of tryptamine (TRYP), octopamine (OCT) and synephrine (SYN) with neurotransmitters. TRYP interactions with 5-HT (Jones, 1982a; Paterson and Boulton, 1988) and ACh (Jones, 1982a) have been studied, but with other transmitters, the effects are unknown. TRYP enhanced the depressant responses to exogenous 5-HT when applied with ejecting currents that did not affect the cell firing rate (Jones, 1982a). In contrast, TRYP inhibited the excitatory responses of 5-HT in the nucleus raphe medianus at low

concentrations, or converted them to inhibitory effects (Jones, 1982a). OCT enhances the effect of NA with no effects on DA and 5-HT (Jones, 1983).

Table 1: Interactions of Neurotransmitters and Trace amines

	PE	TYRA	TRYP	OCT	SYN
DA	+	+		-	
NA	+	+		+	
5-HT	-	-	+/-	-	
ACH	-		-		
GABA	-/+	-/+			
GLUTAMATE	-	-			

+ = Neurotransmitter response potentiated.

+/- = Neurotransmitter response potentiated or decreased.

- = No Effect.

Blank = Effects not studied.

These effects of trace amines are post-synaptic. The exact mechanism is not known but when the vesicular stores of neurotransmitters are depleted by pre-treatment with reserpine, the trace amines still potentiated the responses of iontophoretically applied neurotransmitters without affecting the spontaneous firing rate (Paterson, 1993). This suggests the effects of trace amines are not dependent on endogenous neurotransmitter stores and as such are not due to the indirect sympathomimetic effects of high doses.

Effects are also seen if pre-synaptic terminals are lesioned, providing direct evidence that the effects are post-synaptic (Paterson, 1988).

Effects are also seen if endogenous levels of PE are altered. Trace amines are not stored in the synaptic vesicles (Juorio et al., 1988) and are thought to be released directly upon their synthesis and this could be mainly because they were thought to diffuse across membranes (Paterson et al., 1990; Tchercansky et al., 1994; Berry, 2004). Thus synaptic levels of trace amines can be modified by altering their synthesis and degradation. Endogenous levels of PE were increased by inhibiting the degradative enzyme MAO-B with deprenyl (Paterson et al., 1991; Berry et al., 1994). The increase in endogenous levels did not affect the spontaneous neuronal activity but in the presence of dopaminergic activity, elevated PE levels significantly affected neuronal responsivity, seen as increased sensitivity to dopamine (Paterson et al., 1991; Berry et al., 1994). Further, on application of an AADC inhibitor the levels of endogenous PE decreased, and the neuronal sensitivity was returned to the pre-deprenyl administration levels (Paterson et al., 1991; Berry et al., 1994). Post-synaptically PE is hypothesized to interact with a TAAR (Berry, 2004), but it is not known which one(s).

The electrophysiological effects, combined with the previously described regulation of trace amine synthesis in response to neurotransmitter receptor activation, suggest that trace amines can function as an endogenous mechanism for regulating neurotransmitter activity in order to maintain basal neuronal tone.

2.3.3 Trace Amine Associated Receptor Mediated Effects

Two separate groups (Borowsky et al., 2001; Bunzow et al., 2001) discovered the existence of GPCRs activated by trace amines. This discovery has highlighted many new dimensions regarding the trace amines, by providing a target through which trace amine effects can be mediated. TAARs are a sub family of the rhodopsin class of GPCR (Lindemann et al., 2005) that are distinct from the invertebrate tyramine and octopamine receptors (Niimura and Nei, 2005; Hashiguchi and Nishida, 2007; Hussain et al., 2009) and also other biogenic amine receptors (Lindemann et al., 2005; Hussain et al., 2009).

The most recent classification (Hussain et al., 2009) has identified 28 sub-families of vertebrate TAAR. TAAR1-9 are seen in multiple vertebrate species and TAAR10-28 are fish specific. Multiple isoforms of individual TAAR are present, many of which appear species specific (Hussain et al., 2009). These subtypes can be further divided into major clades referred to as class I to III. Class I (TAAR1, 10-11,21,27) and class II (TAAR2-9,12-13) contain both tetrapod and teleost genes and class III (14-20, 22-26, 28) are restricted to teleosts (Korsching, 2009). All TAAR are single exon genes (Lindemann et al., 2005) and in non-aquatic vertebrates they are clustered onto a single chromosome (Lindemann et al., 2005). In fish which have a much larger pool of TAAR, these genes are scattered over multiple chromosomes (Hussain et al., 2009).

Large species variation between the vertebrates also exists in terms of different numbers of isoforms as well as pseudogenes (Table 2). Humans have 6 functional TAAR genes with 3 pseudogenes, while the chimpanzee has only 3 functional genes but 6 pseudogenes

(Lindemann et al., 2005). Similarly, in rat and mouse there are 17 and 15 functional TAAR and 2 pseudogenes, respectively (Lindemann et al., 2005).

Table 2: Species variation in TAAR showing number of functional and pseudogenes in different species.

	Functional TAAR Genes	Pseudogenes
Human	6	3
Chimpanzee	3	6
Rat	17	2
Mouse	15	2

Of the different TAAR only TAAR 1 and TAAR 4 have so far been shown to be activated by endogenous trace amines (Bunzow et al., 2001; Borowsky et al., 2001; Lindemann and Hoener, 2005; Grandy, 2007; Hussain et al., 2009), with TAAR1 the only receptor studied in detail. Endogenous ligands for the other TAAR have not been identified.

TAAR are distributed throughout the body. TAAR1 distribution is described in detail below (Section 2.3.3.1). TAAR 6 and TAAR 8 are found in the kidney and amygdala (Borowsky et al. 2001) and TAAR 9 found in kidney, pituitary and skeletal muscles

(Vanti et al., 2003). TAAR have also been classified as a novel class of olfactory receptors in vertebrates (Liberles, 2009).

2.3.3.1 TAAR1

TAAR1 is activated by PE, tyramine, catecholamine-O-methyl metabolites of catecholamine neurotransmitters and iodothyronamines (Borowsky et al., 2001; Bunzow et al., 2001; Scanlan et al., 2004; Tan et al., 2008). Drugs of abuse like d-amphetamine, l-amphetamine, methamphetamine, and 3,4-methylenedioxymethamphetamine (MDMA; ‘ecstasy’) also potently activate TAAR1 (Bunzow et al., 2001; Lindemann and Hoener, 2005) along with the hallucinogenic dihydroergotamine and *N,N*-dimethyltryptamine (Bunzow et al., 2001). Unlike neurotransmitter receptors, TAAR are intracellular (Bunzow et al., 2001; Miller et al., 2005; Wolinsky et al., 2007; Grandy, 2007; Reese et al., 2007; Wainscott et al., 2007; Barak et al., 2008). This requires membrane passage of endogenous ligands for post-synaptic effects to be mediated. The level of expression of TAAR is also lower than neurotransmitter receptors (Borowsky et al., 2001).

TAAR1 is coupled to the G_s protein leading to activation of adenylate cyclase and cAMP production (Borowsky et al., 2001; Bunzow et al., 2001). Recent studies suggest coupling through G-protein-gated potassium (GIRK) channels (Bradaia et al., 2009) also occurs. TAAR1 signaling is also enhanced by monoamine transporters (Xie et al., 2007; Xie et al., 2008) and it was suggested that intracellular TAAR1 receptors may redistribute to the cell membrane as part of their signal transduction cascade (Miller, 2011).

In mouse CNS TAAR1 is distributed across the limbic system, monoaminergic cell body regions and their projection areas like the locus coeruleus, substantia nigra, ventral

tegmental area, dorsal raphé, striatum and basal ganglia (Borowsky et al., 2001, Lindemann and Hoener, 2005). In rat CNS TAAR1 is also present throughout the brain, with highest expression in the olfactory bulb, nucleus accumbens, cortical regions, substantia nigra, ventral tegmental area, cerebellum and medulla (Lindemann and Hoener, 2005). In the periphery TAAR1 is expressed in liver, kidney, gastrointestinal tract, spleen, pancreas and heart (Bunzow et al, 2001, Lindemann and Hoener, 2005). TAAR1 have also been found in blood vessels (Borowsky et al., 2001; Broadley, 2010) and leukocytes (Nelson et al., 2007). In rhesus monkey TAAR1 is expressed in the primary dopaminergic areas such as the substantia nigra and striatum (Xie et al., 2007). Of these various locations the amygdala region in rat, mouse and human CNS contain the highest levels of TAAR1 mRNA (Borowsky et al., 2001). Thus TAAR1 is widely expressed in primary dopaminergic areas of brain and suitably located to modulate the locomotor, emotional and behavioural responses which are related to dopaminergic activity in these areas.

Several studies have determined the potencies of trace amines at TAAR1. PE and tyramine are the more potent having EC₅₀ values in the range of 400-900 nM for PE and 70-1100 nM for tyramine (Borowsky et al., 2001; Bunzow et al., 2001; Lindemann and Hoener, 2005; Miller et al., 2005; Navarro et al., 2006; Wolinsky et al., 2007; Grandy, 2007; Reese et al., 2007; Wainscott et al., 2007; Barak et al., 2008; Lewin et al., 2008). Octopamine and TRYP show relatively weaker activity at rat and human TAAR1 (Borowsky et al., 2001; Bunzow et al., 2001; Lindemann and Hoener, 2005; Miller et al., 2005; Navarro et al., 2006; Wainscott et al., 2007; Barak et al., 2008). PE is moderately

more potent than tyramine at mouse and human TAAR1 whereas tyramine is the more potent in the rat (Grandy, 2007).

Bunzow et al. (2001) also observed that the O-methyl metabolites of catecholamine neurotransmitters like 3-methoxytyramine (3-MT), 4-methoxytyramine (4-MT), normetanephrine and metanephrine are potent agonists at rat TAAR1. The same finding regarding 3-MT and 4-MT was confirmed at human TAAR1 (Wainscott et al., 2007; Barak et al., 2008). 3-MT is an extracellular metabolite of dopamine formed when dopamine is acted upon by catechol-O-methyl transferase (COMT). These O-methyl metabolites were previously considered inactive compounds and tissue concentrations considered as a marker of extracellular dopamine levels (Kehr, 1976). Activation of TAAR1 by O-methyl metabolites is consistent with the trace amine system responding to the extra-cellular neurotransmitter levels (see section 2.1.1).

Another endogenous agonist is 3-iodothyronamine (T1AM) which is derived from thyroid hormone by decarboxylation and deiodination (Scanlan et al., 2004; Hart et al., 2006; Tan et al., 2007). T1AM is potent at rat, mouse (Scanlan et al., 2004; Tan et al., 2007) and human (Hart et al., 2006; Grandy, 2007) TAAR1 with low nanomolar EC₅₀ values. Other thyronamines such as 3,5-diiodothyronamine and 3,5,3-triiodothyronamine also activate TAAR1 but with much lower potency (Tan et al., 2007; Tan et al., 2008, Tan et al., 2009). T1AM also interacts with a number of other GPCR and membrane proteins with similar affinity (Zucchi et al., 2006; Ianculescu et al., 2009; Ianculescu and Scanlan, 2010) including DA and NA transporters thereby causing inhibition of reuptake and also interacts with vesicular monoamine transporter-2 (VMAT2) (Snead et al., 2007)

and the role of TAAR in the effects of thyroid hormones has not yet been studied in detail.

Amphetamine has a similar molecular structure to the trace amines PE and *p*-tyramine and stimulates cAMP production through rat, rhesus monkey and human TAAR1 (Miller et al., 2005; Wolinsky et al., 2007; Reese et al., 2007; Wainscott et al., 2007; Xie and Miller, 2007; Xie et al., 2007; Barak et al., 2008; Lewin et al., 2008). EC₅₀ values are comparable to PE (210 nM for R-amphetamine and 440 nM for S-amphetamine). Methamphetamine, MDMA and the hallucinogenic amphetamine 2-amino,1-[2,5-dimethoxy-4-iodophenyl]-propane (DOI) are slightly less potent than amphetamine (Bunzow et al., 2001). TAAR1 is now proven to be a validated target for amphetamine and methamphetamine (Reese et al., 2007). The exact role of TAAR1 in the toxicological effects of these drugs of abuse is not yet known. Recently, one TAAR1 antagonist EPPTB (RO5212773) (Bradaia et al., 2009; Stalder et al., 2011) has been identified but this is only active in mouse. A synthetic TAAR 1 agonist, RO5166017, has also been described recently (Revel et al., 2011) which shows high affinity for mouse, rat, primate and human TAAR1. A TAAR1-mediated modulation of dopaminergic and serotonergic activity was seen following administration of RO5166017 to brain slice preparations (Revel et al., 2011).

Presynaptically trace amines bind to TAAR1 receptors and have effects that could lead to enhanced responses to neurotransmitters (Xie and Miller, 2007; Xie and Miller, 2008; Xie and Miller, 2009). A TAAR1 mediated inhibition of dopamine re-uptake via the dopamine transporter has been reported, leading to increases in dopamine levels in the synapse (Miller, 2011). In the presence of a dopamine receptor reserve, this could be

expected to lead to an increase in dopamine receptor activation. As such, trace amines can regulate neurotransmitter re-uptake proteins at low doses via an interaction with TAAR1, and at high doses through a direct inhibition of the transporter. Further, trace amines can therefore potentiate dopaminergic responses both pre-synaptically and post synaptically. Similar TAAR1 mediated effects were also observed at noradrenaline (Xie and Miller, 2007; Xie and Miller, 2008; Xie et al., 2008) and 5-HT (Xie and Miller, 2007; Xie et al., 2008) transporters.

Studies have also demonstrated an interaction between TAAR1 and D2 dopamine receptors (Xie et al., 2007; Bradia et al., 2009; Espinoza et al., 2011). Using TAAR1 knock-out (KO) mice the effects of D2 mediated responses of dopamine were found to be increased (Bradia et al., 2009). On the other hand when D2 receptors were blocked with an antagonist, TAAR1 cAMP-mediated signaling was increased (Espinoza et al., 2011). Thus TAAR1 and D2 receptors can modulate the other's activity. In contrast the TAAR1 ligand PE has been reported to have no effect on agonist and antagonist interactions at D1 dopamine receptors (Berry, 2004).

2.3.4. Toxicologic and Pathophysiological Effects

2.3.4.1 Toxicologic

2.3.4.1.1 Drugs of Abuse

As described above amphetamine-related drugs of abuse are agonists at TAAR1 (Bunzow et al., 2001, Reese et al., 2007). Other drugs of abuse, for example cocaine, however, do not bind to TAAR1 (Revel et al., 2011). TAAR1 KO mice have been shown to have

increased sensitivity to the locomotor stimulating effects of d-amphetamine (Wolinsky et al., 2007; Lindemann et al., 2008) and show higher increases in extracellular monoamine (example dopamine) levels after d-amphetamine administration (Wolinsky et al., 2007). Also methamphetamine induced behaviours in TAAR1 KO mice were seen to increase (Miller, 2011). This suggests a role for TAAR1 as a modulator of amphetamine mediated effects (Miller, 2012).

2.3.4.1.2 Cheese Effect

The “cheese effect” is seen in some patients taking MAO inhibitors. The hypertensive crises (Blackwell, 1963) known as the cheese effect reflects an increase in blood pressure, severe headache, and sometimes brain hemorrhage or heart failure (Smith, 1980). Patients receiving MAO inhibitors will have increased levels of trace amines compared to normal individuals. When such sensitized patients take foodstuffs, such as aged cheeses, that are rich in tyramine, the levels may increase far in excess of physiological levels. Trace amines have been found in fermented products like cheese and especially many manufactured cheese products (El-zayat and El-Bagoury, 1988; Stratton et al., 1991). The discovery of TAAR present in the vasculature (Zucchi et al., 2006; Broadley, 2010) raises the possibility that these effects are TAAR-mediated rather than being through indirect sympathomimetic actions as was previously assumed (Maguire et al., 2009).

2.3.4.1.3 Migraine

Red wine, cheese and chocolate are frequently implicated in precipitating migraine (Peatfield et al., 1984; Fukui et al., 2008). Such food induced migraine has been

hypothesized to be due to foodstuffs which are high in trace amine levels (Hannington, 1967; Vaughan, 1994). For example, red wine has been shown to have non-volatile trace amines like tyramine and TRYP and volatile amines like PE (Ancin--Azpilicueta et al., 2008). As described earlier (see Section 2.3) the trace amines and other amines are metabolized by MAO. In wine, substances like ethanol and acetaldehyde inhibit MAO, and thus increase the effects of trace amines (Brink et al., 1990). Red wine has been shown to contain more trace amines than white (Ibe et al., 1991). Chocolates also contain trace amines especially PE (Ziegler et al., 1992). The most important trace amines and other biogenic amines found in cheese are tyramine, TRYP, PE, histamine, putrescine and cadaverine (Joosten, 1988a,b; Tawfik et al., 1992).

2.3.4.2 Pathophysiological

Trace amines have been associated with various human diseases particularly neuropsychiatric diseases like schizophrenia, depression and drug abuse and addiction (Berry, 2007). The physiological effects suggest they may also have relevance for other disorders like Parkinson's disease which affects dopaminergic transmission.

2.3.4.2.1 Schizophrenia

TAAR genes are tightly clustered on human chromosome 6q23.2 (Borowsky et al., 2001) a site which is close to the schizophrenia susceptibility locus SCZD5 (Duan et al., 2004). PE increases have been suggested to cause schizophrenia-like episodes (Davis et al., 1991; Kitamura et al., 2008). Sensorimotor gating deficits are seen in schizophrenia (Quednow et al., 2008; Moriwaki et al., 2009). In animal models prepulse inhibition of startle is an indicator of sensorimotor gating deficits (Braff et al., 1978). Prepulse

inhibition is a neurological phenomenon in which a weaker prestimulus (prepulse) inhibits the reaction to a subsequent strong startling stimulus (pulse). The reduction of the amplitude of startle reflects the ability of the nervous system to temporarily adapt to a strong sensory stimulus when a preceding weaker signal is given. TAAR1 KO mice show deficits in prepulse inhibition (Wolinsky et al., 2007) and thus were proposed as a new model for schizophrenia.

Schizophrenia patients also show an enhanced sensitivity to amphetamines (Breier, 1997; Laruelle et al., 1999) and an increase in the high affinity state of D2 receptors is seen (Corripio et al., 2006; Hirvonen et al., 2005). As described above amphetamines and D2-receptors are both suggested to interact with TAAR1 and thus an important role may be played by TAAR in the pathophysiology of schizophrenia. Olfactory deficiencies are often seen during the symptomatic phases of schizophrenia (Hurwitz et al., 1988; Kopala et al., 1989; Serby et al., 1990; Warner et al., 1990; Seidman et al., 1991; Kopala et al., 1992; Wu et al., 1993; Kopala et al., 1994; Houlihan et al., 1994; Malspina et al., 1994; Kopala et al., 1995; Brewer et al., 1996). TAAR are now known to be olfactory receptors (Liberles and Buck, 2006) and thus TAAR could be a possible common molecular substrate for psychiatric and sensory symptoms associated with schizophrenia.

2.3.4.2.2 Depression

Depression is thought to be caused by a deficiency of biogenic amine neurotransmission (Schildkraut, 1967; Lapin, 1969; Sandler, 1980; Davis and Boulton, 1994; Sabelli, 1996). Trace amines regulate neurotransmission (see Section 2.3.2) and therefore may play a role in depression. Clinically, decreased trace amine levels are suggested to correlate with

depressive symptomatology (Sandler, 1980; Davis and Boulton, 1994). Trace amines like PE and its precursor L-phenylalanine can improve the affective state of depressed individuals (Sabelli, 1996). Antidepressant drugs interact with the re-uptake proteins and MAO (Jarema et al., 2011). TAAR regulate re-uptake proteins (see Section 2.3), and MAO inhibitors cause increases in trace amine levels. Thus a role of trace amine systems in mood control is possible.

2.3.4.2.3 Parkinson's Disease

Parkinson's disease is caused by degeneration of the dopaminergic neurons in the substantia nigra (Dauer and Przedborski, 2003; Phani et al., 2012), which leads to a deficiency of dopamine (DA). Thus levodopa (3,4-dihydroxy-L-phenylalanine), the immediate precursor of DA, is used as a treatment to alleviate the symptoms of the disease (Katzenschlager and Lees, 2002). Continuous treatment with L-dopa has been questioned as it leads to other effects like dyskinesia (Cenci et al., 2009; Onofrij et al., 2009) and the 'On-Off' phenomenon (Nutt et al., 1984; Nutt, 1987; Menza et al., 1990). This is an increase in refractoriness to L-dopa's ability to control the smooth skeletal muscle movement in Parkinson's disease, where periods of excess abnormal movements alternate with periods of prolonged immobility or freezing, or symptom control followed by loss of control in response to L-dopa. Trace amines increase dopaminergic transmission, therefore can act as a novel therapeutic target. AADC is responsible for the conversion of externally administered L-dopa to DA. The level of trace amines also depends on the activity of AADC. AADC activity is altered depending on the stimulation of DA receptors (Rossetti et al., 1990; Hadjiconstantinou et al., 1993; Zhu et al., 1993). AADC may act as a rate limiting enzyme in the conversion of L-dopa to DA in

Parkinson's disease due to the large loss of AADC protein associated with degeneration. Administered L-dopa is converted to DA which in turn stimulates DA receptors and thus symptoms are controlled. However the increase in DA receptor stimulation causes less AADC activity so less conversion of L-dopa to DA and less DA receptor stimulation and loss of symptom control. AADC activity now starts to increase again due to decreased receptor activation and again conversion of L-dopa to DA and again control of symptoms. TAAR ligands which regulate DA receptor sensitivity may help control such "on-off" symptoms.

2.4 MEMBRANE PERMEABILITY OF NEURONAL MONOAMINES

In general trace amines do not appear to be actively stored in synaptic vesicles (Wu and Boulton, 1975; Philips and Boulton, 1979; Durden and Philips, 1980) although there may be both vesicular and non-vesicular pools of *p*-tyramine (Dyck, 1989). Consistent with this general lack of vesicle storage, trace amines also do not appear to be released in an activity dependent manner (Dyck et al., 1983). Since they are also predicted to be more lipophilic than the monoamine neurotransmitters (Oldendorf et al., 1971; Blakeley and Nicol, 1978; Mack and Bonisch, 1979) it has been assumed that trace amines readily diffuse across neuronal membranes (Berry, 2004). Consistent with this, Tcheransky et al., (1994) studied the transfer of tyramine across rat intestinal membrane and concluded it to be via simple diffusion. In contrast, Blakeley and Nicol (1978) concluded tyramine entry into erythrocytes occurred by facilitated diffusion. In brain slices, trace amine membrane passage was concluded to be most consistent with simple diffusion (Dyck et al., 1983, Dyck, 1989). Recent computer simulation studies, however, suggested a high

energy barrier for tyramine passage across a lipid bilayer (Berry et al., 2011) raising the possibility that the passage of trace amines is not merely passive.

In biological systems there are mainly two types of carrier mediated transport processes that are prevalent, facilitated diffusion and active transport. It is well known that many of the nutrients and chemical messengers in the body are transported using the two mechanisms. Transporters are present in all organs of the body. Thus due to the presence of transporters in the cells of these organs, homeostasis is maintained via exchange of chemicals needed for normal functioning. Transport of materials needs to occur in two directions, with distinct processes being present for the release of molecules from cells and for the entry of metabolites or their pre-cursors. The following sections will focus on these two processes as they relate to events at the synaptic cleft, which is the major site of action of the trace amines.

2.4.1 Release mechanisms

The release mechanism of neuronal monoamine neurotransmitters involves the fusion of vesicles filled with monoamine neurotransmitters with the plasma membrane through the process of exocytosis (Burger and Schafer, 1998; Ales et al., 1998). This requires adenosine 5' triphosphate (ATP) for initial priming of the apparatus and then the release is triggered by calcium whose cytoplasmic levels rise in response to membrane depolarization. In the case of trace amines the release is somewhat different as they do not appear to be stored in vesicles (Juorio et al., 1988) and K^+ -induced depolarization does not increase trace amine release (Dyck, 1989). In contrast, veratridine-induced depolarization of brain slices did increase tyramine release (Dyck, 1989), and on this

basis it was suggested that multiple synaptic pools of tyramine may exist, with a minor, veratridine-sensitive pool present.

2.4.2 Uptake processes

Synaptic cleft monoamine neurotransmitters are taken up back into the synaptic terminals by two active mechanisms referred to as uptake 1 and uptake 2. Uptake 1 relates to re-uptake and storage of monoamines in synaptic vesicles for re-use whereas Uptake 2 relates to the re-uptake and catabolism of monoamines (Lightman and Iversen, 1969). Uptake 1 depends on three conditions (Iversen, 1973): i] absence of bulky N-substituent group, ii] absence of methoxyl groups on the aromatic ring, and iii] presence of at least one phenolic hydroxyl group (Iversen, 1971). The re-uptake of synaptic monoamines is mediated through various transporters as described below.

The first monoamine transporter identified was the noradrenaline transporter (NAT) (Hertting and Axelrod, 1961) which was shown to play a role in the re-uptake mechanism into neurons. This was followed by the discovery of dopamine transporter (DAT) and serotonin transporter (SERT) (Iverson, 1971). These three transporters are important in the mechanism of psychostimulants and antidepressants, as well as in the physiological control of synaptic neurotransmitter levels (Barker and Blakley, 1995; Amara and Sonders, 1998; Miller et al., 1999). While the transporters show selectivity toward their preferred substrate, they also non-specifically transport a variety of compounds, including other neurotransmitter amines when their levels are elevated above physiological values (Giros et al., 1994).

DAT, NET and SERT are located on the pre-synaptic plasma membrane of neuronal terminals where they act to pump neurotransmitter out of the synapse back in to the pre-synaptic terminal (Torres et al., 2003). They have also been identified in the cell membranes of various organs such as the stomach, kidney, platelets and adrenal glands (Talvenheimo and Rudnick, 1980; Wade et al., 1996; Eisenhofer, 2001). All three transporters are dependent on a Na^+ concentration gradient, which is ultimately maintained by the plasma membrane Na^+/K^+ -ATPase (Rudnick and Clark, 1993; Gu et al., 1994), with Na^+ and Cl^- being co-transported with the monoamines (Torres et al., 2003). Cocaine acts as an inhibitor of monoamine transporters with moderate selectivity for DAT (Harris and Baldessarini, 1973; Heikkila et al., 1975; Kuhar et al., 1991) while amphetamine acts as a substrate of all the transporters, with little selectivity between the three (Torres et al., 2003). Amphetamines also cause the transporters to 'invert' and pump substrate amines out of the neuron into the synaptic cleft (Fleckenstein et al., 2007). As such a number of drugs of abuse can increase the synaptic monoamine levels through interaction with membrane transporters (Torres et al., 2003), resulting in inhibition of re-uptake and stimulation of efflux.

Trace amines are structurally related to amphetamine and are thus able to interact in a similar way with the monoamine transporters. PE, like amphetamine, can inhibit the uptake of DA, NA and 5-HT (Dyck et al., 1983). Amphetamine and PE are shown to increase DA synaptic levels through its interaction with DAT and vesicular storage transporters (Parker and Cubeddu, 1988). An inhibition of DA uptake by PE with an IC_{50} of 4.5 μM has been described (Raiteri et al., 1977), however, this value is not physiologically relevant. Similarly an inhibition of NA uptake by PE has been described

with an IC_{50} of 0.7 μ M (Raiteri et al., 1977). PE-induced inhibition of 5-HT uptake is less potent with an IC_{50} of 89.5 μ M (Raiteri et al., 1977). Similarly, tyramine also inhibits the uptake for DA, NA and 5-HT with IC_{50} values of 2.5 μ M, 0.3 μ M and 9 μ M (Raiteri et al., 1977). Octopamine is in general less potent with IC_{50} values at the DA, NA and 5-HT transporters of 15.7 μ M, 2 μ M and 64.1 μ M (Raiteri et al., 1977). A number of TRYP and amphetamine derivatives such as MDMA have been shown to interact with SERT with affinities ranging from 0.1 μ M to 100 μ M (Horn, 1973; Baumgarten et al., 1975; Horn, 1978; Rudnick and Wall, 1992; Hilber et al., 2005).

Separate transporter proteins are responsible for the loading of neuronal monoamines into synaptic vesicles. Vesicular amine transporter (VAT) includes the Vesicular acetylcholine transporter (VACHT) and the vesicular monoamine transporter (VMAT) which has two isoforms VMAT1 and VMAT2 (Eiden et al., 2004). VMAT transports monoamines like DA, serotonin, noradrenaline and histamine from cytoplasm into storage vesicles. Of the two isoforms, VMAT2 is the only vesicular monoamine transporter expressed in the CNS neurons (Eiden and Weihe, 2011). The transport mechanism is based on a proton gradient where the ratio is one amine into the vesicle for two protons leaving the vesicle (Eiden et al., 2004). Endogenous trace amines such as PE, tyramine and T1AM have been identified to be substrates for VMAT2 (Borowsky et al., 2001). Tyramine shows a similar affinity for VMAT 2 as DA with K_m values of 0.82 μ M (DA) and 0.20 μ M for tyramine (Partilla et al., 2006). In addition amphetamines interact with VMAT2 in a similar manner to that seen with DAT (Fleckenstein et al., 2007) while cocaine does not appear to interact directly with VMAT2 (Brown et al., 2001).

A large number of organic cation transporters are also present in most cell membranes that trace amines could conceivably interact with by virtue of their charged amine component. These transporters belong to a number of different families and are responsible for transporting various biologically important organic cations and amino acids across cellular membranes with varying degrees of specificity (Koepsell et al., 2003). For example the SLC-6 sub-family transports a variety of monoamines in a Na^+ and Cl^- dependent manner (Koepsell et al., 2003), while SLC-5 serves as a choline transporter, SLC-19 as a thiamine transporter and SLC-22 a general organic cation transporter (Koepsell et al., 2003). PE membrane transport has been reported to be independent of all organic cation transporters so far investigated (Fischer et al., 2010) while tyramine can act as a substrate for SLC22 (Iseki et al., 1993; Takahashi et al., 1993; Breidert et al., 1998) at 100nM. Although T1AM membrane transport has characteristics suggesting it is transporter mediated this did not appear to be via any characterized organic cation transporters (Ianculescu et al., 2009).

2.4.3 Methods for studying membrane passage

Non-cell based model systems have been extensively used for the prediction of membrane passage in the absence of transporter proteins. Historically this has often relied on simple polarity based predictions of the distribution of compounds of interest between polar and non-polar solvents. In addition, several more refined systems that utilize artificial membrane systems have been described.

2.4.3.1 Polarity-based predictions

Partition coefficient (log P) values are the ratio of the **distribution of a compound of interest in octanol to the distribution in water**. Log P values have been widely used to predict membrane permeability as it defines the lipophilicity of the drugs and is an important tool for prediction of *in vivo* absorption (Buchwald and Bodor, 1998; Avdeef, 2001). The major disadvantage is that log P does not provide an accurate measurement of the lipophilicity of ionizable compounds under physiological conditions as it describes the partition coefficient of molecules in non-buffered solutions. However, many thousands of compounds have been studied and data correlated to *in vivo* membrane passage. On this basis a number of on-line software tools have been developed to allow the prediction of log P scores (<http://www.vcclab.org/lab/alogps>; <http://www.acdlabs.com/resources/freeware/chemsketch/logp>; <http://www.chemdbsoft.com/Physical-Property-Prediction.php>).

Topological polar surface area (TPSA) scores are proposed to provide a superior prediction of drug transport properties based on the summation of surface contributions of polar fragments or atoms (Ertl et al., 2000). Again freely available on-line software (<http://www.molinspiration.com>) is available for the prediction of TPSA scores.

2.4.3.2 Artificial membrane based systems

Immobilized artificial membrane chromatography has been used as an *in vitro* screening technique for predicting drug membrane permeability (Pidgeon et al., 1995). Immobilized membranes consist of phosphatidylcholine residues which are covalently bound to silica propylamine HPLC beads with on column retention time taken as a measure of

membrane permeability (Thurnhofer et al., 1991; Liu et al., 1995). Large volume screening of drugs is theoretically possible. However, it is limited by the need to detect each compound of interest. As such, in order for this to be of general use it requires a generally available analytical technique that exhibits a general and sensitive response profile to a wide variety of chemical families. In reality this necessitates the use of mass spectrometer detectors. Further, while the system allows relative permeabilities to be determined it does not readily provide parameters such as lipid bilayer permeability (and/or diffusion) coefficients and diffusion half-life across a lipid bilayer.

Parallel artificial membrane permeability assays (PAMPA) have also been used to predict the permeability through lipid membranes. Here transport across multi-well plate inserts consisting of a hydrophobic membrane impregnated with egg lecithin are determined (Kansy et al., 1998). Again this has a high throughput capacity to screen large number of drugs while at the same time giving information regarding the lipophilicity, ionization and the solubility status of compounds (Kansy et al., 1998). It is limited by the necessity to perform end-point analytical detection for each compound of interest at a variety of time points. Again, for general, sensitive detection, this most often requires mass spectrometric facilities. Further, it is not readily modified to a continuous monitoring protocol.

Fluorosomes® are phospholipid vesicles containing a trapped fluorescent dye (Melchior, 2002). The entry of any compound of interest into these vesicles is detected by the quenching of the probe fluorescence. The only requirement is the compound of interest should quench fluorescence of the probe and the availability of a fluorescence spectrometer. This system has been proposed to be useful for the detection of the passage

of drugs across lipid bilayer membranes (Melchior, 2002), but has not been widely reported in the literature.

2.4.3.3 Cell culture

While the above techniques can provide information with respect to the ability of compounds to passively cross lipid bilayers, they provide no information about the passage across membranes under physiological conditions where active transport processes are also present. To obtain such information cell-based assays must be conducted. Initial screening of compounds is often done with one of two cell culture systems, Caco-2 or MDCK cells.

Caco-2 cell culture is a proven method that is widely used for membrane permeability studies (Artursson, 1990; Artursson et al., 1996; Hidalgo, 1996; Delie and Rubas, 1997). Caco-2 cells are human epithelial colorectal adenocarcinoma cells and are particularly useful for predicting the intestinal absorption of compounds of interest following ingestion. Since trace amines are found in a number of foodstuffs, it was of interest to determine their ability to cross Caco-2 cell membranes. The major disadvantage is the time required for monolayer formation is 21 days, a low throughput and sensitivity to the expression levels of active transporter systems which can lead to incorrect predictions of membrane permeability (Chong et al., 1996).

MDCK (Madin-Darby canine kidney) cells are an alternative cell-based system used to assess drug permeability (Irvine et al., 1999). These also form tight junctions (Misfeldt et al., 1976; Cereijido et al., 1978) and have been used as a model to assess drug transport (Cho et al., 1990; Cho et al., 1996). The major advantage over Caco-2 cells is that

MDCK cells grow faster giving a higher throughput as it requires 3 days to form a monolayer as compared to 21 days taken by Caco-2.

2.4.3.4 Synaptosomes

Synaptosomes are nerve cell endings which can be readily prepared from homogenized rat brain (Booth and Clark, 1978; Dodd et al., 1981). They are used to study the transport across neuronal membranes, and may have more relevance to trace amines than the above described cell culture methods. Synaptosomes retain all the necessary components required for neurotransmission which includes storage and release processes (Whittaker, 1993). The calcium internal resting concentrations (Verhage et al., 1988) as well as normal membrane potential regulated by a Na^+/K^+ -ATPase is maintained which together bring about the release of neurotransmitters (Verhage et al., 1991). Hence, synaptosomes are a useful model system for the study of membrane permeability of neurally active compounds (Breukel et al., 1997).

2.5 AIMS AND HYPOTHESIS

The trace amines were thought to diffuse across biological membranes easily with little energy barrier present. Recent computer simulation studies suggest that other mechanisms might exist (Berry et al., 2011) and that a significant energy barrier might be involved. The lipid bilayer permeability of trace amines has not previously been systematically studied in the absence of membrane transporters. Since membrane passage is required for post-synaptic effects, the nature of passage across membranes by trace amines will be directly measured. The lack of vesicular storage and depolarization-

induced release of trace amines previously reported suggests that trace amines may use different processes for membrane passage than neurotransmitters. Therefore, the membrane permeability of trace amines in presence and absence of transporters will be compared to the corresponding neurotransmitters.

2.5.1 Hypothesis

Trace amines membrane transport is by passive diffusion. The major objectives are to a) compare the ability of trace amines to diffuse across lipid bilayer membranes in the absence of membrane proteins to that of the structurally related monoamine neurotransmitters and b) examine the effect of the presence of membrane proteins in physiological membranes on the lipid bilayer permeability of a trace amine and its structurally related monoamine neurotransmitter.

3 MATERIAL AND METHODS

3.1 FLUOROSOMES

Membrane permeability of trace amines was measured using Fluorosomes® (GL Synthesis Inc. Worcester, MA). These are phosphatidylcholine vesicles with covalently trapped fluorescent dye. Studies were conducted using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) operating in kinetic mode and using black walled 96-well plates (Corning, Canada). The Fluorosomes® kit consists of a proprietary fluorophore, proprietary H. buffer and a standard solution of amiloride. Drug solutions were prepared in 18 MΩ water. All assays were conducted at 37°C, pH 7.4. Tyramine and octopamine were used at 46.9 mM, PE was used at 85 mM, DA and noradrenaline at 93.8 mM, TRYP 9.4 mM and 5-HT 2.3 mM. Preliminary studies were performed to determine whether individual compounds quenched fluorescence and what concentration of the individual compound would be required to produce quenching using the fluorophore alone. Fluorescence was measured with an excitation wavelength of 494 nm and emission at 523 nm. To determine whether quenching occurs with each compound, the fluorophore was added to individual wells of black walled 96-well plates. First 15 μL of fluorosome probe was added to 145 μL of buffer. Fluorescence was followed for 25 seconds after which 15 μL of compound of interest was added and fluorescence followed for a further 25 seconds. As per manufacturers instructions, only compounds causing a greater than 10% decrease in fluorescence were used in subsequent assays.

To determine membrane permeability 15 μL of Fluorosome solution was added to 145 μL of buffer and baseline fluorescence determined for 50 seconds at 1 second intervals. Fluorescence was determined for another 350 seconds at 2 second intervals after addition of 15 μL of compound of interest. Post-addition data was fit to a first-order decay function using GraphPad Prism 5.0 (La Jolla, CA). Equation parameters were then entered into the manufacturer's provided software algorithm to obtain permeability coefficients and diffusion half-lives. The mean \pm SEM of 6-8 independent experiments, each conducted on a different day, was determined for each compound. Differences between the corresponding neurotransmitter and trace amines were determined using an unpaired *t*-test with Welch correction using GraphPad InStat 3.10 software (La Jolla, CA) and statistical significance taken at a level of $p < 0.05$. On each day of assay, the permeability coefficient and diffusion half-life of the manufacturer's positive control (amiloride) was determined and confirmed to be within 10-15% of accepted values.

3.2 PARTITION COEFFICIENT, TOPOLOGICAL POLAR SURFACE AREA AND DISTRIBUTION COEFFICIENTS.

Log *P* values and TPSA values were obtained from online software (www.molinspiration.com). Although log *P* values have been used to predict membrane transport, under physiological conditions trace amines and neurotransmitters are in a buffered solution. Thus a distribution coefficient (log $D_{7.4}$) was also determined for compounds distribution between octanol and phosphate-buffered saline (PBS; 50 mM, pH 7.4).

A modification of the shake flask (OECD guideline section 1) method was used to determine $\log D_{7.4}$ values. Compounds were dissolved in 2 mL octanol and then 2 mL 50mM PBS (pH = 7.4) was added in 15 mL Falcon tubes (VWR International, Mississauga, ON). The solution was incubated at 37°C for 6 hrs with constant shaking. The octanol and the PBS layers were separated and the concentration of compound in each phase determined by measuring absorbance at 280 nm using an Agilent 8453 UV diode spectrophotometer (Agilent, Brockville, Ontario) (single beam) with collimator and comparison to standard curves prepared the day of each experiment. Quartz cuvettes (Hellma Quartz Semi micro Cells, 10 mm path length rectangular cells) were used for all determinations. Preliminary experiments determined the solubility of each compound in the two phases and the linear range for determination of standard curves. Suitable starting concentrations based on the anticipated distribution for each compound and linear range of each standard curve, were determined to be 600 μ M for DA, tyramine, octopamine and TRYP, and 1 mM for noradrenaline.

3.2.1 Data Analysis

Log $D_{7.4}$ data represent mean \pm SEM of 9-14 independent experiments. Data for a trace amine and its corresponding neurotransmitter were compared by unpaired *t*-test with Welch correction for unequal standard deviations. Significance was taken at a level of $P < 0.05$.

3.3 Caco-2 CELL CULTURE

3.3.1 Materials

DMEM High with sodium pyruvate, T-75 Flasks and HBSS were obtained from Fisher Scientific (Ottawa, Ontario); Fetal Bovine Serum, MEM-NEAA, Transwell CL 6.5 mm plates, Versene, and Trypan blue were obtained from Gibco (Burlington, Ontario); Trypsin 2.5%, pargyline, sodium diethyldithiocarbamate trihydrate, Lucifer yellow and HEPES were obtained from Sigma-Aldrich, (Oakville, Ontario); OR-486 was obtained from Tocris Bioscience (Ellisville, MO); [³H]-tyramine from American Radiochemicals Company (St. Louis, MO); and [³H]-dopamine from Perkin Elmer (Boston, MA). Caco-2/TC-7 cells were a kind gift from Dr. Wolfgang Koester, Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan (Saskatoon, SK) having a passage number of 40-45.

3.3.2 Cell Culture

Caco-2 cells were cultured as previously described (Sun and Pang, 2008). Complete media was prepared by adding 50 mL of FBS and 5.5 mL MEM-NEAA to 500 mL of DMEM media. Frozen stocks of cells were thawed quickly at 37°C and were transferred into a centrifuge tube containing 5 mL of complete media. The tube was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended into 10 mL of fresh media by pipetting up and down several times. A T-75 cell culture flask was filled with 15 mL of media and 0.5 mL of cell suspension was added to the T-75 flask containing the media. Flasks were incubated at 37°C in 5% CO₂ until cells were

confluent. The media was changed every 2-3 days and the confluent cells were then sub-cultured as described below.

3.3.2.1 Subculture of Cells

Media from confluent cells was removed and cells rinsed with 5 mL of Versene. After rinsing the flask with Versene, 2 mL Versene was added again and 4-5 drops of 2.5% trypsin solution (final concentration of trypsin is 0.5%) was added. The flask was incubated at 37°C and 5% CO₂ for 3-4 minutes and 5 mL of complete media was added to stop the reaction. The cell suspension was collected in a centrifuge tube and centrifuged at 1000 rpm for 5 minutes. Medium was decanted and cells suspended in 10 mL of complete media by pipetting up and down several times. Another T-75 flask containing 15 mL of complete media was taken and 0.5 mL of the cell suspension was added.

3.3.2.2 Culture into Transwell® Plates

Cells, at passage number 40-45, were harvested when they were more than 90% confluent as described above. Cell suspension (90 µL) was added to 10 µL of trypan blue solution and cell viability was determined as follows. A Neubar's chamber was loaded with 10 µL of mixture. Four quarters containing 16 squares were counted and the average was taken. This average was multiplied by 1.11×10^4 to determine the number of cells per mL. The basal compartment of a transwell plate was filled with 600 µL of complete media. The apical compartment was filled with 200 µL of cell suspension containing 1×10^5 cells as previously described (Sun and Pang, 2008). The plate was incubated at 37°C and 5% CO₂ and the media was changed every alternate day for 21 days. On the 21st

day Trans-epithelial electric resistance (TEER) values were measured as described below.

3.3.2.3 Trans-epithelial Electrical Resistance

TEER electrodes (Millipore, Billerica, MA) were dipped into 70% alcohol for 2 minutes and then transferred into media for 10-15 minutes. In the voltage mode, the voltage was adjusted to zero. Similarly in resistance mode, the resistance was adjusted to zero. Electrodes were inserted into the apical and basal sides of the transwell plate and the resistance measured. TEER values between 500-900 Ω indicates integrity of the monolayer.

3.3.2.4 Lucifer yellow (LY)

Post-assay Lucifer yellow (LY) rejection rate was measured to assess monolayer integrity at the end of the permeability assay. Caco-2 monolayers were washed three times with the transport medium. After a 30-min equilibration period, transport media on the apical side was replaced with 0.2 mL of Lucifer yellow solution (100 $\mu\text{g}/\text{mL}$). Blank solution (600 μL , in this case HBSS + inhibitors) was added to the top row wells of the plate and bottom row wells consist of 400 μL blank plus 200 μL Lucifer yellow and the plate was incubated for 1 h at 37°C under 5% O₂, 95% CO₂ and 95% humidity for 1 h. Lucifer Yellow fluorescence emission (excitation wavelength = 485 nm, emission wavelength = 535 nm) in the basal compartment was read using a Biotek Synergy HT microplate reader (Fisher Scientific, Nepean, ON, Canada) and the concentration determined by comparison of fluorescence emission of basal compartment against standards after background subtraction. Percent Lucifer yellow rejection was calculated from the

fluorescence values and Lucifer yellow rejection was calculated using the following equation.

$$\text{LY rejection} = 100 - \% \text{ LY rejection}$$

3.3.2.5 Permeability Experiment

Modified Hank's balanced salt solution (HBSS) consists of HBSS (CaCl₂·H₂O 186 mg/mL, KCl 400 mg/mL, KH₂PO₄ 60mg/mL, MgSO₄·7H₂O 200 mg/mL, NaCl 8 mg/mL NaHCO₃ 350 mg/mL, Na₂HPO₄·7H₂O 90 mg/mL and glucose 1000 mg/mL) with 20 mM HEPES, pH 7.4. All compounds were prepared in this modified HBSS. All solutions also contained three inhibitors of monoamine metabolism. Pargyline was used at 10 μM concentration to inhibit MAO-A and MAO-B (Murphy et al., 1998). OR-486, which is a COMT inhibitor, was used at 2.5 mM concentration (Nissinen et al., 1988). Sodium diethyldithiocarbamate trihydrate was used at 100 μM to inhibit dopamine-β-hydroxylase (Szmigielski, 1975). The monolayers were rinsed three times with 200 μL of modified HBSS on the apical side and 600 μL on the basal side. [³H]-Tyramine and [³H]-dopamine additions were either done on the apical (upper chamber) or the basal (lower chamber) side. For apical side addition, after washing the cells, the apical side was filled with 190 μL of modified HBSS containing the inhibitors. The basal side received 600 μL of modified HBSS with inhibitors. Similarly for the basal side addition, after washing the cells, the apical end was filled with 200 μL of modified HBSS with inhibitors and the basal side was filled with 590 μL of modified HBSS with inhibitors. [³H]-Tyramine or [³H]-dopamine was added to either the apical or basal sides in 10 μL volume to give final concentration of 100 nM for tyramine and 10 μM for DA. Cells were incubated for

varying time periods (5, 10, 20, 30, 45 and 60 minutes) at 37°C in 5% CO₂ atmosphere following the addition of radiolabelled compound. After incubation, the apical and the basal solutions were transferred into scintillation vials. Fresh modified HBSS containing inhibitors, 200 µL for the apical side and 600 µL for the basal side, was added to each side of the insert. The apical wash solution was transferred to a separate scintillation vial and replaced with Lucifer yellow solution (100 µg/mL in HBSS). After 1 hour of incubation at 37°C, inserts were removed, and the plate was taken for fluorescence reading at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The insert solution was combined with the apical wash solution in a scintillation vial and replaced with NP-40 cell lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP-40, 2 mM EDTA) in another 24-well plate and incubated for 30 minutes at 37°C. After incubation this was combined in the scintillation vial with the Lucifer yellow and wash solutions.

Ten mL of scintillation cocktail (Ready safe®) was added to each scintillation vial and after at least 4 hours the vials counted for tritium using a Beckman beta counter (Mississauga, Ontario) having a counting efficiency of 0.6. The amount of radioactivity present in the apical solution, basal solution and cells at each time point was converted to moles of tyramine or DA by comparison to a standard curve.

3.4 SYNAPTOSOMES

3.4.1 Preparation of Synaptosomes

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Male Wistar rats (200-250g) were deeply anesthetized with urethane (1 g/kg i.p.). They were then decapitated and the brain was dissected out and kept in 0.9% ice cold NaCl. Synaptosomes were prepared as described by Miller et al., (2005). The brain was diced on ice and homogenized using a Teflon-coated manual homogenizer in 10 volumes of 35 mM sucrose solution. Then, the homogenate was centrifuged at 1000g for 10 minutes at 4°C. The supernatant was collected and the pellet discarded. The supernatant was centrifuged again at 10,000g for 20 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 2 mL of ice-cold assay buffer. The assay buffer consists of 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 10 µM pargyline, 100 µM diethyldithiocarbamate, 2.5 µM OR-486, 2 µg/mL glucose, 0.2 µg/mL ascorbic acid and pH at 7.5.

3.4.2 Uptake Assay

Eppendorf tubes (1.5 mL) were filled with 50 µL synaptosomes and either 10 µL of [³H]-dopamine (specific activity = 38 Ci/mmol) or [³H]-tyramine (specific activity = 40 Ci/mmol). Tyramine was added at a concentration of 100 nM and DA was added at a concentration of 10 µM. The tubes were then incubated for 1, 2, 3, 4, 5, 10, 20 and 30 minutes in a water bath at 37°C. The reaction was stopped by adding 1 mL ice-cold buffer. The tubes were then centrifuged at 10,000g for 3 minutes at 4°C. The supernatant

was collected in scintillation vials for counting for radioactivity. The pellet was re-suspended in 0.5 mL NP-40 lysis buffer and incubated at 37°C for 30 minutes. The lysis solutions were transferred to scintillation vials for counting as previously described.

3.4.3 Release Assay

Eppendorf tubes with 50 µL of synaptosomes were incubated with 10 µL of radiolabelled tyramine or DA for 10 minutes and the reaction was stopped with 1 mL ice-cold assay buffer as described above. The tubes were then centrifuged at 10,000g at 4°C for 3 minutes. The supernatant was discarded and the pellets were re-suspended in 0.5 mL of ice-cold buffer and all the tubes centrifuged again. Supernatants were discarded and one of the pellets, time 0 minute tube was re-suspended in 0.5 mL buffer and immediately centrifuged as described above. The supernatant was collected and the pellet was resuspended in lysis buffer and incubated for 30 minutes and then counted for radioactivity. The remaining pellets were resuspended in 0.5 mL incubation buffer and incubated for 1, 2, 3, 4, 5, 10, 20 and 30 minutes at 37°C. After incubation, tubes were centrifuged at 10,000g for 3 minutes at 4°C and supernatants collected in separate scintillation vials and pellets kept on ice. All of the pellets were then re-suspended with 0.5 mL lysis buffer and incubated for 30 minutes at 37°C and then collected in separate vials for counting.

3.4.4 Release in Depolarized Synaptosomes Assay

The studies were conducted as above with the exception that the pellets were suspended in a high potassium (25 mM) assay buffer for the release assay.

3.4.5 Protein Assay

Bradford's protein assay kit (Bio-Rad, Hercules, CA) was used in which standard serum albumin was used to make different concentration of standards from 1000 μg to 50 μg protein. All assays were conducted using 96-well assay plates. For determination of the protein content of synaptosomes, 5 μL of synaptosome preparation was used and 200 μL of the Bradford reagent added and the plates incubated in the dark for 15 minutes at room temperature. Absorbance was measured at 595 nm wavelength. The protein concentration in each sample was calculated from the standard curve. All assays were conducted in triplicate and the average taken for each sample.

3.4.6. Data Analysis

The uptake studies were compared by plotting the amount of compound of interest in supernatant and synaptosomes against time. For the release data the amount of compound released was plotted against time and a one-phase decay function was applied using Graphpad Prism. Rate constants and half-lives were obtained from the line fit parameters. Rate constants were statistically compared by an F-test using GraphPad Prism and significance taken at $P < 0.05$.

4 RESULTS

4.1 FLUOROSOMES

Preliminary studies determined the concentration of the compounds required to quench the fluorescence of the probe (Figures 3 and 4). PE was found not to quench the fluorescence of free probe at any concentration used (Figure 5) and, hence, could not be investigated further. Compounds showing greater than 10% quenching of the free fluorophore were investigated further. Representative graphs of the quenching of fluorosome fluorescence by amiloride, DA and tyramine, NA and octopamine, and for 5-HT and TRYP are shown in figures 7-9, respectively.

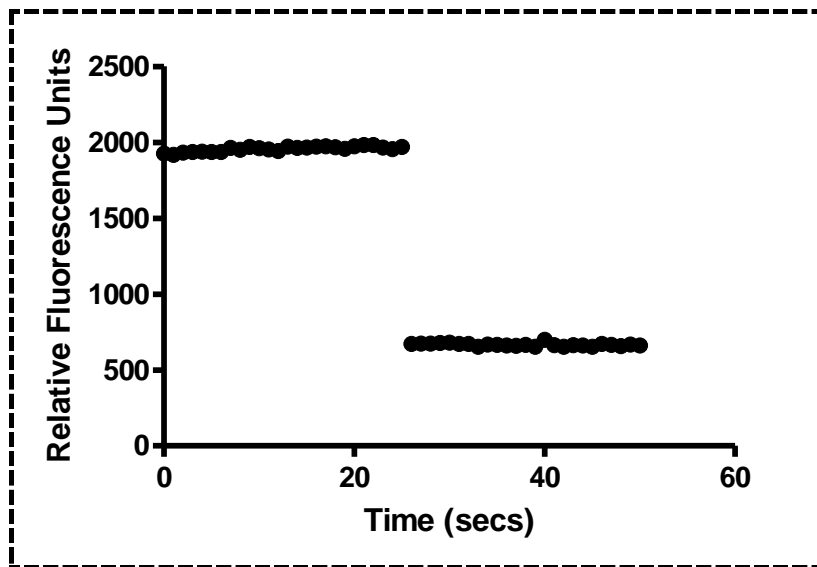


Figure 3 : Effect of 9.4 mM TRYP on free fluorosome probe fluorescence.

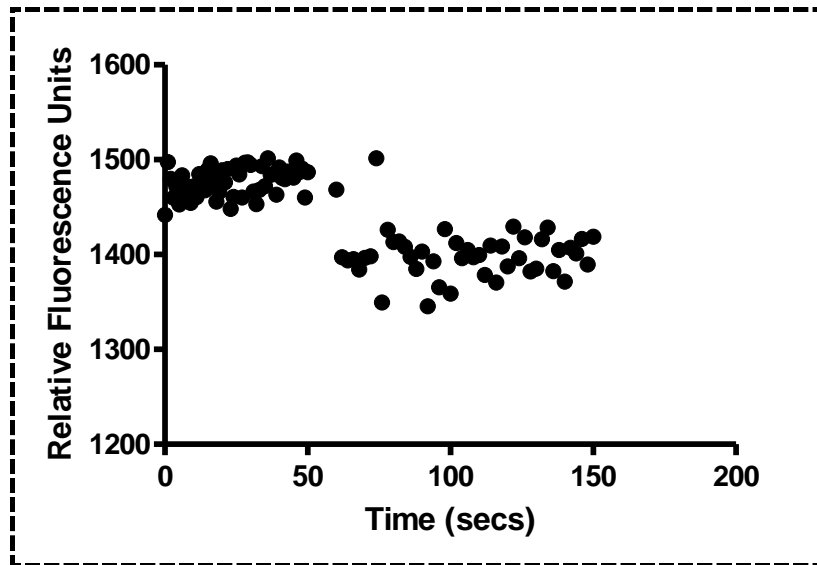


Figure 4 : Effect of 6.25 mM tyramine on free fluorosome probe fluorescence.

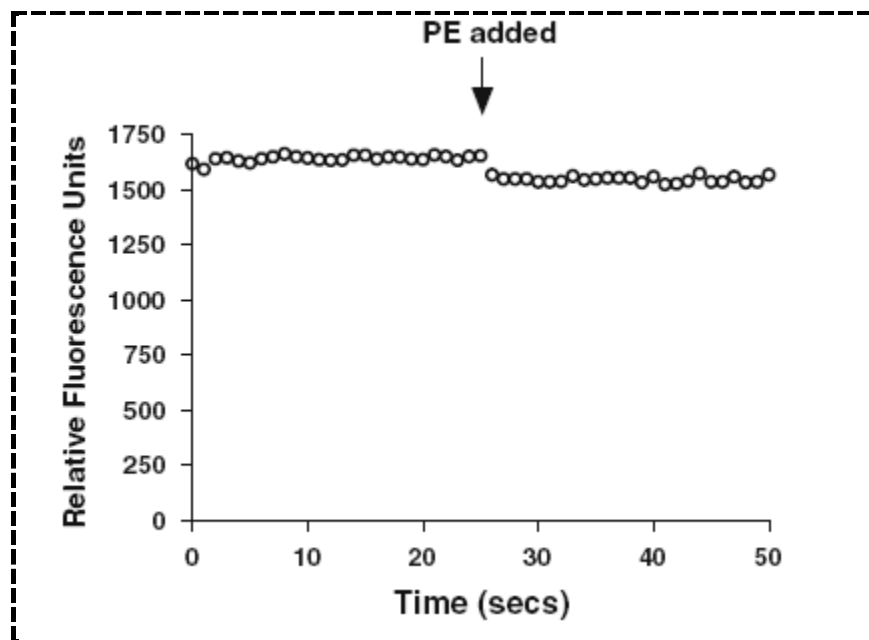


Figure 5: Effect of 85 mM PE on free fluorosome probe fluorescence.

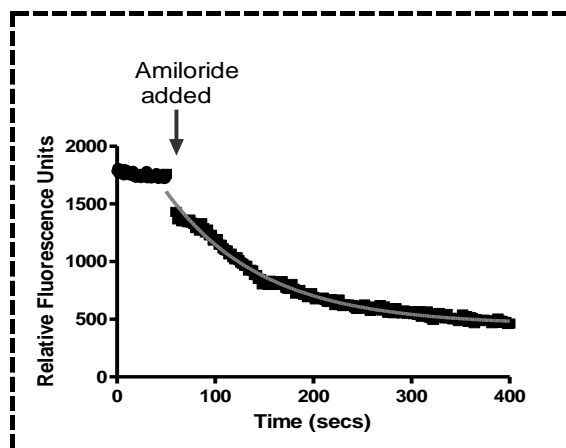


Figure 6: Effect of amiloride on fluorosome fluorescence.

On addition of amiloride the compound quenched fluorescence as seen as a drop from baseline depicted by the curve over 400 seconds.

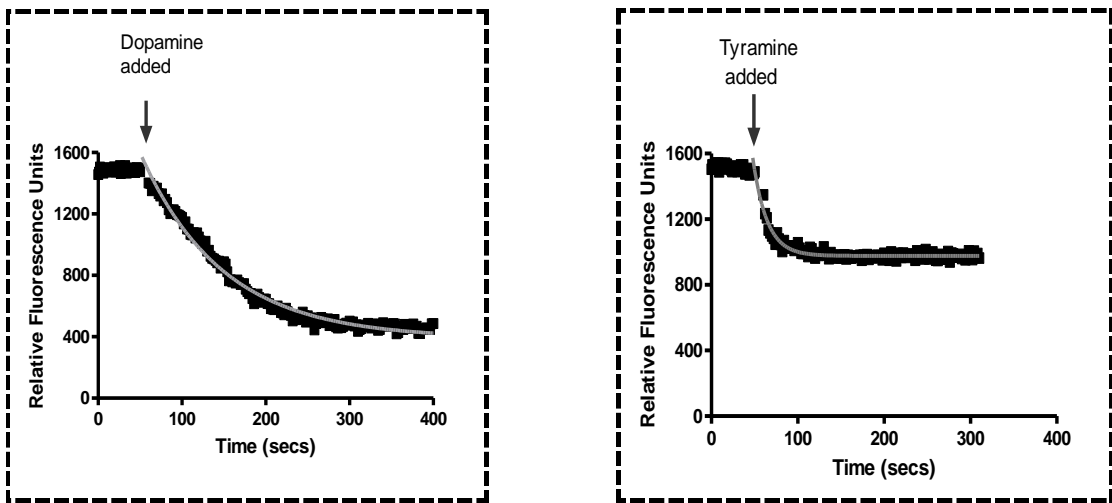


Figure 7: Quenching of Fluorosome fluorescence following addition of 93.8 mM dopamine and 46.9 mM tyramine.

DA showed a slower quenching of fluorescence from the baseline as compared to tyramine which quenched relatively fast.

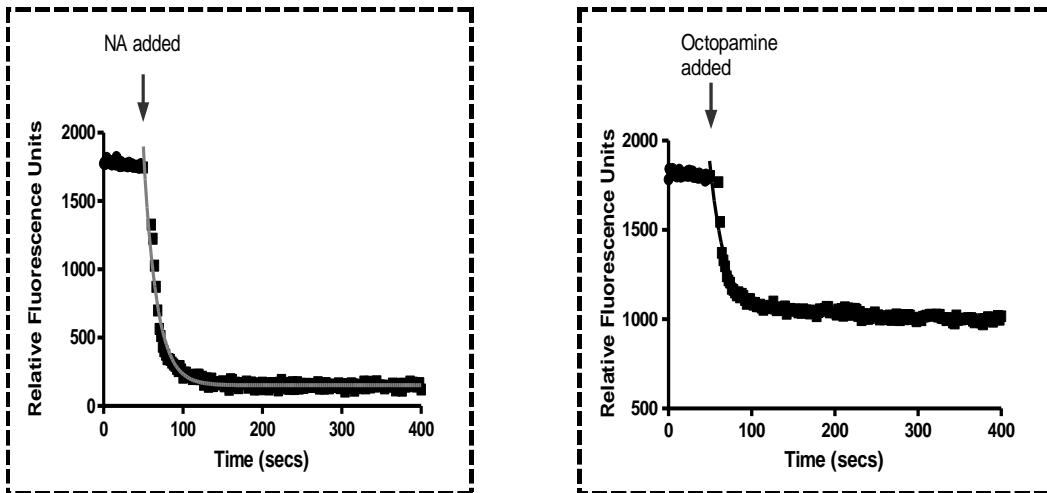


Figure 8: Quenching of Fluorosome fluorescence following addition of 93.8 mM NA and 46.9 mM OCT.

NA and OCT showed almost similar quenching pattern over 400 seconds with no significant differences from each other.

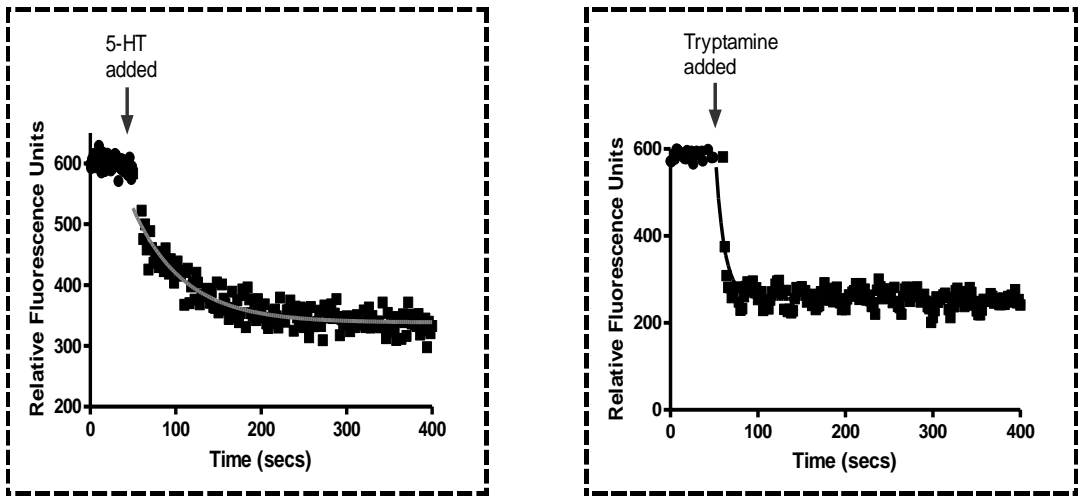


Figure 9: Quenching of Fluorosome fluorescence following addition of 2.3 mM 5-HT and 9.4 mM TRYP.

5-HT and TRYP both quenched fluorescence. TRYP quenched almost as soon as it was added as compared to a slower quenching shown by 5-HT.

The permeability coefficient and the diffusion half-lives obtained for each compound are shown in Table 3. A significant ($p < 0.01$) three-fold reduction in permeability coefficient was observed when DA was compared to *p*-tyramine. Similarly, 5-HT permeability coefficient was significantly ($p < 0.001$) smaller (7 times) than TRYP permeability coefficient. Half-lives were correspondingly changed with DA showing a 2.6-fold greater half-life than *p*-tyramine ($p < 0.01$), and 5-HT a 7-fold greater half-life than TRYP ($p < 0.001$). NA permeability coefficient and half-life were not statistically different from those of OCT.

Table 3: Biogenic amine permeability coefficient and diffusion half-lives across Fluorosome lipid bilayer membranes.

COMPOUNDS	PERMEABILITY COEFFICIENT (Å/sec)	HALF-LIFE (sec)
DOPAMINE	7.7 ± 1.2**	35.9 ± 7.0**
TYRAMINE	22.6 ± 4.3	13.5 ± 4.1
NORADRENALINE	20.3 ± 3.8	14.7 ± 3.4
OCTOPAMINE	19.1 ± 2.9	12.5 ± 1.7
5-HT	4.8 ± 0.6***	48.2 ± 5.9***
TRYPTAMINE	33.2 ± 3.3	6.8 ± 0.7
AMILORIDE	2.2 ± 0.4	134.1 ± 36.7

**Significantly different at $p < 0.01$

***Significantly different at $p < 0.001$. Data represent mean ± SEM, n = 6-9. Pairs of compounds were compared by unpaired t-test with Welch correction for unequal standard deviations.

The permeability coefficient and half-lives of the three trace amines were compared by one-way ANOVA revealing a marginally significant ($p < 0.05$) difference between groups in permeability coefficient, but not half-life. Differences between groups were tested with a Bonferroni multiple comparisons post-hoc test, with a significant difference ($p < 0.05$) observed between TRYP and OA permeability coefficients. Since the permeability coefficients of trace amines in general showed greater values and the diffusion half-lives were smaller than the neurotransmitters, all the values were pooled together and compared using a Mann-Whitney U-test (Figure 10). Trace amines as a group showed statistically ($p < 0.0001$) greater permeability coefficient and correspondingly lower diffusion half-lives than the neurotransmitters.

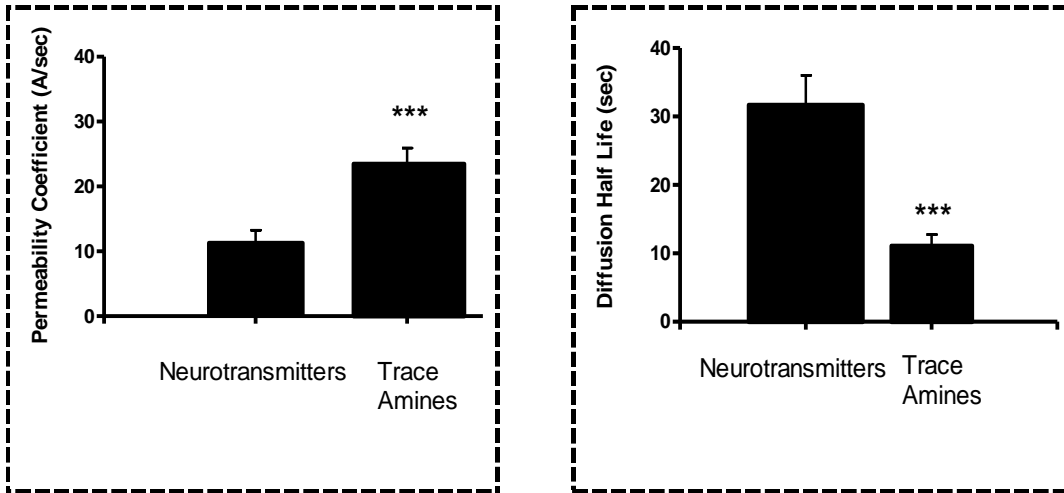


Figure 10: Comparison of pooled neurotransmitter and trace amine membrane permeability measures.

Pooled data compared using Mann-Whitney U-test.

*** Trace amines as a group show statistically ($p < 0.0001$) greater permeability coefficient and corresponding lower diffusion half-life than the corresponding neurotransmitters.

4.2 PARTITION COEFFICIENT, TOPOLOGICAL POLAR SURFACE AREA AND DISTRIBUTION COEFFICIENTS.

Predicted log P and TPSA values were obtained from the molinspiration website (<http://www.molinspiration.com/cgi-bin/properties>) as a potential predictor of distribution between aqueous and lipid phases, and are shown in Table 4. 5-HT log $D_{7,4}$ value could not be determined as 5-HT did not dissolve in octanol at a concentration that could be detected by UV spectroscopy. The predicted values from Table 4 were plotted against the permeability coefficients determined from Fluorosome studies and examined for signs of a relationship (Figure 11). There was no obvious trend observed.

Table 4: Comparison of Predicted log P, Predicted TPSA and experimentally determined log $D_{7.4}$ values.

	Predicted log P	Predicted TPSA	log $D_{7.4}$
Dopamine	-0.047	66.479	-0.68 ± 0.07
p-Tyramine	0.442	46.251	-0.79 ± 0.03
Noradrenaline	-1.038	86.707	-0.90 ± 0.05
p-Octopamine	-0.549	66.479	-0.65 ± 0.04**
5-HT	0.568	62.042	
Tryptamine	1.071	41.814	0.21 ± 0.02
Amiloride	-1.244	156.803	

Log $D_{7.4}$ data represent mean ± SEM of 9-14 independent experiments. Pairs of log $D_{7.4}$ data were compared by unpaired t-test with Welch correction for unequal standard deviations. ** $p < 0.001$.

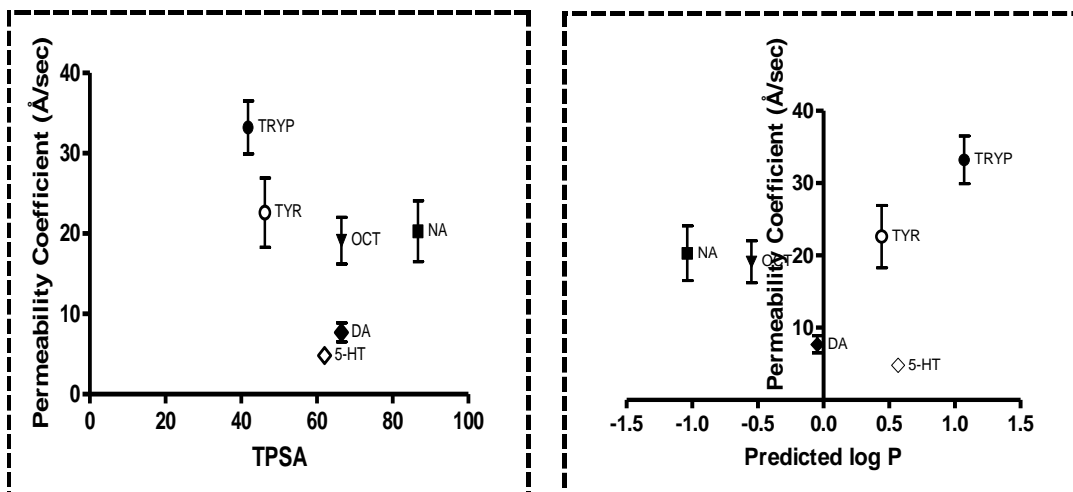


Figure 11: Relationship of predicted TPSA and log P scores to Fluorosome membrane permeability coefficients.

Since there was no obvious correlation between either predicted log P or TPSA scores and Fluorosome permeability data, the distribution between octanol and a PBS solution buffered to physiological pH was determined. Standard curves in octanol and PBS were made for each compound by using a wide range of concentrations and measuring their absorbance (Figures 12-15). From these data, the distribution of compounds between octanol and PBS phases could be determined allowing the calculation of log $D_{7.4}$ (Table 4). Pair-wise comparison was done between DA and tyramine, noradrenaline and octopamine of which noradrenaline and octopamine were significantly different ($p < 0.001$). TRYP log $D_{7.4}$ values could not be determined as the compound was not sufficiently soluble in octanol and PBS to allow its detection by UV spectroscopy.

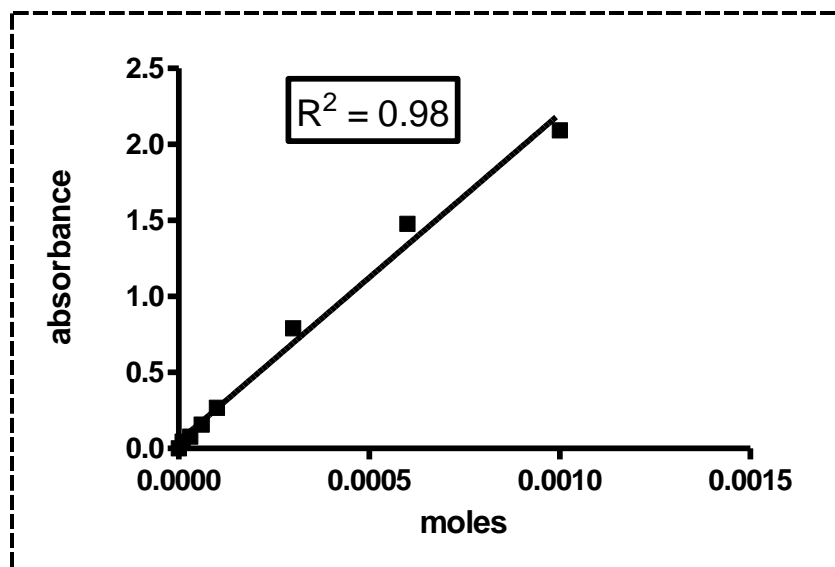


Figure 12: Representative standard curve of dopamine in PBS.

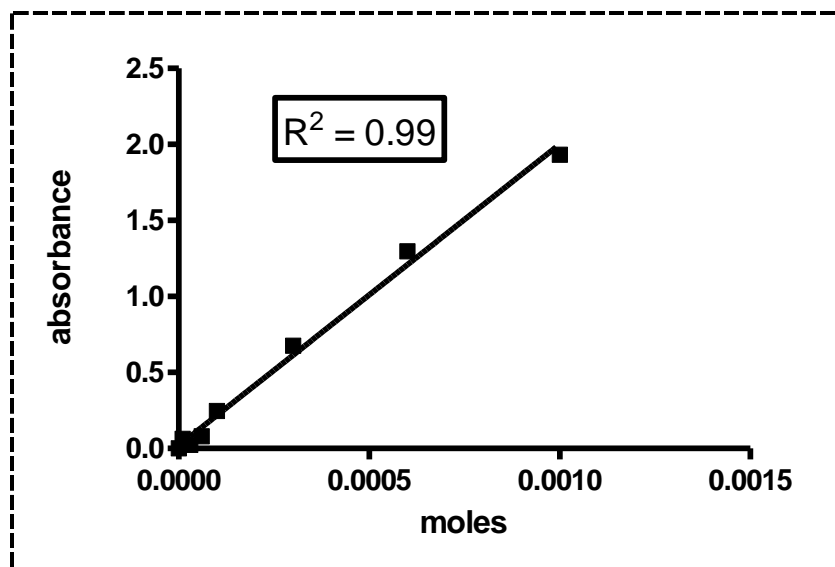


Figure 13: Representative standard curve of dopamine in octanol.

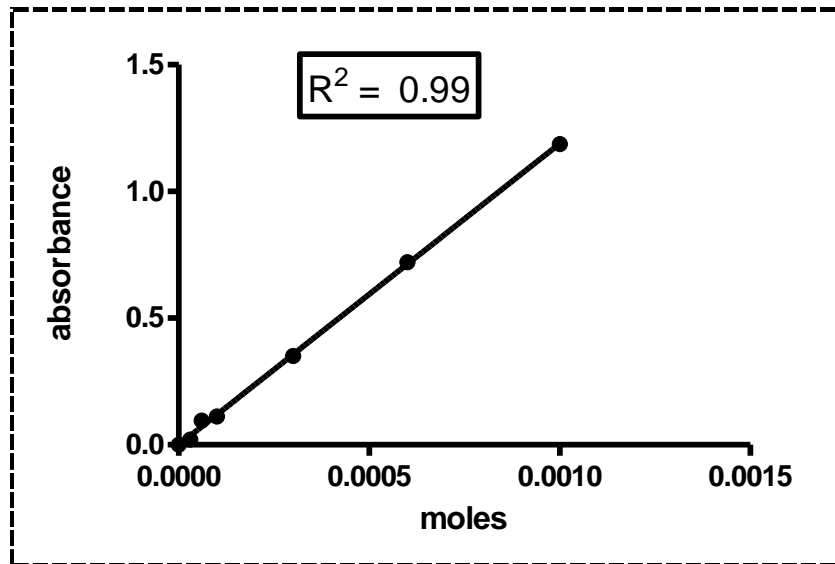


Figure 14: Representative standard curve of tyramine in PBS.

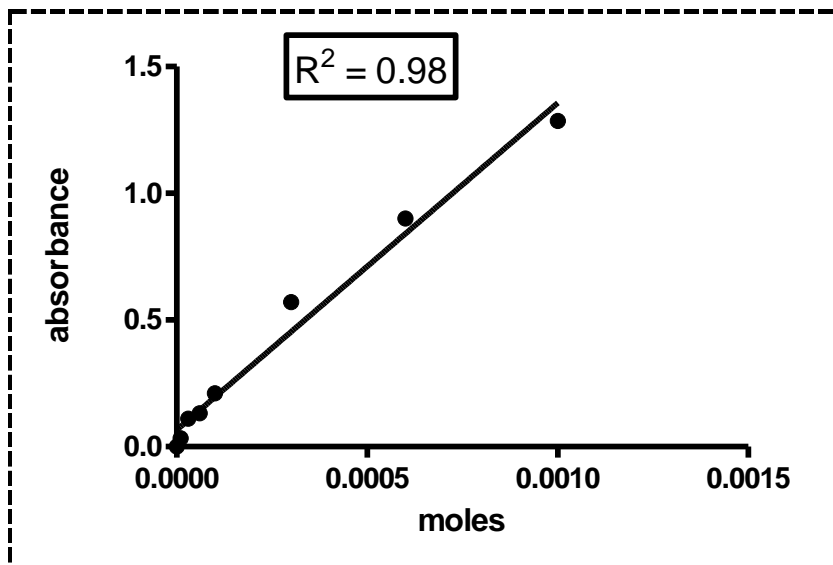


Figure 15: Representative standard curve of tyramine in octanol

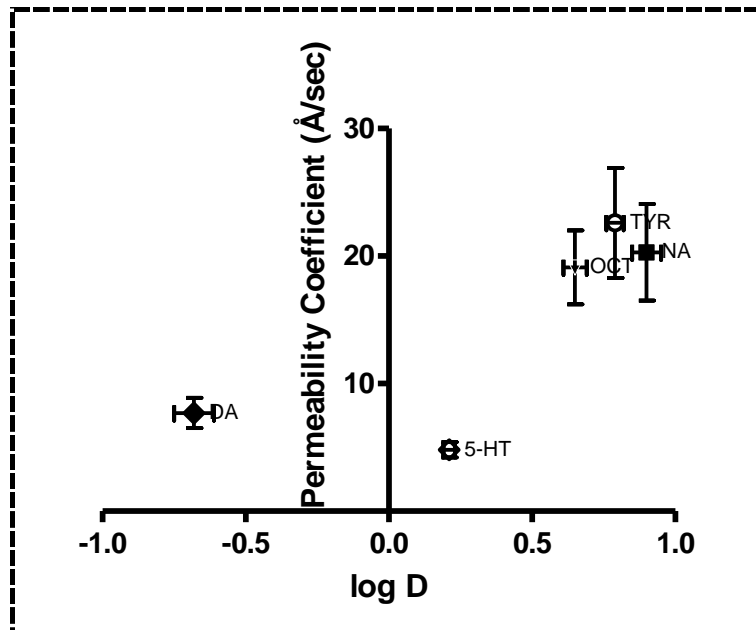


Figure 16: Relationship of $\log D_{7.4}$ to Fluorosome membrane permeabilities.

The calculated $\log D_{7.4}$ values were also plotted against the permeability coefficient values determined from Fluorosome studies as shown in figure 16. This showed more evidence of a predictive relationship than was observed with $\log P$ and TPSA scores.

4.3 CELL CULTURE

Caco-2 cell culture was used to determine the permeability of one neurotransmitter [^3H]-dopamine and one trace amine [^3H]-tyramine. These were selected as they were readily available and cost effective as compared to others. Tyramine accumulation (~7-8% of administered concentration) was approximately twice that of DA (3-4%). From Figures 17 and 18 it can be seen that equilibration of both tyramine and DA occurred in less than 10 minutes. Initial studies were conducted with 5-minute sampling with equilibration already evident (data not shown). The flux constant could not be determined as equilibration occurred at such short time points that precluded accurate sampling. Representative standard curves to allow the calculation of tyramine and DA concentrations are shown in figures 19 and 20.

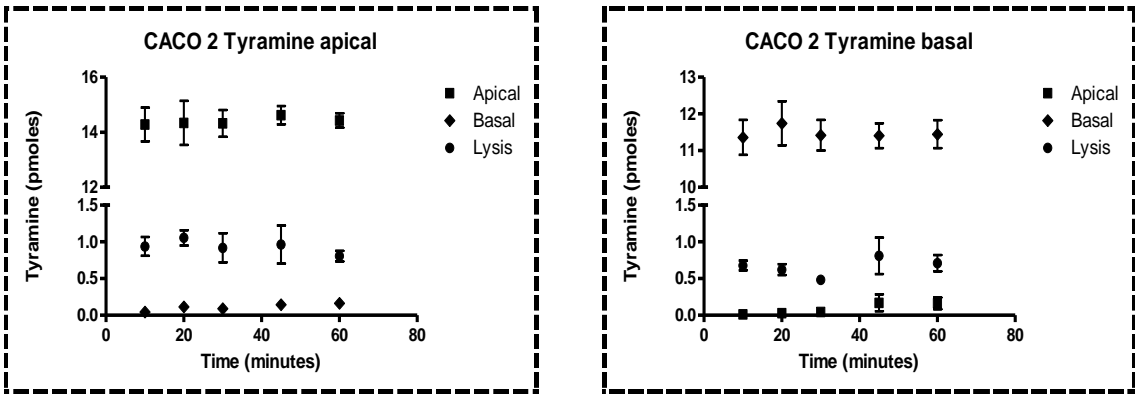


Figure 17: Permeability of [³H]-tyramine in Caco-2 cell culture upon apical and basal additions.

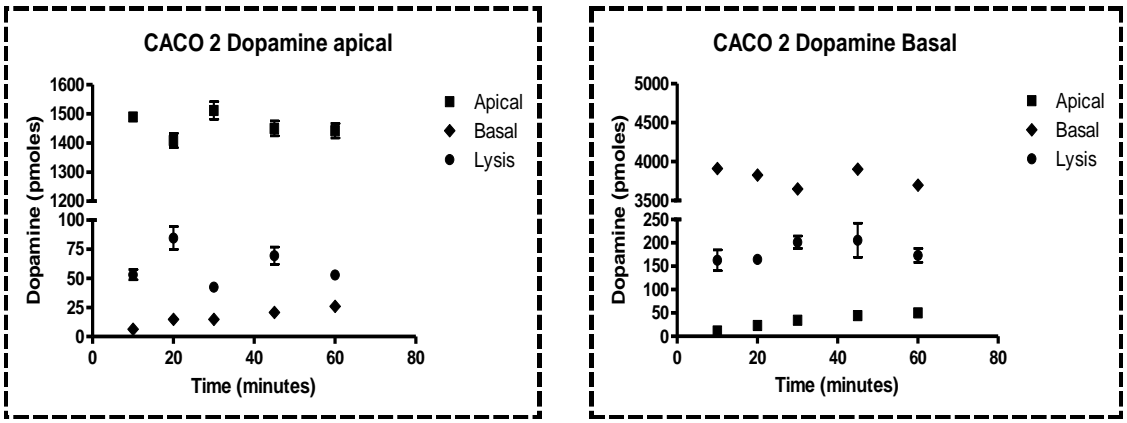


Figure 18: Permeability of [³H]-dopamine in Caco-2 cells following apical and basal addition.

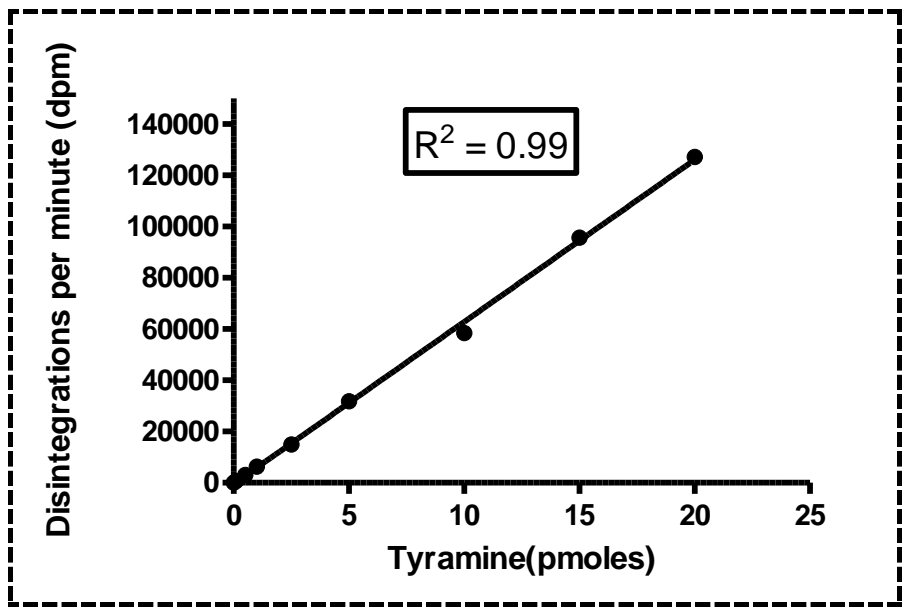


Figure 19: Standard curve for [³H]-tyramine application.

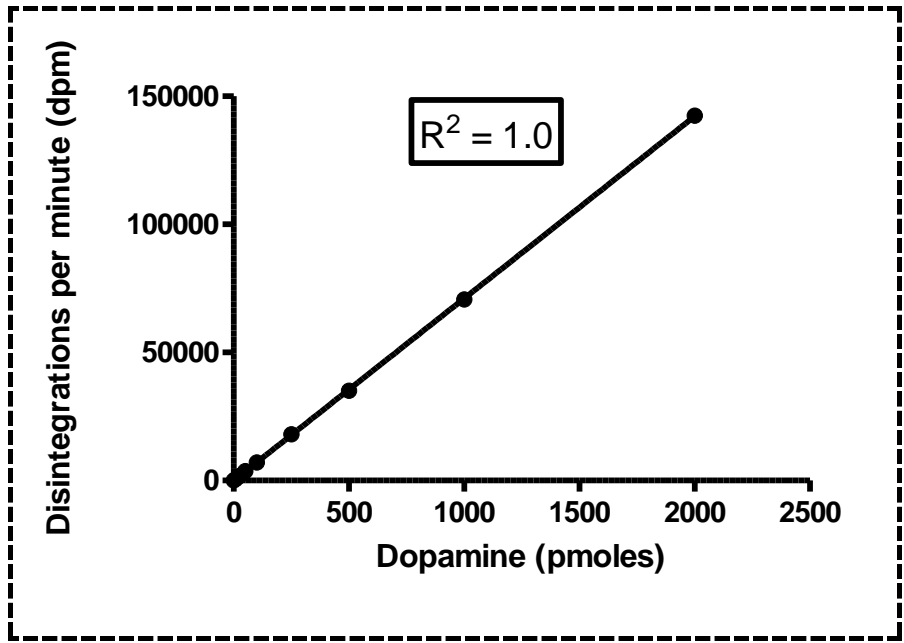


Figure 20: Standard curve for [³H]-dopamine application

4.4 SYNAPTOSOMES

Caco-2 cell culture predicts the intestinal permeability whereas synaptosomes can provide a measure of neuronal permeability. Since trace amines are synthesized neuronally as well as being ingested in foodstuffs, the uptake and release of [³H]-dopamine and [³H]-tyramine were also determined in rat brain synaptosomes. The radioactivity from the supernatant and synaptosomes was counted at individual time points and the amount of the radioactive drug determined by comparison to standard curves. Total protein present in the synaptosome pellets was determined by Bradford's protein assay (Figure 21). The amount of each compound taken up into synaptosomes, corrected for total protein present, was plotted against time of incubation (Figure 22 and 23). Equilibration was reached within 5 minutes for both tyramine (Figure 22) and DA (Figure 23), although a trend to a slower equilibration time may have been present for DA.

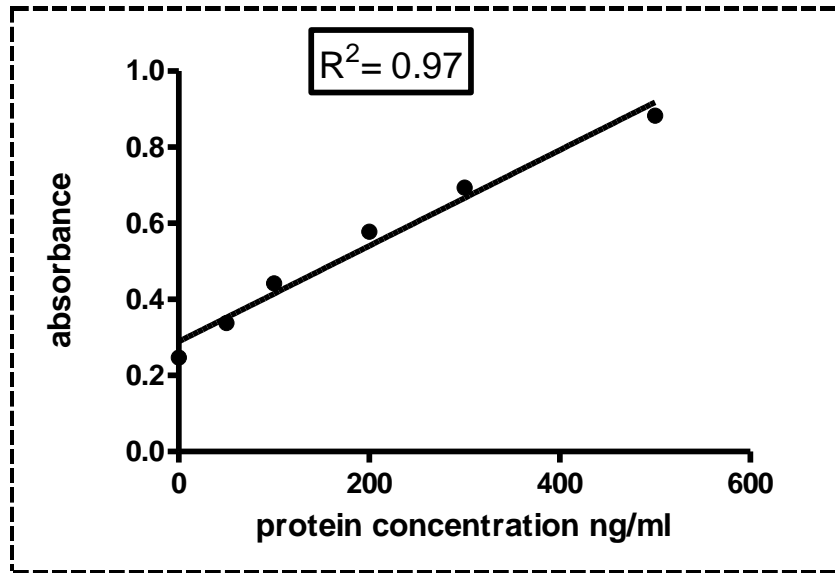


Figure 21: Standard curve for protein assay.

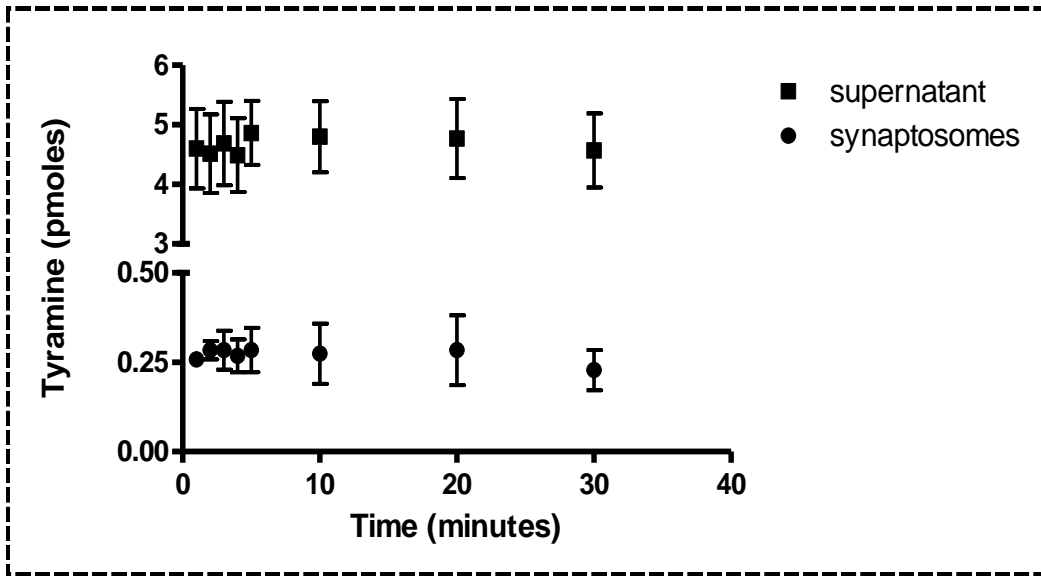


Figure 22: Uptake of [^3H]-tyramine in rat brain synaptosomes.

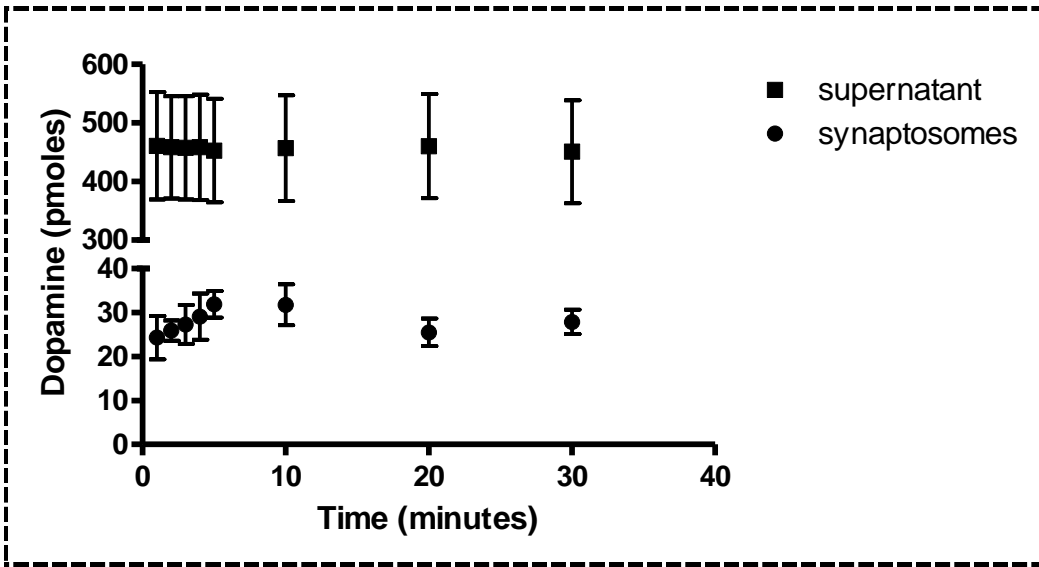


Figure 23: Uptake of [³H]-dopamine in rat brain synaptosomes.

The release of [³H]-dopamine and [³H]-tyramine was determined following loading of synaptosomes with [³H]-tyramine and [³H]-dopamine for 10 minutes. Release was measured at different incubation times under normal and depolarizing conditions (Figure 24 to 25). The first-order rate constant and the half-lives were calculated by fitting the release data to a one-phase exponential decay function (table 5).

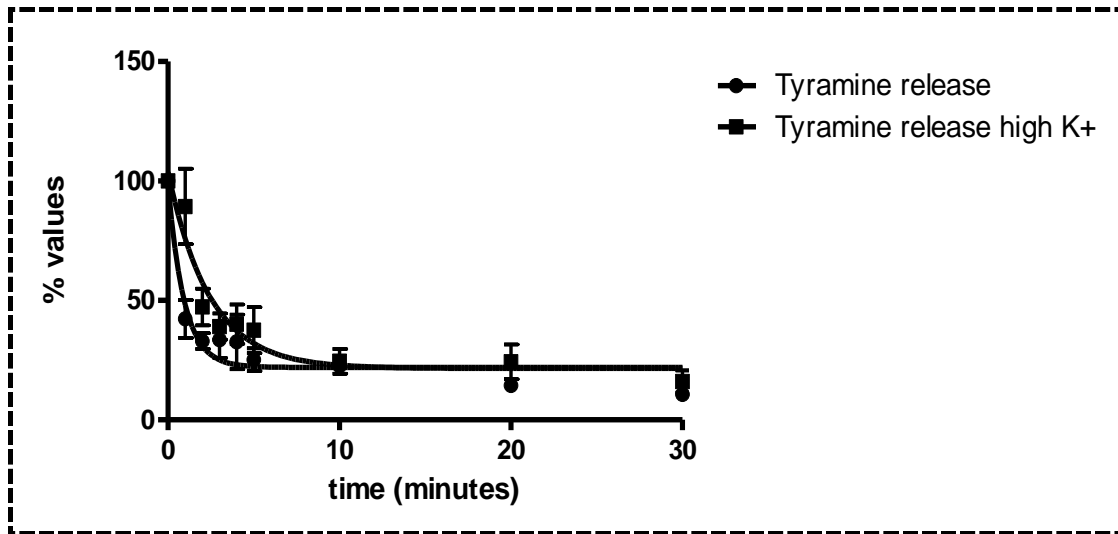


Figure 24: Release of $[^3\text{H}]$ -tyramine under basal and high K^+ -induced depolarization conditions.

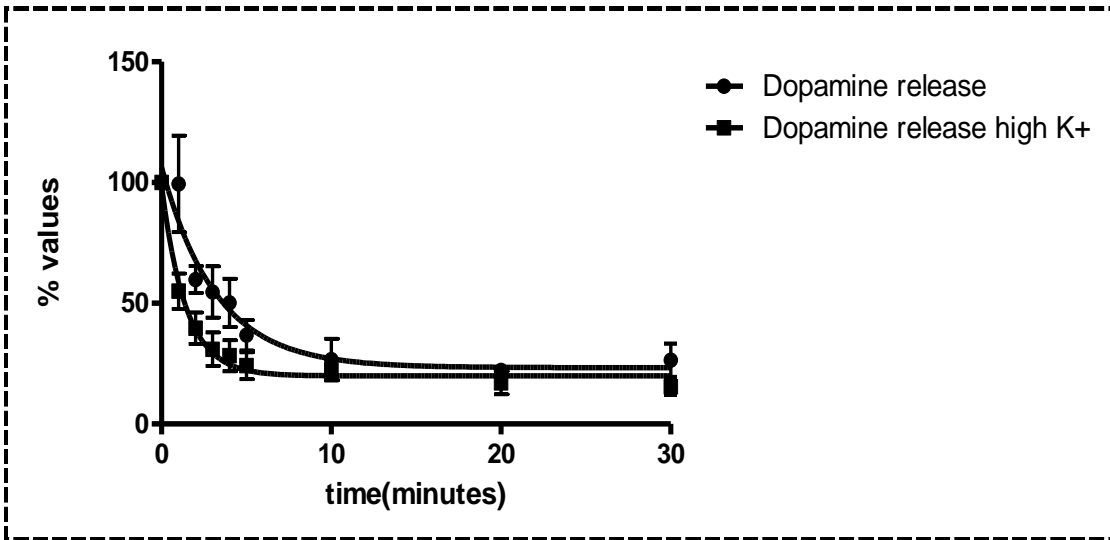


Figure 25: [³H]-Dopamine release from synaptosomes under basal and high K⁺-induced depolarization conditions.

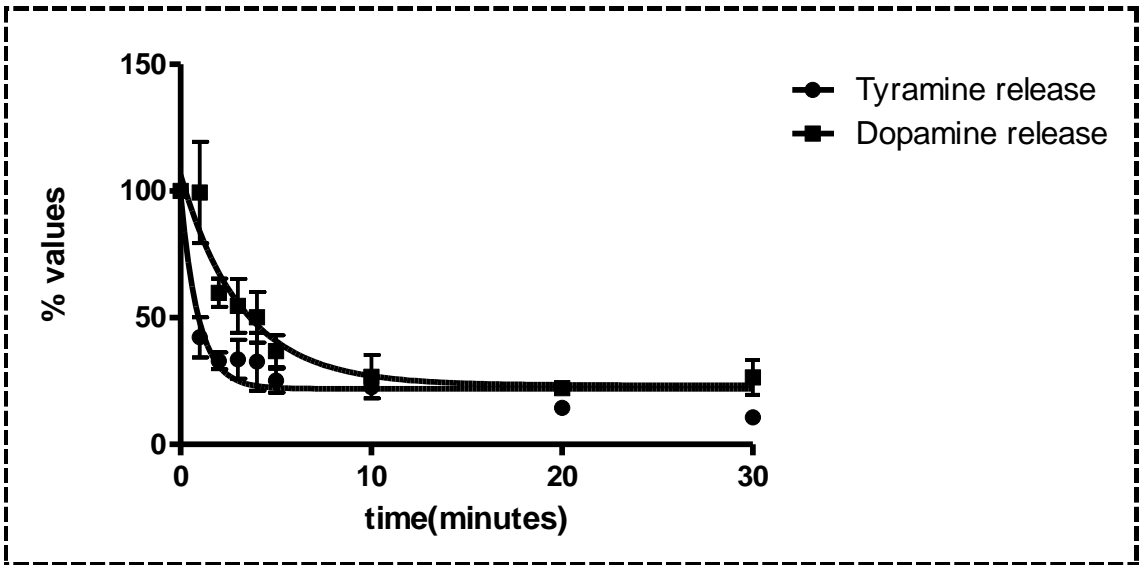


Figure 26: Comparison of the release of [³H]-tyramine and [³H]-dopamine from synaptosomes under basal conditions

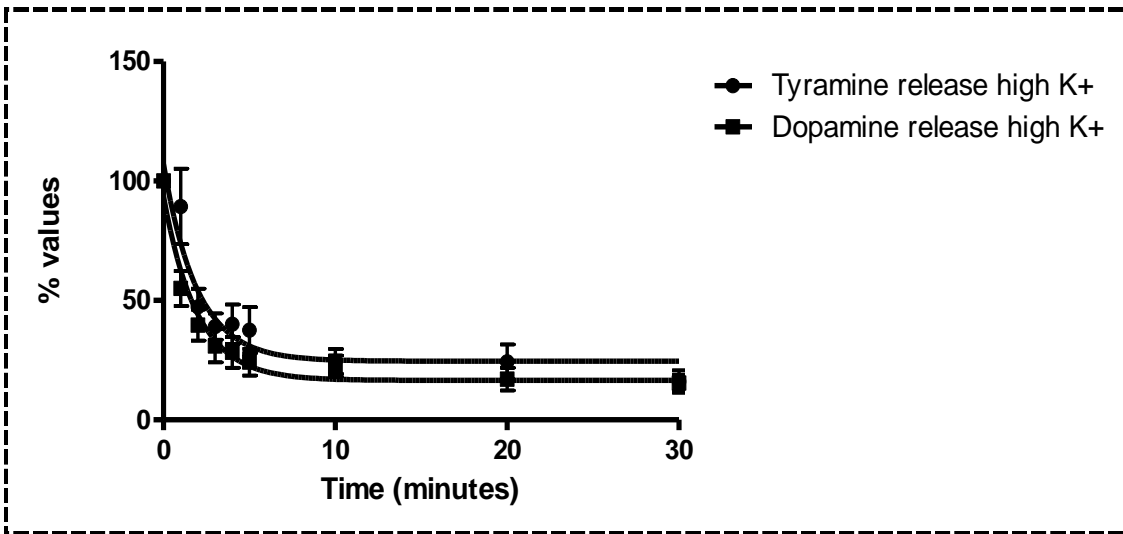


Figure 27: Comparison of the release of [³H]-tyramine and [³H]-dopamine from synaptosomes following high K⁺-induced depolarization.

Table 5: Rate constants and half-lives for [³H]-tyramine and [³H]-dopamine release under various conditions.

	K (Rate constant 1/minute)	HALF-LIFE (minute)
TYRAMINE RELEASE	1.064 ± 0.23**	0.65
TYRAMINE RELEASE HIGH K	0.4109 ± 0.09 [#]	1.69
DOPAMINE RELEASE	0.3113 ± 0.07 ^{##}	2.23
DOPAMINE RELEASE HIGH K	0.70 ± 0.11	0.99

**Tyramine release was significantly ($p < 0.001$) faster than dopamine release.

[#] Tyramine high K release was significantly slower than tyramine release ($p < 0.05$)

^{##} Dopamine release significantly ($p < 0.01$) slower than dopamine release in high K.

The results were analyzed by fitting a one-phase exponential decay function to the data and the resultant curves compared with an F-test. Tyramine release occurred significantly ($p = 0.0042$, $F = 8.77$) quicker than DA (figure 26). High K⁺ caused a significant ($p = 0.01$, $F = 6.95$) increase in the rate of DA release and a significant decrease for tyramine ($p = 0.02$, $F = 5.86$) shown in Figures 25 and 24. There was no significant difference between DA and tyramine release under high K⁺ induced depolarization.

Tyramine release between 2 and 4 minutes time points under basal and depolarizing conditions showed an apparent plateau which was not seen with DA and could suggest multiple pools of tyramine release. However, a two-phase exponential curve fit was not significantly better than the one-phase curve ($p > 0.05$, $F = 0.588$).

Tyramine synaptosome release data and tyramine fluorosome data was compared using an F-test. Tyramine membrane passage was significantly faster ($F = 13.63$, $p = 0.0002$) in Fluorosomes than in synaptosomes. Similarly DA synaptosome data and DA fluorosome data was compared and membrane passage in Fluorosomes was again significantly faster ($F = 56.77$ and $p = 0.0001$).

Table 6: Comparison of the rate constants and half-lives for tyramine and dopamine in synaptosome and Fluorosome assay systems.

	K (Rate constant 1/second)	HALF-LIFE (seconds)
Tyramine synaptosome	$0.0178 \pm 0.0038^{**}$	38.9
Tyramine fluorosome	0.0587 ± 0.0080	13.9
Dopamine synaptosome	$0.0052 \pm 0.0013^{***}$	133.6
Dopamine fluorosome	0.0167 ± 0.0009	35.9

** Tyramine synaptosome release was significantly ($p < 0.0002$) slower than tyramine fluorosome entry.

*** Dopamine synaptosome release was significantly ($p < 0.0001$) slower than dopamine fluorosome entry.

5 DISCUSSION

The trace amines are biogenic amines which are structurally similar to monoamine neurotransmitters. The synthesis of trace amines is regulated by AADC (Paterson et al., 1990) and they alter responses of neurotransmitters (Jones, 1981; Paterson et al., 1990; Berry, 1994). In general trace amines do not appear to be actively stored in synaptic vesicles (Wu and Boulton, 1975; Philips and Boulton, 1979; Durden and Philips, 1980). Consistent with this, trace amines also do not appear to be released in an activity-dependent manner (Dyck et al., 1983). Since they are also more lipophilic than the monoamine neurotransmitters (Oldendorf, 1971; Blakeley and Nicol, 1978; Mack and Bonisch, 1979), it has been assumed that trace amines readily diffuse across neuronal membranes (Berry, 2004) and furthermore that their release is not *via* exocytosis. The TAARs are GPCRs (Borowski et al., 2001; Buzow et al., 2001), but unlike neurotransmitter GPCRs these are thought to be intracellular (Bunzow et al., 2001; Lindemann and Hoener, 2005; Xie et al., 2008). Thus, for trace amines to exert post-synaptic effects at these receptors they have to cross cell membranes.

Previous studies have suggested that trace amines can cross the lipid cell membrane by simple diffusion (Blakeley and Nicol, 1978; Tchercansky et al. 1994). The entry of NA, 5-HT and tyramine was rapid into erythrocytes and it was concluded that this occurred by facilitated diffusion (Blakeley and Nicol, 1978). Tchercansky et al., (1994) examined the transfer of tyramine across rat intestinal membranes and concluded that this occurred by simple diffusion. Both of these studies, however, used biological membranes with transporter proteins present which could complicate determination of permeability

coefficients related to simple diffusion. Thus, the current studies provide the first systematic examination of membrane permeability of trace amines in both the absence and presence of membrane transporters and compare this to that of monoamine neurotransmitters.

Fluorosomes® are phospholipid vesicles containing a trapped fluorescent dye. The entry of any compound of interest into these vesicles is detected by the quenching of the fluorescence. From table 3 it can be seen that trace amines enter Fluorosomes statistically quicker than the corresponding neurotransmitters. The permeability coefficient of tyramine was 65% greater than that of DA while TRYP's permeability coefficient was 85.5% greater than that of 5-HT. The half-lives for DA and 5HT membrane passage were correspondingly greater than those of tyramine and TRYP.

The average thickness of a cell membrane is 30-60Å (Andersen and Koeppe, 2007). Membrane permeabilities for trace amines were between 19 and 33 Å/second suggesting that membrane passage by simple diffusion can occur in less than 3 seconds. In contrast, neurotransmitter membrane permeabilities were 4 to 20 Å/second suggesting the membrane passage by simple diffusion would require up to 10 seconds. These data for trace amines are consistent with previous *in vivo* electrophysiology studies, where neurotransmitter modifying effects of trace amines were seen to occur very rapidly (Jones and Boulton, 1980; Paterson, 1988; Berry, 2004). PE permeability characteristics could not be determined as it did not quench the fluorescence of free fluorosome probe. Chemically PE is dehydroxylated tyramine and is therefore expected to be less polar. As such it would be expected that this will result in increased lipophilicity and therefore even greater membrane permeability than was seen with tyramine.

Log P, log $D_{7.4}$ and TPSA scores were compared to Fluorosome permeability data. There was no indication of any trend toward a predictive relationship between membrane permeability in the fluorosome assay and either log P or TPSA scores (Figure 11). Log $D_{7.4}$ was expected to be a better indicator of membrane permeability as the pH was maintained at physiological pH. When log $D_{7.4}$ data were compared with permeability coefficient values (figure 16) the graph was more suggestive of a possible predictive relationship. However, both of these studies evaluated only 6 compounds and far greater than 6 values would be required for a full statistical comparison.

Trace amines have been found in high levels in various foodstuffs, most notably cheese (El-zayat and El-Bagoury, 1988; Joosten, 1988a,b; Stratton et al., 1991; Tawfik et al., 1992), red wine (Ibe et al., 1991; Ancin-Azpilicueta et al., 2008) and chocolates (Ziegleder et al., 1992). Such foodstuffs have been implicated in food-induced toxicity, in particular food-induced migraine (Hanington, 1967; Vaughan, 1994) and the 'cheese'-effect associated with certain antidepressant therapeutics (Blackwell, 1963). As such, it is of interest to determine the ability of trace amines to cross intestinal epithelial membranes.

Caco-2 cell culture provided a model of the absorption of the compounds through an intestinal membrane. Both tyramine and DA distribution between cellular, apical and basal compartments reached equilibrium within 10 minutes, with no evidence of a difference between basal and apical additions (Figures 17 and 18). Preliminary studies showed that this was also true at 5 minutes. Shorter time points could not be studied as it was not physically possible to accurately isolate the three required compartments in such short time spans. A different sampling method would be required in order to determine

distributions at such short incubation times. As a result of these limitations, rate constants and flux values could not be determined in Caco-2 cells. However, it was found that tyramine accumulation in the acceptor side (\approx 7-8% of administered) was approximately twice that of DA (3-4%) at equilibrium. Such a low fraction of administered concentration being accumulated suggested that in the presence of membrane proteins both tyramine and DA were either actively pumped out of Caco-2 cells or actively prevented from entering. The greater accumulation of tyramine than DA appears consistent with the permeability coefficient values from fluorosome data showing tyramine to be more permeable than DA (table 3).

Caco-2 cells express a variety of transporters (Sun and Pang, 2008). These are both uptake and efflux transporters and belong to two major super families, one is ATP-binding ABC transporters and solute carrier (SLC and SLCO) family of transporters (Smetanova et al., 2011). Tyramine and DA interact with these transporters at concentrations of 100 nM through the organic cation transporters 1 (Iseki et al., 1993; Takahashi et al., 1993; Breidert et al., 1998; Koepsell et al., 2003). These are similar to the concentrations used in the current study and hence these transporters could play a role in modulating the membrane passage of these amines. A proton driven, ATP-independent, active transport process for PE has been demonstrated in Caco-2 cells (Fischer et al., 2010). This was concluded to occur *via* an as yet unidentified orphan transporter, as the characteristics of the transport were not consistent with known transporters of organic cations and amino acids. The kinetics of the PE transport showed some similarities to those observed here, with equilibration of transport being very rapid, occurring within 5 minutes (Fischer et al., 2010). However, there was no effect of other trace amines

(including tyramine) or monoamine neurotransmitters on the transport of PE. This suggests that there are different transport processes involved in PE and tyramine membrane passage, at least in intestinal epithelial cells.

Tyramine and DA are endogenously synthesized in neurons (section 2.1). Therefore the passage of these compounds across neuronal synaptosome membranes was also investigated. The uptake into the synaptosomes was very quick for both tyramine and DA. The equilibrium occurred within 3 minutes for tyramine, with a trend for a slower rate of uptake with DA (Figures 22 and 23). However, in both instances this was sufficiently quick that rate constants for uptake could not be determined.

Tyramine release from synaptosomes occurred significantly ($p = 0.0042$, $F = 8.77$) quicker than DA (figure 26) with a 71% faster rate of release. This is consistent with previous studies showing DA to be stored in synaptic vesicles in nerve terminals, but that the same does not hold for tyramine (Juorio et al., 1988). Further it is consistent with the previous suggestions (Paterson et al., 1990; Berry, 2004) that tyramine is released by simple diffusion and synaptic concentrations are in a steady state determined by the relative rates of synthesis and degradation. This is also consistent with the greater rate of membrane permeability for tyramine seen in Fluorosomes.

High K^+ caused a significant ($p = 0.01$, $F = 6.95$) increase in the rate of DA release but a slowing of the rate for tyramine ($p = 0.02$, $F = 5.86$) as shown in figures 24 and 25. Tyramine release in non-depolarized membranes was 61% faster than tyramine release under high K^+ -induced depolarization. DA release in high K^+ was 55% greater than DA release across non-depolarized membranes. Under depolarizing conditions there was no

longer a significant difference between the rates of release of tyramine and DA. Depolarization of the synaptosomes results in the opening of voltage-gated calcium channels (Hall, 2011). The increased calcium then initiates the release of vesicle stores by exocytosis. As such it would be expected that a greater release of vesicle-stored compounds would occur under depolarizing conditions. This was seen with DA. The greater release of tyramine under basal conditions and lack of increased release following depolarization is consistent with earlier studies of Dyck (1989) showing a lack of effect of depolarization on tyramine release. However, in the current study a slowing of tyramine release was observed under depolarizing conditions. The mechanism of such an effect is unknown and requires further study. No other reports of a decreased release of compounds from synaptosomes under depolarizing conditions is currently known.

A plateau region for tyramine release appeared to be present between 2 and 4 minutes (Figure 24). This could indicate two neuronal pools of tyramine. Dyck (1989) previously suggested multiple pools of tyramine were present that responded differently to membrane depolarization. It was suggested that one of these pools may represent a distinct sub-form of synaptic vesicles. Although data in the current study was suggestive of multiple pools, a significant improvement was not observed when the data was fit to a 2-phase exponential function. Again further studies would be required to clarify the nature of these apparent multiple pools of synaptic tyramine. Interestingly a similar apparent biphasic temporal response to trace amines in synaptosomes has been reported by Xie et al. (2008) during studies of the effects of trace amines on monoamine transporters. Whether these observations are related is unknown at present.

Finally when tyramine fluorosome data was compared with tyramine synaptosome data, membrane passage was significantly faster ($F = 13.63$, $p = 0.0002$) in Fluorosomes. A similar greater release was also observed for DA ($F = 56.77$ and $p = 0.0001$). This suggests that the presence of transporters modifies the passage of both trace amines and neurotransmitters across membranes. In fluorosomes which are merely phospholipid vesicles there are no transporters involved and it merely shows the passage of compounds through the membrane by passive diffusion. Synaptosomes express all of the monoamine transporters associated with synaptic terminals. Proteomic studies by Bai and Witzmann (2007) identified the various proteins present in neuronal terminals. With respect to DA the DAT and VMAT2 are likely the most important and are well documented to act to limit synaptic concentrations of DA (Eiden et al., 2004; Bai and Witzmann, 2007). For tyramine there are no known selective tyramine transporters. Tyramine has however been reported to interact non-specifically with a number of transporter proteins. At the concentration used here (100 nM) tyramine has been reported to interact to a minor extent with NET and VMAT (Raiteri et al., 1977; Partilla et al., 2006), but is unlikely to interact with either DAT or SERT (Raiteri et al., 1977; Parker and Cubeddu, 1988). As such NET and VMAT are the most likely transporters responsible for the modification of tyramine transport in the current study. In addition there are a number of amino acid transporters present in cells (Kanai and Hediger, 2004; Palacin and Kanai, 2004). Due to the close structural similarity between trace amines and amino acids, it is possible that trace amines are transported across cell membranes by one or more of these amino acid transporters. Other trace amines also can interact to a minor extent with neurotransmitter transporters at physiological concentrations. PE interacts with DAT (Baker et al., 1976; Raiteri, 1977;

Parker and Cubeddu, 1988), while TRYP interacts with 5-HT (Horn, 1973; Horn, 1978; Baumgarten et al., 1975; Rudnick and Wall, 1992; Hilber et al., 2005). It is likely that these non-specific interactions will also modify the membrane transport properties of these trace amines.

Trace amines have been shown to modify monoamine transporter function specifically through TAAR 1 binding (Xie et al., 2008; Xie and Miller, 2008). Whether TAAR1-mediated modulation alters monoamine neurotransmitter transporter affinity for trace amines such as tyramine would require specific study. This could mean that at physiological concentrations the trace amines are substrates for membrane transporters following TAAR1-mediated modulation of transporter functioning. This would allow membrane transporters to at least partially control synaptic trace amine levels.

Trace amines have been implicated in a number of neurological and psychiatric disorders, such as schizophrenia and depression (section 2.3.4). These diseases are marked by poor therapeutic management in comparison to other disorders. Understanding the basal functioning of the trace aminergic system is therefore of potential clinical utility. The current data begins to address this by clarifying the mechanism by which endogenous ligands at TAAR can cross lipid bilayer membranes and thereby interact with TAAR.

6 SUMMARY AND CONCLUSION

In this thesis trace amine membrane permeability was compared to the corresponding neurotransmitters in the presence and absence of membrane transporters. First fluorosomes were used to determine the permeability coefficients and diffusion half-lives in the absence of any transporters. Trace amines in general were significantly more permeable than the corresponding neurotransmitters. Further log P, log $D_{7.4}$ and TPSA markers of lipophilicity were examined for their ability to predict the permeability of trace amines and neurotransmitters. Log $D_{7.4}$ appeared more predictive than log P and TPSA as the ionization of compounds is maintained at pH 7.4 which also occurs physiologically. Caco-2 cell culture and synaptosomes were used to determine the permeability of [^3H]-tyramine and [^3H]-dopamine in the presence of membrane transporters. With Caco-2 cell culture a rate constant or flux could not be determined due to limitations of physical sampling at early time points. The results obtained with Caco-2 cell culture did suggest that only a small fraction of administered tyramine was accumulated, although this was approximately twice that of DA, suggesting a role of membrane transporters in modifying membrane passage. In synaptosomes uptake equilibration was reached within 5 minutes for both tyramine and DA, although a trend to a slower equilibration time may have been present for DA. Tyramine release occurred significantly quicker than DA. In depolarized membranes there was an increase in the rate of DA release but not for tyramine release. All these results suggest that trace amines in general are more permeable than the corresponding neurotransmitters and release does not occur primarily through exocytosis. Finally when the transport in the absence and presence of transporters was directly compared (table 6) a significantly slower membrane

passage was observed in the presence of transporters for trace amine (tyramine) and neurotransmitter (DA). This means the membrane proteins or transporters limit the passage of trace amines across membranes under physiological conditions.

It is possible to conclude that in general, trace amines are more permeable than the corresponding neurotransmitters both in the presence and absence of transporters, which was seen in all the systems used. Membrane passage of trace amines was in general consistent with physiological 'release' occurring by simple diffusion. However, there is evidence that this can be modified by the presence of membrane transporters. The nature of the transporters involved in this modulation of trace amine transport requires further study. This could be addressed by the systematic study of trace amine transport across isolated biological membranes such as synaptosomes, following the selective knock-out of genes encoding candidate transporters. Such candidate transporters could include, but are not limited to, DAT, NET, SERT, and the various amino acid transporters.

7 FUTURE DIRECTIONS

Though it can be seen that the trace amines are more permeable than the corresponding neurotransmitters, the exact mechanism of membrane passage is not known. The slower permeability in biological membranes than in Fluorosomes suggests an involvement of membrane transporters. Future studies should systematically characterise the transporter systems involved in trace amine membrane passage. Fluorosomes can be custom made such that they contain a specific transporter. In addition, transporter inhibitors could be used to examine the role of established monoamine transporters such as DAT, NET, SERT and VMAT2. More compounds should be analysed for log P, TPSA and log $D_{7.4}$ to allow a statistical comparison of correlations to fluorosomes permeability data. Finally, tyramine release from synaptosomes showed evidence of two pools. Previous studies have shown release of tyramine to be sensitive to the method used for inducing depolarisation. Thus, different depolarization methods can be used to begin to examine the nature of the putative multiple synaptic pools of tyramine.

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