I. Advanced Fluorescent False Neurotransmitters for the Study of Monoamine Transporter Activity and Synaptic Transmission

II. New Small-Molecule Inducers of Glial Cell Line-Derived Neurotrophic Factor (GDNF) from C6 Glioma Cells

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Abstract

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This thesis details two projects at the interface of chemistry and neuroscience. Part I (consisting of chapters 1 – 5) focuses on the development and characterization of fluorescent optical tracers of monoamine neurotransmitters for use in the study of monoamine transporter activity and distribution, as well as synaptic transmission in the brain. The second section details early studies concerning experimental therapeutics recently identified by our group to induce the synthesis and release of glial cell line-derived neurotrophic factor (GDNF) from a model astroglial cell line.

In Part I of this thesis, Chapter 1 provides a brief introduction to the relationship between chemistry and neuroscience, while explaining the motivation and background behind the development and study of Fluorescent False Neurotransmitters (FFNs). Chapter 2 discusses the characterization of new fluorescent substrates for the Vesicular Monoamine Transporter 2 (VMAT2) for applications in high throughput screening of VMAT activity and inhibition, as well as for imaging aminergic synaptic transmission in brain tissue. Chapter 3 describes the discovery of fluorescent probes FFN201 and AGH093 as dual substrates for the norepinephrine transporter (NET) and VMAT, with potential applications in the
imaging of noradrenergic modulation of cortical signaling networks. NG54, a FFN with substrate activity at each of the monoamine transporters was also identified, and represents a new structural class of FFNs. In Chapter 4, APP+, a known fluorescent substrate for the monoamine transporters, is evaluated as a potential tracer of monoamines in the brain. It has been determined that while this molecule may have utility as a marker for monoaminergic neurons, APP+ is not an appropriate FFN due to high background labeling of mitochondria and other intracellular structures. Chapter 5 discusses the discovery of fluorescent substrates of the Organic Cation Transporter 3 (OCT3) and Plasma Membrane Monoamine Transporter (PMAT). Mounting evidence suggests that these transporters play a significant role in clearing extracellular space of monoamines and are likely involved in neurological disease. As such, fluorescent substrates of OCT3 or PMAT would be useful as imaging agents in the brain, as well as for the development of fluorescence-based quantitative inhibition assays.

Part II of this thesis (Chapter 6) discusses N-arylethyl isoquinuclidines as releasers of glial cell line-derived neurotrophic factor (GDNF) from a model glial cell line. Initial characterization of GDNF release and its dependence on protein synthesis and MAPK/ERK signaling is described. Preliminary studies indicate that at least two experimental compounds described herein modulate fibroblast growth factor receptor (FGFR) signaling.
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II. New Small-Molecule Inducers of Glial Cell Line-Derived Neurotrophic Factor (GDNF) from C6 Glioma Cells

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Part I:

Advanced Fluorescent False Neurotransmitters for the Study of Monoamine Transporter Activity and Synaptic Transmission
Chapter I

Imaging Synaptic Communication, and the Significance of New Methods in Neuroscience
“In the same way that chemistry grew from the ashes of alchemy, neuroscience, a field still in its infancy, may one day subsume psychology.”

-Neil deGrasse Tyson

I: Introduction

Mother nature has hidden some truly wondrous complexities within the brain, and humankind is currently on the threshold of a new understanding of the physical basis of our rich human experience. It is the biochemical control of information that constitutes our entire experience of the universe: our perception, our emotions, our ability to learn and communicate, and the autonomic control of our physical bodies. Indeed, biochemical processes underlie the function, structure, plasticity, and disease etiologies of the brain. Because of this truth, infinitesimal biochemical deficiencies or divergences within the brain can have tremendous influence over our personalities, physical health, and overall interaction with the world. Humankind is beginning to understand the complex intracellular and extracellular signaling networks involved in the control of information in the brain, and we are using this new knowledge to describe the brain with the rigorous, empirical language of science. Neuroscience is approaching a leap of similar scale and impact as when chemistry arose to describe the physical world.

We can imagine a future where medicine has the ability to rapidly and accurately diagnose every disease or disorder of the central nervous system with minimally invasive techniques. We can also envision a day when we have at our disposal targeted therapies that overcome the genotypic or phenotypic deficiencies characteristic of and causal of many of these disorders. In order for the
development of these technologies to be realized, both diagnostic imaging and therapeutics, we have much to learn about the structure and function of the brain. It was only recently in human history when Ramón y Cajal, employing the Golgi stain and a light microscope, visualized the discrete nature of neurons in brain tissue, leading ultimately to the understanding of the neuron as the principal signaling element of the brain, with functional connections through synapses.\(^1\) Cajal’s example is an excellent and fundamentally simple one: he employed new techniques at the interface of chemistry, brain science, and microscopy in order to see what had yet been elusive. The development and application of new methods generates knowledge.

**Chemistry and Neuroscience**

The field of chemistry is uniquely positioned to create new methods for the study of the brain. Fluorescent small molecules have been developed as selective labels for distinct cells, organelles, proteins, and other structures. Additionally, functional fluorescent probes have been developed for the purpose of imaging key cellular parameters including membrane potential and intracellular calcium concentrations, both of which are used in the study of the brain.\(^2,3\) Nanoparticles and functional materials are also being developed using the tools of chemistry in order to sensitively detect structures and processes in the brain,\(^4,5\) and drugs which target specific tissues or cells have been developed into imaging agents for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging.\(^6,7\)
The development of imaging agents is not the only role that chemistry plays in neuroscience. In the context of neuropharmacology, chemistry continues to expand our understanding of the function of the brain. A classic example is the serendipitous discovery of thorazine as a first generation antipsychotic drug, which made the draconian surgical transorbital lobotomy procedure obsolete while simultaneously igniting a curiosity about chemical neurotransmitters and their centrality in signaling. Thorazine (chlorpromazine) and other early antipsychotic drugs interact with a number of receptors in the central nervous system (CNS) and cause myriad side effects, and the early and widely-prescribed antipsychotic haloperidol is in fact metabolized to toxic pyridinium species (See Chapter 4 for a discussion of pyridiniums and neurotoxicity). The neuropsychopharmacological examination of small molecules with well-characterized pharmacological profiles is emerging as a means of understanding the complex interplay between receptor agonism/antagonism and behavior.

Furthermore, once the brain is sufficiently understood such that the pathophysiologies of disease states are well characterized, chemistry will likely continue to play an important role in the development of small molecules specifically targeted to compensate for phenotypic and genotypic deficiencies which underlie disease. These future therapies would not rely on the serendipitous discovery of efficacious drugs working through unknown and uncharacterized mechanisms with potentially devastating side effects.

It is evident that we need a more clear and sound understanding of the function and structure of the human brain and its disease states in order to create
more selective, cleaner, more advanced, and more efficacious therapies. The specific example of thorazine’s accidental launch of neuropharmacology, while an early step in the progression of our understanding of the brain, proves the principle that such steps lead to meaningful improvement in treatment of neuropsychiatric disorders. Moreover, thorazine and other later pharmaceuticals serve as probes of the relationship between receptor activity/expression and physiology/behavior. It is up to interdisciplinary neuroscientific research, for which chemistry and chemical neuroscience plays a central role, to engage in the methodological research to develop a modern toolbox of approaches to better study the brain.

Signaling Networks in the Brain: Functional Connectomics and Optical Imaging Methods for the Study of Neural Circuits

In order to completely understand the brain, its disease states, and its remarkable plasticity, we must understand the interconnected networks of billions of neurons that constitute its structure and underlie its function. In an emerging area of research termed connectomics, some groups are employing methods at the interface of neuroscience, imaging, and computer engineering to gather detailed information on the structure, function, and connections of neurons, with the ultimate goal of understanding how every neuron in the human brain is connected to every other neuron.\(^{9-11}\) This presents an enormous challenge to science reminiscent of the Human Genome Project in its bold aspirations, as the technologies and paradigms for data acquisition, storage, and analysis have not clearly emerged.
The general motivation behind connectomics relies on the assumption that invaluable information will be learned about the function of the brain through the mapping of its physical connections. There is debate as to whether a solved connectome will serve as a useful tool for understanding the function of the brain, as a connectome tells us little about the strength of these connections, and its definition of these connections may not be the most clear and precise (based on morphology and distance rather than the strength and nature of signals conveyed). What is necessary beyond a solved connectome is detailed information about the functionality of the connections and the networks discovered, how and why those networks signal, and how those signals change in response to stimulus or disease. Recently, an advanced, collaborative superproject termed the Brain Activity Map (BAM) was proposed and has been supported by the White House and funded by Congress as a project called the Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative. This project envisions an international, interdisciplinary program to map the functional connectome of the human brain, and must in the process develop and cultivate the methods for collection and analysis of data on an unprecedented scale.

Much of the technology used in early pursuits toward the BAM has been developed from fluorescent reporters of cellular conditions, namely calcium concentrations and membrane potential. Small molecule fluorescent calcium reporters have been found to be sufficiently sensitive to the changes in intracellular calcium that result from action potentials to enable imaging of individual cell bodies propagating single action potentials. Similar small molecule-based environmental
sensors with electrochemically-sensitive fluorescence properties have proven useful in detecting action potentials. Both of these technologies are employed today in mapping functional networks of neurons in living brain tissue by allowing observation and characterization of arrangements of neurons signaling together in concert, in patterns thought to represent local signaling networks.\textsuperscript{(3, 14)} As such, it has been shown that imaging technologies with the spatial resolution of single cells, and temporal resolution sufficient to detect individual action potentials, will play a key role in the discovery of the functional organization of signaling networks in the brain.

While it is important to observe how clusters of neurons signal in concert, as well as how these patterns of collections of signaling neurons change with response to stimulus, there is an additional need to resolve activity of individual synapses within the functional network. It is well understood that synaptic hypertrophy, dystrophy, or general dysfunction are characteristic of the pathophysiology of most neurological diseases. Imaging synaptic events in neuronal signaling will prove useful for the characterization of signaling networks in the brain, both to fully characterize the synaptic nature of observed signaling events within the network, and to image direct communication between neurons where the cell bodies in direct communication are not located in the imaging frame. A hypothetical example would be the study of monoaminergic signaling on the dendritic trees and cell bodies of cortical neurons. Using fluorogenic calcium sensors, it is currently possible to resolve patterns of neurons signaling in the cortex. It is also known that monoamines such as dopamine and norepinephrine modulate these signaling
networks, resulting in observable differences in cortical signaling upon stimulation by aminergic signaling or direct application of neurotransmitter to the brain. Because the cell bodies of the modulatory aminergic neurons are spatially separated from the cortical neurons that they modulate, it is not possible with current methods to observe with one microscope how these aminergic neurons directly affect cortical signaling networks. As such, a tracer of monoaminergic synaptic activity could prove useful in this hypothetical example and in the pursuit of functional connectomics in general.
II – Imaging Neurotransmitter Release in the Brain

Detecting Neurotransmitters

As neurotransmitters are the fundamental signaling components of the chemical synapse, the ability to sensitively detect neurotransmitter release, concentration, and distribution in the brain has become centrally important to neuroscience. Microdialysis and electrochemical methods including cyclic voltammetry are widely-used methods used to study neurotransmitter release.\(^{15,16}\) Although these methods, particularly voltammetry, are quite sensitive and are capable of detecting neurotransmitter concentrations with excellent temporal resolution, their poor spatial resolution — limited in large part by the physical dimensions of the probes — leads to the collection of information from a number of synaptic inputs from numerous different cells. We have estimated that for voltammetric measurements of dopamine in the striatum, hundreds of synapses are measured at once, based on the density of dopamine synapses and the dimensions of the microelectrode probes commonly used (Figure 1).\(^{17}\)
**Figure 1.** While voltammetric measurement of neurotransmitter (NT) release provides excellent temporal resolution and kinetic data, this method is limited spatially as a function of the size of the probe, as illustrated in the left panel, representing a microelectrode in 1 mm³ of brain tissue. The central panel represents the area from which the voltammetric probe records, and is represented by a 3D projection of a fluorescent false neurotransmitter, FFN102, in the dorsal striatum with an accompanying illustration of a microelectrode measuring an ensemble of hundreds of terminals (microelectrode is represented by the cylinder of the indicated dimensions, scale bars represent µm, FFN102 image is courtesy of Matt Dunn). The right panel is a representation of a striatal dopaminergic presynaptic release site, which is known to modulate excitatory (glutamatergic) synaptic inputs to dendritic spines of medium spiny neurons (MSN). The small black circles represent dopaminergic synaptic vesicles.

**Direct Imaging of Neurotransmitter Release**

Optical imaging of neurotransmission is a relatively new approach to the study of neuronal signaling. Neurotransmitters have attracted much attention recently as potential targets of fluorogenic or fluoromorphic sensors. The potential benefits of optically measuring synaptic communication and neurotransmitter concentrations in brain tissue are innumerable, and a number of related approaches have been developed recently as steps towards this greater understanding.
Because of its centrality to brain function, glutamate has become an early target of optical methods. An early fluorescence-based approach to the detection of the glutamate was an indirect one. The experimental system was treated with L-glutamate dehydrogenase and NAD(+), which was reduced to the fluorescent NADH in the presence of glutamate. This allowed for indirect fluorescence imaging of “waves” of glutamate release.\textsuperscript{(18)} Later, glutamate optical sensors (EOS) were engineered from scaffolds of glutamate receptors.\textsuperscript{(19-21)} These EOSs are designed to, upon binding of glutamate, induce detectable photophysical changes in a bound, environmentally sensitive fluorophore (Figure 2). The binding of glutamate induces allosteric changes of the receptor-sensor, to which the environmentally sensitive, bound fluorophore dye responds in a measurable manner. The EOS technique is only capable of labeling tissues via a chemical or biochemical linker, such as a biotin-streptavidin bridge. This requires the biotinylation of local tissue being studied, and could significantly alter the behavior of systems, although it provides an excellent readout of extrasynaptic glutamate, and by association, local glutamate release.

\textbf{Figure 2:} Various extracellular fluorescence-based sensors have been developed for the purpose of detecting neurotransmitter release. Such fluorescent sensors can
be applied via biotin-streptavidin linkage to nonspecifically-biotinylated extracellular structures, or can be genetically encoded via transgenic approaches. To date, glutamate is the only neurotransmitter capable of detection by these means, although one can envision sensors for other neurotransmitters acting similarly, or these sensors acting in concert with FFNs (introduced below).

Genetically encoded glutamate sensors have also been used to detect glutamate release,\cite{22} and these sensors function in a conceptually similar manner to EOS (Figure 2), with some notable advantages in comparison. These sensors utilize fluorescence resonance energy transfer (FRET) to detect neurotransmitter release. A bacterial glutamate binding protein was engineered with fluorescence reporters capable of FRET on two regions of the protein, which undergoes a large conformational change upon glutamate binding, leading to a detectable FRET response. Recently, an advanced sensor called an intensity-based glutamate-sensing fluorescent reporter (iGluSnFR) was reported.\cite{23} As this sensor can be genetically encoded and directed to specific cell populations, the problematic need for functionalization of tissue and physical application of the sensor to the brain region of interest is eliminated, allowing for facile \textit{in vivo} imaging. This sensor was found to have a sufficient signal to background ratio to detect glutamate signaling events in the brain, and allowed synaptic resolution of task-dependent signaling events in behaving mice. The iGluSnFR has also been used to study glutamate release at the synaptic level with physiologically relevant (1-5) pulses of electrical stimulation.

A third approach developed recently has been the use of specially engineered cells that respond fluorogenically to extracellular acetylcholine (ACh).\cite{24} Termed cell-based neurotransmitter fluorescent engineered reporters (CNiFERs), these cells express the M1 muscarinic receptor as well as a genetically encoded fluorescent
calcium sensor, which responds to changes in intracellular calcium concentrations as a function of receptor activation (Figure 3). Drawbacks include the invasiveness of this method and the indirect nature of the detection. While it is conceivable that CNiFERs could be developed for the detection of any number of neurotransmitters simply by overexpressing different neurotransmitter receptors, the reported application has been limited to acetylcholine.

Figure 3: A representation of the CNiFER motif for detection of neurotransmitter release. Specially implanted “CNiFER” cells are engineered to respond fluorometrically to stimulation of intracellular signaling pathways by action of extracellular neurotransmitters like glutamate.

Chemosensors for dopamine (DA) and norepinephrine (NE), as well as other primary biogenic amines, have been recently reported. These compounds have the ability to penetrate catecholamine-containing vesicles, react with monoamines, and change their fluorescence properties accordingly. Thus far, fluorogenic “turn on” sensors have been developed to be selective for dopamine/norepinephrine over epinephrine in large dense core vesicles (LDCVs) of adrenal chromaffin cells and
have not been reported in brain tissue or primary neuronal culture. These sensors represent an advance over classical histochemical techniques for detection of catecholamines in cultured cells, as fixation is not required.

**Figure 4:** Fluorogenic/fluoromorphic chemosensors have been developed to react with and detect monoamine neurotransmitters.

**Endocytic Dyes and SynaptopHluorins Enable the Study of Individual Synapses but not Neurotransmitters**

Two related optical methods have been developed recently for imaging of synaptic vesicle fusion, but not neurotransmitter release. These are the fluorescent endocytic dyes and the synaptopHluorins.

Fluorescent endocytic dyes enable visualization of synaptic activity with sufficient spatial and temporal resolution to monitor vesicular fusion at the synaptic level. One of the most well known of these endocytic dyes is FM1-43 (**Figure 5**).\(^{27, 28}\) FM1-43 and related endocytic probes functions by labeling all treated membranes, at which point local electrical stimulation is applied to induce synaptic recycling. What results after washing the residual plasma membrane-bound dye is a population of labeled, active synapses, which can be studied fluorometrically. For example, FM1-43 has uncovered differential effects of dopamine on sets of corticostraital connections in the dorsal striatum.\(^{29}\) Potential drawbacks to FM1-43 include the necessity to stimulate tissue with long pulses of electrical stimulation,
which may affect the system being studied, as well as the lack of specificity of the label among the heterogeneous population of active synapses.

**Figure 5:** Structure of FM1-43. This and related amphipathic endocytic dyes bind to the plasma membrane of all cells without crossing the membrane (by the dicationic nature of the polar head). FM1-43 can be endocytosed via electrical stimulation to label active synaptic zones, and the remainder of extracellular label can then be removed by washing with cyclodextrins.

A genetically-encoded pH-sensitive variant of green fluorescent protein (GFP), known as pHluorin, has been developed to image synaptic vesicle fusion.\(^{30,31}\) The pH-sensitive pHluorin was generated as a fusion protein with synaptobrevin, a vesicular SNARE protein, which localizes the pHluorin on the interior of the vesicular membrane, where it is photophysically quenched in the low pH of the vesicular lumen (~5.5-6). Upon synaptic vesicle fusion, the synaptopHluorin experiences a rapid increase in pH and fluoresces more intensely. Because the signal is bound to the membrane, synaptopHluorins are quite useful for the study of synaptic activity, although they do not directly detect neurotransmitter release or concentrations.
III - Fluorescent False Neurotransmitters as Optical Tracers of Monoamine Neurotransmitters

Monoamine Neurotransmitters: Life Cycle

The monoamine neurotransmitters associated with CNS signaling include dopamine (DA), serotonin (5HT), and norepinephrine (NE). These neurotransmitters are responsible for modulating both excitatory glutamatergic and inhibitory GABAergic synapses,\(^{29, 32, 33}\) and are associated with mood, reward and learning, cognition, motor function, sleep and wakefulness, and homeostasis.\(^{33-35}\) Histamine has also shown to have similar modulatory effects in the CNS.\(^{36}\) Dysfunction of monoaminergic signaling has been implicated in or associated with numerous diseases. Because of their involvement in behavior and disease, monoaminergic neurons and monoamine neurotransmitters are of interest. The life cycle of monoamine neurotransmitters has been studied in significant detail, and is introduced below with respect to the role of these signaling molecules in neurotransmission.

Monoamine neurotransmitters are principally synthesized in the cell bodies and axons of their cognate neurons. Cell bodies of dopaminergic neurons are located in the zona compacta of the substantia nigra (SN), as well as in the nearby ventral tegmental area (VTA), and project primarily to the striatum and cortex.\(^{37}\) Serotonergic cell bodies reside in the dorsal and median raphe nuclei (DR and MR), and project to the CA1 and CA3 regions of the hippocampus, as well as the cerebral cortex. Noradrenergic cells originate from the locus coeruleus (LC) and other areas of the brainstem, and also project to the hippocampus and cortex.
Figure 6: A) Biosynthesis of dopamine and norepinephrine. B) Biosynthesis of serotonin. TH = tyrosine hydroxylase, AAAD = aromatic amino acid decarboxylase, DBH = dopamine β-hydroxylase. HTP = tryptophan hydroxylase.

Dopamine and norepinephrine are derived from the aromatic amino acid tyrosine (Figure 6A), which is transported into the brain across the blood brain barrier via amino acid transporters. The enzyme Tyrosine Hydroxylase (TH) is principally responsible for the ω-hydroxylation of tyrosine to form the catechol amino acid DOPA. DOPA is decarboxylated by the enzyme aromatic amino acid decarboxylase (AAAD) to form dopamine. In noradrenergic neurons, dopamine further undergoes β-hydroxylation by dopamine β-hydroxylase in the synaptic vesicles to form norepinephrine. Serotonin is metabolized from tryptophan (Figure 6B), which is also transported into the brain via amino acid transporters and hydroxylated in the 5-position of the indole ring by action of tryptophan hydroxylase (HTP) to form 5-hydroxytryptophan (5-HTP). Decarboxylation by AAAD affords serotonin.

In the neurons, the cytosolic population of freshly synthesized neurotransmitter (Figure 7A, dopamine as a representative monoamine
neurotransmitter) is sequestered at high concentration into synaptic vesicles by action of the neuronal vesicular monoamine transporter (VMAT2, Figure 7B, see Chapter 2 for a full discussion of VMAT). Once an action potential arrives at the synapse, a complex series of signaling events occurs, leading to fusion of the vesicular membrane with the plasma membrane and subsequent release of the packaged monoamine into the synaptic cleft (Figure 7C). The monoamine neurotransmitter signals on presynaptic or postsynaptic receptors before diffusing away or being transported back into the neuron via high affinity plasma membrane transporters (MATs, Figure 7D), either the dopamine transporter (DAT) on dopaminergic neurons, the norepinephrine transporter (NET) on noradrenergic neurons, or the serotonin transporter (SERT) on serotonergic neurons (see Chapter 3 for discussion of MATs). After reuptake, the monoamine can be re-vesicularized by VMAT2 (Figure 7B) or metabolized by monoamine oxidase (MAO) (Figure 7E).
Figure 7: The life cycle of dopamine, as a representative monoamine neurotransmitter. A) Dopamine is synthesized by dopaminergic neurons in the cell bodies or axonal projections. B) Dopamine is vesicularized by VMAT2 (blue ovals). C) Upon arrival of an action potential, synaptic vesicles fuse with the plasma membrane and release the packaged neurotransmitter into the synaptic cleft. D) Reuptake via DAT (gray boxes) controls the duration of the dopamine signal and allows the neuron to recycle a large proportion of the dopamine. E) Oxidative deamination by monoamine oxidase (MAO, orange structure) is a principle mechanism of dopamine metabolism.

False Neurotransmitters

The concept of false neurotransmitters (FNs) arose in the mid to late 1960s with the discovery that certain small ethylamine analogs are actively transported across the plasma membrane analogous to monoamine neurotransmitters, and can be packaged and released alongside neurotransmitters by action of the then
uncharacterized vesicular transporter. False neurotransmitters may have pharmacological activity at neurotransmitter receptors, may induce efflux of neurotransmitter from synaptic stores, or have other effects including replacement of presynaptic neurotransmitter with molecules with reduced or altered signaling capability.

Early experiments demonstrated the ability of simple analogs of monoamine neurotransmitters to act as false neurotransmitters. This effect was demonstrated with metaraminol in cardiac tissue of cats.\(^{(40)}\) It was later found that tyramine and α-methyltyramine can be accumulated by sympathetic noradrenergic nerve terminals, converted to β-hydroxy analogs octopamine and α-methyloctopamine in synaptic vesicles, and can be released with electrical impulse as is norepinephrine.\(^{(41)}\) Further, it was demonstrated that phenethylamine, amphetamine, metatyramine, and α-methyl-metatyramine can act similarly, to various extents. \(p\)-Hydroxyamphetamine was found to deplete releasable norepinephrine from peripheral noradrenergic synaptic elements, causing a commensurate decrease in blood pressure, as \(p\)-hydroxyamphetamine is a less active “false neurotransmitter.”\(^{(42)}\) While tyramine is a false neurotransmitter known to increase blood pressure due to its activity at adrenergic receptors, it was found that this effect can be abolished by treatment with \(p\)-hydroxyamphetamine, which depletes catecholamines (or tyramine) from terminals while having little pressor activity compared to tyramine or norepinephrine.\(^{(42)}\) These pieces of evidence highlight the possibility that false neurotransmitters can drastically alter the biological system that they are administered to, for example if their substrate
activity is high enough to outcompete endogenous neurotransmitters at plasma membrane or vesicular transporters, or if they have other neuropharmacological activities.

It was postulated that if a novel false neurotransmitter could be identified to be sufficiently fluorescent for 2-photon microscopy, it could act as a tracer of monoamine neurotransmitters in tissue and cultured cells. An analog of serotonin, 5,7-dihydroxytryptamine (5,7-DHT), has been shown to be a fluorescent substrate for the dopamine transporter, and has been used to image neurons in culture.\(^{[43]}\)

5,7-DHT is a poor choice as an imaging candidate, however, as it oxidizes readily to form toxic peroxide species, is not a particularly bright probe, and photobleaches readily, but proves the principle that fluorescent monoamine-like substrates can be used in imaging applications. Our search for optimal FFNs has led to some success (Figure 8), and has matured into the current research program into which this dissertation fits.
Figure 8: Structures of monoamine neurotransmitters, some select false neurotransmitters capable of being transported into neurons alongside neurotransmitters, and previously reported fluorescent false neurotransmitters developed in the Sames group. Our approach involves employing the neuron’s endogenous transporters to selectively load fluorescent reporters into synaptic vesicles.

Fluorescent False Neurotransmitters

When our collaboration with the Sulzer group initiated the FFN program, it was hypothesized that fluorescent false neurotransmitters could serve as valuable research tools in the study of monoamine neurotransmission, synaptic physiology, etc. Potential fluorescent false neurotransmitters are prepared in our lab by functionalizing small, fluorescent compounds with a recognition element for transporters of interest, such as an ethylamine moiety (Figure 9). The resulting novel monoamine-like fluorescent compounds are then evaluated for photophysical properties and substrate activity at VMAT2 (see Chapter 2 for a discussion), MATs (Chapter 3), and polyspecific Uptake2 transporters (Chapter 5). After potential fluorescent false neurotransmitters are identified in our lab using heterologous cell-
based studies of transporter-dependent uptake, the behavior of these lead probes is evaluated in mouse brain tissue in the lab of Professor David Sulzer (Columbia University Medical Center) for ability to label neuronal structures, selectivity of labeling, and ability to visualize exocytotic release from vesicular stores, among other characteristics.

**Figure 9:** Concept of FFNs. A) Our approach has been to combine the ethylamine moiety, a key recognition element of binding and transport through MATs and VMAT, with a small fluorescent core. The resulting libraries of novel monoamine-like fluorophores are evaluated for activity at relevant monoamine transporters, and leads are studied in brain tissue. B) At the synapse, FFNs are loaded into presynaptic terminals by action of plasma membrane transporters or by passive diffusion followed by concentration by VMAT2. FFNs can then be used to study morphology and synaptic activity of labeled neurons.

For a FFN to be useful in cultured cells or primary tissue applications, it must exhibit a number of specific traits. First, FFNs must be sufficiently bright and photostable for fluorescence microscopy applications. To date, all FFNs share substrate activity at VMAT and use either this activity or additional activity as a dual substrate for VMAT and a MAT on the plasma membrane to load aminergic neurons selectively. Once inside the cell, FFNs must not become heavily localized in
intracellular compartments other than vesicles, to avoid background uptake. FFNs must also have carefully-tuned polarity, allowing them to either cross the plasma membrane sufficiently enough to become sequestered by VMAT but without labeling all cells nonspecifically, or polar enough to be excluded by all other cell types except those actively transporting the FFN by a plasma membrane transporter. Finally, while FFNs must have sufficient kinetic properties at the relevant transporters such that they selectively label target aminergic neurons, the kinetics of transport must not be so high that the FFNs displace endogenous neurotransmitters and concomitantly alter the signaling characteristics of the synapse (as some false neurotransmitters are known to do: see above). One benefit of fluorescence microscopy is that it is sufficiently sensitive to image a small number of vesicularized fluorophores; it is not necessary to displace endogenous monoamines in order to image the synapses.

**FFNs Visualize Synaptic Activity**

Our lab is currently involved in pursuing FFNs that can selectively load aminergic neurons via MATs or through action of VMAT2, depending on the specific parameters of the FFN. When imaged in the dorsal striatum of acute mouse brain slice, a dopaminergic FFN will appear as points of fluorescence within each frame, herein referred to as “puncta.” These puncta can represent loaded aminergic presynaptic elements or other structures within the neuron or brain slice preparation, with each FFN offering unique labeling characteristics. A FFN with a well-characterized mechanism of loading will serve as a tool to study how that
uptake mechanism responds to various drugs, and how it behaves in disease models.

**Figure 10:** Two different detection motifs enabled by Fluorescent False Neurotransmitters for detection of synaptic activity and neurotransmitter release. A) Probes including FFN200 have been developed to provide a reduction in signal upon vesicular fusion, and are herein referred to as “destaining FFNs.” B) pH-sensitive FFNs afford an increase in brightness upon release from the synapse, and are referred to as “flashing FFNs.”

Once the brain slice has been loaded with the FFN, release studies can be carried out. Neurotransmitter release via vesicular fusion with the plasma membrane can be induced by chemical means (KCl) or direct electrical stimulation, and the resulting synaptic release events can be modulated or inhibited by various drugs. A functional tracer of monoamines can be used to visualize this process with
different readouts. In release studies, a well-characterized FFN can potentially give important information about the kinetics of vesicular fusion and neurotransmitter release under various conditions (drugs, disease models), possibly provide information about the different populations of vesicles in synaptic zones (reserve pool vs. releasable pool), the kinetics of vesicular recycling, and behavioral heterogeneity of distinct presynaptic elements under different conditions (frequency of electrical stimulation, effects of drugs or disease, various environmental stimuli in \textit{in vivo} experiments, etc.).

We are currently pursuing two FFN motifs that provide optical information upon vesicular fusion. The first is a non-pH sensitive FFN family based on aminoethyl-substituted 7-aminocoumarins. These compounds are loaded into aminergic neurons or VMAT2-expressing cells by an indeterminate mechanism, but label synaptic areas in neurons, or VMAT2-expressing organelles in heterologous or peripheral cells, in a VMAT2 dependent manner.\cite{44,45} It is thought that VMAT2 may drive this accumulation or selective retention, although plasma membrane transporters cannot be ruled out at this time. Upon vesicular fusion and exocytotic release from neurons, these FFNs diffuse from their highly concentrated luminal environment into the synaptic cleft, where they dissipate below the detection limit of the fluorescence microscope. The result is a “destaining” of the labeled, punctate presynaptic elements (\textbf{Figure 10A}).

The second motif uses aminoethyl-substituted, pH-sensitive 7-hydroxycoumarins, which are ratiometric pH sensors with tuned pK\textsubscript{a} values between the relatively low pH of the acidic synaptic vesicles (pH~ 5.5-6.0) and the
neutral extracellular environment (pH = 7.4), with an accompanying increase in brightness upon deprotonation.\cite{46,47} These compounds are quite polar, have been found to be substrates for MATs (Chapter 3),\cite{47} and afford quite selective labeling of aminergic neurons. Due to the pH sensitivity of these probes, exocytotic release from vesicular stores results in a visible “flash” (Figure 10B).

The ultimate goal of this body of work is to generate a method with multitudinous applications, including but certainly not limited to the study of aminergic signaling with high spatiotemporal resolution, aminergic modulation of glutamatergic, cholinergic, or other synapses (as well as the modulation of whole signaling networks), the effects of acute and long-term pharmacological treatment on aminergic signaling in the brain, trafficking of loaded vesicles within individual neurons, and the relationship between aminergic signaling and disease. The latter studies could focus on the effects of disease on monoamine release and reuptake within individual neurons, as well as the broader effects of disease on signaling networks involving aminergic neurons. Currently, FFNs exist for the study of dopaminergic synapses in the dorsal striatum of acute mouse brain, as fluorescent ratiometric pH sensors of VMAT2-expressing vesicles or acidic organelles, and as high-throughput amenable screening agents for VMAT2 activity. This thesis details new advancements in these areas (Chapter 2) and work toward the identification of FFNs as fluorescent probes for the study of systems beyond nigrostraital dopaminergic innervations (Chapters 3-5).
IV - Conclusions and Outlook

From the formative days of modern neuroscience when a simple silver stain was used to visualize individual neurons with a light microscope, chemistry has contributed to the methodological engine driving new discoveries about the brain. Neuropharmacology has contributed to our understanding of the brain by helping to elucidate the effects of neurotransmitter receptor agonism or antagonism on behavior or CNS phenotypes. With emerging optical imaging technologies for determining functional connections in neural networks, chemical reporters have allowed for imaging of action potentials in individual neurons and in the context of signaling networks. With an emerging interest in detection of neurotransmitters with special engineered receptors, there is also opportunity for chemistry to develop optical tracers of neurotransmitters to directly image neurotransmitter release and reuptake.

I posit that the use of pharmacologically selective, highly fluorescent tracers of neurotransmitters will contribute to our understanding of synaptic communication and the relationships between individual synapses and their interrelated signaling networks. Chemistry provides us with the tools to generate new optical imaging agents, and chemical biology provides tools to evaluate these agents for further chemical optimization. The problems we must solve concerning the brain lie at the interface of classical neuroscience, neuropharmacology, synthetic chemistry, microscopy and imaging, and chemical biology. The majority of this thesis deals with fundamental biochemical and imaging work toward the
development and optimization of new fluorescent false neurotransmitters for the study of monoaminergic neurotransmission.
References


Chapter II

New FFNs as Substrates for the Vesicular Monoamine Transporter 2 (VMAT2)
I: Introduction

Vesicular Monoamine Transporter (VMAT)

Transport of monoamines from the cytosol to synaptic or secretory vesicles is a centrally important parameter for the function of these signaling molecules. This translocation is accomplished by action of the Vesicular Monoamine Transporter, a member of the solute carrier family 18 of transporters (SLC18), which includes two distinct VMAT isoforms as well as the vesicular acetylcholine transporter (VACHT).\(^1\) VMAT2 plays a key role in monoaminergic neurotransmission: it is responsible for packaging monoamine neurotransmitters into synaptic vesicles for eventual exocytotic release into the synapse.\(^2-5\) VMAT2 also exists in the periphery, to a large degree in the pancreatic β-cells, where transport of monoamines has been shown to play a role in insulin secretion.\(^6\) VMAT1 exists solely in the periphery, most notably in adrenal chromaffin cells and endocrine cells of the gastrointestinal tract, where it packages monoamines into large dense core vesicles (LDCVs) for storage and exocytotic release for endocrine signaling.\(^2, 7, 8\) The two distinct isoforms share approximately 60% sequence homology,\(^9\) both contain 12 transmembrane α-helices, and both are capable of transporting monoamines.\(^1\) Substrate and inhibition profiles are similar but not identical, with reserpine serving as a potent inhibitor of both isoforms and tetrabenazine (TBZ) displaying selectivity towards VMAT2.\(^7, 10\)

VMAT is a monoamine/H\(^+\) antiporter,\(^11\) and functions by transporting monoamine substrates against their concentration gradient by effluxing two protons down a pH gradient, from the low pH of the vesicle to the higher pH of the
cytosol. This pH gradient is generated and maintained by a vacuolar ATPase (vATPase), using energy from the conversion of ATP to ADP to transport protons into the vesicular lumen (Figure 1B).\(^{11,12}\)

**Figure 1:** The vesicular monoamine transporter (VMAT) is responsible for vesicularizing monoamine neurotransmitters in the central nervous system and periphery. A) Monoaminergic presynaptic elements can accumulate FFNs either by action of plasma membrane transporters (shown in light blue) or passive diffusion followed by uptake by VMAT2 (dark blue ovals). B) A representation of a synaptic vesicle, highlighting the relationship between VMAT and a vATPase (light blue), which is responsible for generating the pH gradient across the vesicular membrane, which VMAT uses to drive transport of monoamines. VMAT is capable of translocating a number of substrates, including endogenous monoamines and FFNs. C) A representation of FFNs interacting with VMAT in a peripheral cell (VMAT2 in a pancreatic B-cell or VMAT1 in an adrenal chromaffin cell, for example), or in a heterologous VMAT2-overexpressing cell.

VMAT is quite functionally plastic, tolerating a relatively broad scope of substrates. Transported substrates include all of the monoamine neurotransmitters, various other phenethylamines or tryptamines, the structurally unrelated neurotoxin MPP+, rhodamine 6G, and others.\(^{12-14}\) Relatedly, VMAT2 is involved in neuroprotection for two distinct reasons. First, monoaminergic neurons exploit the
broad substrate scope of VMAT in order to sequester small-molecule toxins into synaptic vesicles for eventual exocytotic release. It has been shown, for example, that VMAT2 activity can rescue cells from MPP+ toxicity (discussed in Chapter 4), and VMAT inhibition has been linked to an increase in cytotoxicity under certain circumstances.\(^{[14-16]}\) Second, VMAT2 is protective of neurons simply by clearing the cytosol of monoamine neurotransmitters after biosynthesis or reuptake. Monoamines are principally catabolized via oxidative deamination, catalyzed by monoamine oxidases (MAOs), resulting in the generation of toxic reactive oxygen species (ROS).\(^{[17,18]}\)

Because of its role in neurotransmission, endocrine signaling, and neuroprotection, VMAT has historically been a drug target and is reemerging as potentially relevant for the treatment of several conditions. Correspondingly, VMAT is also a target for imaging agents.

**VMAT2 as a Drug Target**

Because of its critical role in modulating neurotransmission, VMAT has long been considered as a target for therapeutics. VMAT is the target of reserpine, an early anti-hypertensive and antipsychotic drug (Figure 2A).\(^{[19]}\) Reserpine is thought to interact with the monoamine binding site of VMAT, and its mechanism of action involves depletion of monoamines in aminergic synapses.\(^{[20]}\) The narrow therapeutic index and serious side effects including sedation, anxiety, parkinsonism, and gastrointestinal distress (caused by inhibition of VMAT1 in the gastrointestinal tract), have led to reserpine being phased out as a drug and has cast a negative image on VMAT as a pharmacological target.\(^{[17]}\) However, there has recently been
renewed interest in targeting VMAT, particularly in the context of terminally ill patients\(^{(21)}\) and for diabetes treatment\(^{(6)}\).

![Figure 2: A) Representative inhibitors of VMAT. B) Many of the inhibitors of VMAT have been applied as positron emission tomography (PET) probes.](image)

TBZ (Figure 2A) is a selective VMAT2 inhibitor thought to interact with an allosteric site different from that of reserpine\(^{(19)}\). TBZ has been used previously as an effective treatment of hyperkinetic movement disorder and chorea associated with Huntington’s disease\(^{(22)}\). Side effects resemble those of reserpine, and include depression, drowsiness, fatigue, and Parkinsonism\(^{(22,23)}\). Despite these side effects, the FDA has recently approved TBZ for the treatment of chorea associated with Huntington’s disease\(^{(24)}\). Interest has arisen in more potent TBZ derivatives such as dihydrotetrabenazine (DTBZ) to increase efficacy, reduce requisite doses, and concomitantly reduce off-target side effects\(^{(25)}\).

VMAT2 inhibitors are also of interest for treatment of efflux-inducing psychostimulant abuse and addiction. Lobeline (Figure 2A) is a natural product that is structurally distinct from reserpine and TBZ, and has been shown to inhibit
Lobeline and its derivatives inhibit methamphetamine-induced dopamine release, as well as methamphetamine self-administration in animal models, and these compounds are currently being pursued as novel therapeutics in preclinical and clinical studies for the treatment of methamphetamine abuse.\(^{(26)}\)

Additionally, there has been some interest in developing VMAT2 inhibitors for the treatment of diabetes, as inhibition of VMAT2 in pancreatic \(\beta\)-cells has been shown to increase insulin secretion, increase overall insulin sensitivity, and improve glucose metabolism in animal studies.\(^{(6, 27)}\) As such, there would be interest in developing a potent, selective VMAT2 substrate that does not cross the blood-brain barrier, in order to treat diabetes and insulin resistance without neurological effects or gastrointestinal effects from VMAT1 inhibition in the digestive tract, as seen with reserpine. Further screening and development of inhibitors would be necessary to reach this goal, and a high-throughput (HT) screening method for VMAT2 inhibition would greatly assist this process (Section II).

**VMAT as a Target of Imaging Agents**

Because of its centrality to monoaminergic neurotransmission and endocrine processes, there has been significant interest in the use of imaging agents targeting VMAT. To these ends, various radiotracers have been used to study VMAT. Radiolabeled \(^{11}\)C-TBZ and \(^{11}\)C-DTBZ, \(^{18}\)F-FE-DTBZ have been introduced as PET imaging agents.\(^{(28-31)}\) Figure 2B enable study of VMAT2 in the striatum. Using these tools, different expression levels have been observed in neurological diseases bipolar I and schizophrenia,\(^{(28)}\) and a decrease of VMAT2 in the pancreas, which has
been associated with the onset of diabetes.\cite{30,31} These imaging agents, while useful for \textit{in vivo} studies of VMAT-expressing tissues, are inherently limited by the spatial resolution of PET. The study of VMAT2 expression and activity in individual presynaptic elements or within single pancreatic beta cells would require the development of other imaging techniques. To address this, we have been pursuing a fluorescence-based approach.

\textbf{VMAT2 and FFNs}

VMAT2 was the initial molecular target of the FFN program,\cite{32} and continues to be centrally important to the development of new fluorescence-based optical tracers of monoamine neurotransmitters. Since VMAT2 is the transporter responsible for packaging monoamines into the synaptic vesicles, substrate activity at VMAT2 is essential for any FFN’s ability to label aminergic synapses and image synaptic vesicle fusion.\cite{33} Since no crystal structure has been solved for VMAT, we systematically employ rational molecular design to incorporate small, fluorescent aromatic moieties with known functionalities common to VMAT substrates (aminoethyl groups, hydrogen bond donors/acceptors, etc; \textbf{Figure 3}).

A number of functional VMAT2 probes have been developed in our group, including pH-responsive FFNs based on aminoethyl-substituted 7-hydroxycoumarins.\cite{34} One of these probes has been demonstrated to be a dual functional DAT-VMAT2 substrate in acute mouse brain slice,\cite{35} serving as a proof of principle for dual-substrate activity of FFNs (See Chapters 1 and 3 for discussion). Other FFNs based on a 7-aminocoumarin core have been studied as superior analogs of FFN511, and the development of a HT screening agent for VMAT2 activity
and inhibition in cultured cells has been reported and is described below (Part II).\cite{36} Substrate activity at VMAT2 is centrally important to the behavior of FFNs, and remains the only truly unifying characteristic of each of these FFNs.

**Figure 3:** VMAT substrates, design of FFNs, and two previously reported FFNs.

This chapter describes recent advancements I have contributed to the FFN program with respect to VMAT2. Part II of this chapter describes the validation and final characterization of a FFN for use in high-throughput screening of VMAT2 activity and inhibition in cell culture, with potential application to drug discovery.
programs targeting VMAT2. Part III details some contributions to the search for next generation FFNs as optical tracers of monoamines in brain tissue.
II: A new fluorescent substrate allows for examination and high-throughput screening (HTS) of VMAT2 activity and inhibition in cultured cells.\(^{36}\)

A. Introduction

Within the framework of our FFN program, we hypothesized that a fluorescent VMAT2 substrate could be developed for the study of VMAT2 activity and inhibition in cultured cells. Any such probe would require highly VMAT2-specific cellular uptake, stable photophysical properties, and pharmacological properties similar to endogenous substrates. If identified, this probe could be developed into a fluorescence-based microscopy assay for VMAT2 activity and subcellular location; we also hypothesized that such an imaging agent could be extended to microplate-based systems for HT examination of VMAT2 activity and inhibition. This section details the previous characterization of FFN206, as well as my collaboration with Dr. Adam Henke for the validation of FFN206 as a high-throughput amenable screening agent for VMAT2 in cultured cells.

FFN206 Enables Examination of VMAT2 Activity, Distribution, and Inhibition in Cultured Cells

FFN206 was designed and developed under the rationale of the FFN program, as described above. The synthesis of FFN206 was completed by Dr. Gang Hu as part of a broad screen of approximately 40 aminocoumarin-based probes toward VMAT2. FFN206 was found to have favorable photophysical properties, and was first evaluated in cultured cells with wide-field fluorescence microscopy. The cellular system employed to test VMAT2-dependent uptake was transfected to heterologously express rVMAT2 (VMAT2-HEK). These cells were developed and
fully characterized in the lab of our collaborator, Professor Robert Edwards (UCSF). It was found that FFN206 enabled visualization of VMAT2 activity in individual VMAT2-HEK cells, as well as the intracellular distribution of labeled compartments in a manner strongly sensitive to VMAT2 inhibitors (Figure 4). Treatment of FFN206-labeled VMAT2-HEK cells with the lipophilic weak base chloroquine, which crosses membranes and collapses the proton gradient between the lumen of acidic compartments and cytosol (the “weak base effect”), caused redistribution of the probe from punctate VMAT2-expressing compartments to the cytosol, confirming that FFN206 accumulates in acidic organelles as opposed to simply binding to the protein target. The observation that FFN206 does not considerably label other organelles renders this compound a useful imaging agent for subcellular localization of VMAT2 activity.

Figure 4: Uptake of FFN206 in cultured cells is dependent on VMAT2 activity. Epifluorescence images of HEK cells (VMAT2 transfected and null-transfected controls) treated with FFN206 (5 μM, 2 h incubation) in the absence or presence of TBZ (2 μM, 1 h preincubation followed by coincubation with the probe). (A) FFN206 selectively accumulates in cells expressing VMAT2. (B) Negligible uptake of FFN206 in VMAT2-HEK cells pre-treated with TBZ. (C) HEK293 cells (not expressing VMAT2) pre-treated with DMSO vehicle. (D) Control HEK293 cells pre-treated with TBZ also accumulate negligible amounts of FFN206. Top row = epifluorescence images, bottom row = brightfield images. Images are courtesy of Dr. Gang Hu.
Dr. Hu showed that this wide-field microscopy-based assay for VMAT2 activity and subcellular distribution could be extended to a quantitative fluorometric microplate assay for VMAT2 activity, specifically with examination of dose-response studies of inhibitors and high throughput screening applications in mind. A number of established VMAT2 inhibitors, including reserpine, TBZ, DTBZ, lobeline, fluoxetine, S(+)-methamphetamine, ketanserin, and haloperidol were chosen to validate the assay’s ability to accurately quantify inhibition (IC\textsubscript{50} values) of inhibitors with a wide range of reported potencies (Figure 5).	extsuperscript{(38,39)} Each of these compounds was found to inhibit the uptake of FFN206 in a dose-dependent manner. Inhibition constants (K\textsubscript{i} values) were calculated by substitution of the apparent K\textsubscript{m} value of FFN206 (determined during optimization of this assay platform) and measured IC\textsubscript{50} values of each inhibitor into the Cheng-Prusoff equation (K\textsubscript{i}=IC\textsubscript{50}(1+[S]/K\textsubscript{m})). These K\textsubscript{i} values were found to closely agree with previously reported potencies determined by other methods (Figure 5). Thus, FFN206 enables rapid determination of quantitative inhibition parameters of VMAT2 inhibitors over a wide range of potencies in intact cells using a plate reader.
Figure 5: FFN206 can be used to measure inhibition constants of known VMAT2 inhibitors and can be used as a screening agent for VMAT2 inhibition. IC₅₀ values of known VMAT2 inhibitors were measured in the FFN206 uptake assay. The final concentration for FFN206 is 1 µM. Error bars are standard errors (S.E.) derived from 4 independent experiments. Each experiment was run in triplicate/plate and 2 plates total. IC₅₀ values were listed as [inhibitor] ± S.E. Reserpine: IC₅₀=0.019±0.001 µM; haloperidol: IC₅₀=0.071±0.003 µM; TBZ: IC₅₀=0.32±0.01 µM; Lobeline: IC₅₀=1.01±0.07 µM; DTBZ: IC₅₀=0.017±0.001 µM; Fluoxetine: IC₅₀=1.07±0.05 µM; Ketanserin: IC₅₀=0.105±0.005 µM; S(+) - Methamphetamine (Meth): IC₅₀=4.53±1.42 µM. Results are courtesy of Dr. Gang Hu.

A representative screen of small compounds with various known effects on VMAT2 and vesicular pH was used to characterize FFN206 as a screening agent in practice, and the results of this screen correlated with known behavior of each of the tested compounds (for detailed information, see our report).[^36]

With the invaluable assistance of a Dr. Adam Henke, a postdoc who I trained in mammalian cell culture and cell-based FFN studies, I set out to study a few key
parameters necessary for use of FFN206 in high throughput screening. It was necessary to confirm that the observed kinetics of VMAT2-dependent FFN206 uptake are not limited by either passive diffusion or transporter-dependent accumulation of FFN206 across the plasma membrane. It was also incumbent upon us to establish the Z’-factor, a widely used measure of the quality of a HT assay, of FFN206 for our microplate reader. Because a FFN206-based assay may be adapted to a system requiring repeated measurements, the photostability of FFN206 was also investigated.
B. Results and Discussion:

FFN206 Uptake Kinetics are Not Limited by the Plasma Membrane:

To provide a quantitative measure of FFN206’s interaction with VMAT2, we measured cellular uptake kinetics using fluorometry. Dr. Hu initially characterized the apparent $K_m$ of FFN206 at VMAT2 using this assay, and found it to be $1.16 \pm 0.10 \mu M$, similar to that of dopamine. It was determined that the initial fluorescence uptake velocity of FFN206 in VMAT2-HEK cells remains linear for 12 min for concentrations up to 6 $\mu M$, and the initial rates were measured in the linear interval at various FFN206 concentrations.

A similar $K_m$ to that observed by Dr. Hu was measured using a more modern plate reader ($K_m = 1.12 \pm 0.24 \mu M$, (mean $\pm$ SEM, n = 2 independent experiments; Figure 6A)). Since the vast majority of VMAT2 in these cells is expressed on intracellular compartments, it was necessary to establish whether the observed $K_m$ was in fact due to interaction of FFN206 with VMAT2 or if the measured affinity was actually the rate of diffusion or transport across the plasma membrane. To accomplish this, the apparent $K_m$ was also measured when cells were treated with digitonin to permeabilize the membrane. This experiment was adapted from earlier procedures to study VMAT transport (13) and membrane permeabilization was confirmed in an independent control experiment by trypan blue (0.4%, 10 minute treatment) uptake into cells under permeabilizing conditions. The uptake of FFN206 under these conditions was found to be linear for 9 minutes, and the $K_m$ was determined to be $2.26 \pm 0.92 \mu M$ (mean $\pm$ SEM, n = 3 independent experiments, Figure 6B), suggesting that diffusion across the plasma membrane or transport
through a hypothetical plasma membrane transporter is not the rate-determining step of uptake in the whole cell assay (where the vast majority of VMAT2 protein is intracellular).

To confirm the apparent $K_m$ obtained by the fluorescence assay with intact and permeabilized cells, the affinity of FFN206 for VMAT2 was measured by the inhibition of $[^3H]$-serotonin uptake in membrane preparations obtained from VMAT2-HEK cells in the laboratory of Professor Robert Edwards (UCSF). An IC$_{50}$ value of 1.15 µM was obtained (Figure 6C), showing good agreement with the apparent $K_m$ for FFN206 determined by fluorescence. The modest affinity of FFN206 for VMAT2 compares well with endogenous substrates such as dopamine ($K_m = 0.82 - 0.95$ µM$^{(40, 41)}$ and IC$_{50} = 0.92 \pm 0.05$ µM$^{(40)}$).
Figure 6: FFN206 $K_m$ apparent is not rate-limited by transport or diffusion across the plasma membrane of VMAT2-HEK cells. A) Michaelis-Menten plot of FFN206 as measured by initial rates of accumulation of specific fluorescence uptake in VMAT2-HEK cells (subtracted background is from DTBZ-treated wells, 2 µM); $K_m = 1.12 \pm 0.24$ µM, (mean ± SEM). B) Pretreatment with 10 µM digitonin (to permeabilize the plasma membrane) does not increase the apparent affinity of FFN206 for VMAT2 ($K_m = 2.26 \pm 0.92$ µM). C) FFN206 inhibits $^3$H-5HT uptake in membrane preparations of VMAT2 from the same VMAT2-HEK cells. IC$_{50}$ was found to be $1.15 \pm 0.24$ µM (mean ± SEM). Results for C are courtesy of the Edwards Laboratory (USCF).

FFN206 As a HTS-Amenable VMAT2 Substrate

High-throughput screening assays are evaluated for robustness by determining the dynamic range of the assay and experimental variability (determined as standard deviations of control data sets), and HT assays are held to high standards to minimize occurrences of false positives or negatives arising from statistical variations of the normal distributions of the data. The $Z'$-factor has been
introduced as an important means of quantifying this robustness.\textsuperscript{(42)} A $Z'$-factor greater than 0.5 indicates an assay is acceptable for HT screening. In order to determine the applicability of the FFN206-based VMAT2 assay to high throughput applications, control experiments were conducted from which this parameter was calculated ($Z'$-factor $= 0.68 \pm 0.07$; calculated from the arithmetic means determined from 5 independent experiments, each consisting of 2 identical plates; \textbf{Table 1}, see \textbf{Figure 7} for equations used). Additionally, it was found that by measuring area scans of each well (versus the single-point scans), this parameter can be further improved (\textbf{Table 1}), although area scans considerably slow the reading, making it less attractive for HT application.

\[
Z' = 1 - \frac{3 \times (\sigma_{(-)} + \sigma_{(+)} )}{|\mu_{(-)} - \mu_{(+)}|} \\
CV = \frac{\sigma}{\mu} \times 100 \\
S/B = \frac{\mu_{(-)}}{\mu_{(+)}}
\]

\textbf{Figure 7}: Equations used to calculate high-throughput screening parameters. CV is the coefficient of variation for each data set. S/B is the signal to background ratio. $\mu = \text{control means, positive (+) or negative (-)}$; $\sigma = \text{stdev of controls.}$

The fluorometric assay utilizing FFN206 is well suited for HTS as indicated by the statistical evaluation of its performance ($Z' > 0.5$ is recommended for HTS). It should be noted that automation of the assay, from cell counting to automated
washing and solution delivery, would likely improve these HTS parameters even further.

Other recently described fluorescent VMAT substrates are not as amenable to HT applications. Our group has recently reported FFN202 as a pH-sensitive VMAT2 substrate,\(^\text{(43)}\) although this probe is not well suited for HTS since the pH sensitivity may complicate VMAT2 uptake measurements. Additionally, FFN202 is not as bright as FFN206, yielding a smaller dynamic range of measurement. FFN511, as with many of the screened 7-aminocoumarins (Section III), is not well suited for cell culture applications due to a high background signal. A fluorescent VMAT2 assay has been reported recently using the Neurotransmitter Transporter Uptake Assay Kit (NTUA, Molecular Devices).\(^\text{(44)}\) This report demonstrates that the proprietary fluorescent DAT, NET, and SERT substrate in this kit is also a VMAT substrate. While this assay is readily applicable to HTS format, it requires microscopy. Independent image acquisition, processing, and analysis of each well is required due to VMAT-independent staining of mitochondria and other organelles by the NTUA probe (APP+,\(^\text{(45,46)}\) as discussed in Chapters 3, 4, and 5). While this probe has certain benefits that may be realized in primary culture where DAT is responsible for loading the cells with the fluorescent probe, the FFN206 assay described here enables fluorometric measurement with a standard plate reader, facilitating both the high throughput screening for new VMAT2 inhibitors and quantitative examination of identified hits and lead compounds.
Table 1  High throughput screening parameters as measured by a BioTek H1MF plate reader recoring single points from each well (fastest for HT) compared to area scans of various dimensions. Values are reported as means ± SD. Data were collected in collaboration with Dr. Adam Henke.

<table>
<thead>
<tr>
<th></th>
<th>Single Point</th>
<th>Area Scan (3x3)</th>
<th>Area Scan (5x5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z’</td>
<td>0.68±0.07</td>
<td>0.79±0.03</td>
<td>0.82±0.03</td>
</tr>
<tr>
<td>CV (-)</td>
<td>7.0±2.1</td>
<td>4.6±0.7</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>CV (+)</td>
<td>11.0±1.0</td>
<td>7.2±0.8</td>
<td>7.2±1.0</td>
</tr>
<tr>
<td>S/B</td>
<td>5.4±0.9</td>
<td>6.2±0.9</td>
<td>6.4±1.0</td>
</tr>
</tbody>
</table>

Photostability of FFN206

As some applications of FFN206 in the study of VMAT2 may require repeated measurements, the photostability of FFN206 was tested using our plate reader. In order to determine the photostability of FFN206 under the fluorometric assay conditions, fluorescence emission of a solution of FFN206 (0.5 μM in PBS) was repeatedly measured on a BioTek H1MF plate reader using the same excitation and emission wavelengths as above (λ_{ex} = 368 nm, λ_{em} = 464 nm). Measurements were taken every 15 s for 10 min. No significant decrease in fluorescence intensity was observed over this time period.
Figure 8: Photostability of FFN206. No significant attenuation of FFN206 signal was observed after repeated measurements using a plate reader. Data are representative of 2 independent experiments.
C. Conclusions and Outlook (Part II)

We have introduced FFN206 as a new optical probe with several applications in the study of VMAT2 in cell culture. This small, highly fluorescent coumarin analog is capable of sensitively detecting VMAT2 activity on wide-field microscopic or fluorometric platforms. FFN206 is capable of determining subcellular location of VMAT2-expressing organelles with high selectivity; it does not considerably label non-VMAT2-expressing structures or other cellular components, including mitochondria. It is also capable of reporting on VMAT2 activity in a fluorometric format highly amenable to high throughput screening; the assay features simple operations amenable for automation, excellent \( Z' \)-factor, as well as reproducibility. As such, FFN206 represents an excellent HTS candidate capable of facilitating the search for next generations of VMAT2 modulators including inhibitors, substrates or enhancers.

As is the case for all HTS campaigns, secondary assays are needed to address and filter false positives. It would be most important to have secondary assays to address potential collapse of pH gradient across the vesicular membrane by action of experimental compounds via the weak base effect or inhibition of v-ATPase. Such a secondary assay could take the same format as the FFN206 assay, using a lysotracker probe or acridine orange. These dyes are amphiphilic weak bases that accumulate in acidic organelles independent of transporters, in a manner proportional to the pH of the compartments, allowing for measurement of pH of acidic compartments within the cell, and in this case, providing a secondary screen for pH collapse by hits identified by FFN206.
We have shown that FFN206 uptake by VMAT2 is not limited by the plasma membrane, although a hypothetical plasma membrane transporter may ultimately be responsible for transport of FFN206 into the cell. Due to the vast number of potential transporters that could facilitate FFN206 conveyance across the plasma membrane, it was not practical for us to conduct a systematic pharmacological or gene silencing study to address this issue. Thus, it is impossible to rule out the possibility that hit compounds resulting from a HT screen may be identified by action as inhibitors of such a conjectural plasma membrane transporter. Adaptation of the digitonin-induced permeabilization used above may serve well as an additional secondary assay addressing direct inhibition of VMAT2 after confirmation of pH-independence of any initial hits.
III: New FFNs for Imaging VMAT2 in Brain Tissue

A. Introduction –

As discussed in the introduction, our lab is focused on developing FFNs as optical tracers of monoamine neurotransmitters in brain tissue, and VMAT2 substrate activity is an essential parameter for these probes. Within the FFN program, there is always the need to find superior VMAT substrates with optimized kinetic parameters ($V_{\text{max}}$, $K_m$), tuned lipophilicity, and low nonspecific uptake by secondary transporters or other mechanisms. The interplay between these parameters is manifest in selectivity of the FFN for aminergic terminals, signal to background ratio, and destaining characteristics of the FFN in the overall observed behavior of the FFN in acute brain slice, primary neuronal culture, or other advanced experimental systems.

In search of high-quality FFNs, a number of probes have been synthesized and screened for activity at VMAT2, either previously by former members of the Sames group or concurrently with this work by Dr. Adam Henke. The results of these screens are briefly summarized in Figure 9. Of the numerous compounds screened in VMAT2-HEK cells, promising leads were tested in acute mouse brain slice preparations by Sames Lab members working in the lab of Professor David Sulzer (CUMC), or were sent to other collaborators for characterization in other advanced systems. A number of these compounds are currently used to study VMAT2 activity, distribution, and pharmacology in cultured neurons (Dr. Yvonne Schmitz, Sulzer Group), and monoaminergic neurotransmission in whole brains of *d. melanogaster* (Dr. Mark Sonders and Dr. Zachary Freyberg, Javitch Group) and acute
mouse brain slice (Dr. Daniela Pereira, Sulzer Group; Matt Dunn and Paolomi Merchant, Sames Group). The 7-hydroxycoumarin-based FFN102 has previously been described by our groups for the latter purpose, and functions as a “flashing FFN,” affording a signal increase upon exocytotic release into the synapse, as described in Chapter 1.\textsuperscript{(33,35)}

\textbf{Figure 9:} Summary of previous and contemporaneous substrate mapping of VMAT2 with respect to coumarin-based FFNs. A) In parallel with the work reported here, Dr. Adam Henke has been completing a structure-activity relationship study of aminoethyl hydroxycoumarins initiated by Dr. Minhee Lee.\textsuperscript{(34)} Screened compounds include approximately 30 hydroxycoumarins, including regioisomers with the aminoethyl group in the 3-, 4-, 6-, and 8-positions. B) Previous studies conducted by Dr. Gang Hu have identified SAR relationships in 7-aminocoumarins, although this library included only 3- and 4-aminoethyl regioisomers.
The Need for a Better 7-Aminocoumarin for Use in Acute Mouse Brain Slice

The Sames and Sulzer groups have been pursuing studies in the brain with FFN200, as it is the most selective “destaining” FFN currently available, although labeling and destaining properties are not optimal. We are yet unsure what drives FFN200 uptake into aminergic neurons in acute mouse brain slice, although it is possible that plasma membrane transporters are involved. Conversely, VMAT2-driven uptake or VMAT2-dependent selective retention of FFN200 during washout could be responsible for the observed labeling selectivity for dopaminergic neurons, as determined by anatomical coincidence with GFP expressed under the control of the promoter for tyrosine hydroxylase (TH-GFP) in the dorsal striatum or midbrain of acute mouse brain slice. Dr. Daniela Pereira and Paolomi Merchant have shown an approximate 25% reduction in the number of labeled puncta, thought to be dopaminergic terminals, in the presence of VMAT2 inhibitors, and similar reductions are noticed in VMAT2 hypomorph mice. This contrasts with the approximate 80% colocalization with TH-GFP. I hypothesized that if we could find a VMAT2 substrate similar to FFN206 that was more selective in culture, we could identify an aminocoumarin-based “destaining” FFN that is more useful in examining aminergic innervation and synaptic activity in the brain.

We have been motivated to search for a probe with superior VMAT2 uptake kinetics with minimal background labeling in mouse brain, as this would increase selectivity toward aminergic synapses as well as possibly result in a probe with more ideal destaining kinetics (assuming vesicular recycling is necessary for
complete destaining of puncta). Completion of the structure-activity mapping of the 7-aminocoumarin library was one approach to meet this goal.

**Screened Libraries of Coumarin-Based Probes**

7-Aminocoumarin and 7-hydroxycoumarin-based FFNs with aminoethyl groups in the 3- and 4- positions have been previously synthesized by members of the Sames group, due to the readily accessible nature of these compounds (Dr. Paul Vadola & Dr. Mini Lee, unpublished results). I proposed that completion of these libraries with regioisomers containing aminoethyl groups in the 6 and 8 positions would afford us with a more complete understanding of structure-function relationships between these probes and VMAT, as well as afford the potential to discover a superior probe. Both the 7-hydroxycoumarin and 7-aminocoumarin libraries were expanded with the help of Dr. Adam Henke. An expanded 7-hydroxycoumarin library has been screened for substrate activity at VMAT2 by Dr. Henke (paper in progress), including regioisomers containing the aminoethyl group in the 3-, 4-, 6-, and 8-positions, and some select aminomethyl analogs. I have screened this library for DAT, NET, SERT, and Uptake2 substrate activity (for structures of compounds and results, see Chapter 3 for DAT/NET/SERT and Chapter 5 for Uptake2).

From this expanded library, we have identified and introduced a new 7-aminocoumarin-based VMAT2 substrate that may prove useful as a “destaining” FFN in the brain. I have also uncovered substrate activity at VMAT2 of a new class
of FFNs, which has become a promising lead FFN at DAT, NET, and SERT (Chapter 3).
B. Results and Discussion

**AGH180 is a Substrate of VMAT2 in Cultured Cells**

In a continuing effort to search for VMAT2 substrates as FFN leads, I was curious to test other regioisomers of 7-aminocoumarins. With the help of Dr. Adam Henke, AGH180 was synthesized as a lead. AGH180 is a 6-aminoethyl regioisomer of FFN200 (Figure 10A). Our screening studies in VMAT2-HEK cells confirmed that AGH180 exhibits punctate labeling of VMAT2-expressing compartments, similar to results obtained with FFN206.

In each of three fluorescence microscopy experiments conducted by myself and Dr. Adam Henke, it was found that AGH180 compares favorably to FFN206, and is a considerably brighter label than FFN200 (5 µM, 60 min, Figure 10 B,C). When incubated in the presence of DTBZ (2 µM), the labeling of AGH180 is significantly attenuated. These results were confirmed on a microplate reader by comparing the total uninhibited and DTBZ (2 µM)-inhibited uptake of FFN206, FFN200, and AGH180. As seen in Figure 10D, FFN206 is best for use in cell culture with the highest specific uptake, and AGH180 displays an intermediate to high uptake compared to FFN200 under standard treatment conditions (5 µM probes, 15 minute treatment, n = 2 plates).
Figure 10: Comparison of some advanced 7-aminocoumarin FFNs. A) Structures of FFN206, FFN200, and FFN180, with indicated properties including $K_m$ at VMAT2 (mean ± SEM, see Figure 11), and the calculated topological polar surface area (tPSA; ChemBioDraw Ultra). B) Direct comparison of uninhibited VMAT2 uptake (5 μM probe, 60 minutes) and C) DTBZ (2 μM)-inhibited uptake of these probes in VMAT2-HEK cells, in the same order as listed in A. While FFN200 is a VMAT substrate with low background uptake, it is nearly invisible when uptake images are contrasted identically with FFN206 and AGH180. Images are representative of 3 independent experiments, at least 3 images per well. D) Representative comparison of FFN206, FFN200 and AGH180 uptake and inhibition using a plate reader (5 μM probe, 15 minute treatment. Error bars represent SEM, n = 2 plates).
Although FFN200 is a clear VMAT2 substrate, when compared directly alongside FFN206 and AGH180 in cultured VMAT2-HEK cells, the uptake of FFN200 is nearly invisible after 60 minutes as determined by epifluorescence microscopy (Figure 10B). If the images were contrasted independently for each probe relative to its inhibited uptake, VMAT2-specific uptake would become visible for FFN200, as background uptake (as measured in the presence of TBZ 2 µM) at 5 µM is lowest for FFN200 (Figure 10C,D). The topological polar surface area (tPSA) of AGH180 is similar to that of FFN200 (as predicted by ChemBioDraw Ultra, Figure 10A), suggesting that lipophilic uptake may be comparable for AGH180 and FFN200, although nonspecific uptake of AGH180 appears closer to that of FFN206, and can be rationalized by the amphipathic nature of AGH180, where both moieties capable of carrying charge are on the same side of the molecule.

Comparison of Apparent $K_m$ of FFN206, FFN200, and AGH180 for VMAT2

The $K_m$ for each compound was determined by initial rates experiments within the linear region of time-dependent accumulation. These results are summarized in Figure 11, showing representative Michaelis-Menten plots for FFN200 and AGH180. Indeed, FFN206 has the highest apparent affinity for VMAT (results from above, $1.16 \pm 0.1 \mu M$, $n = 5$ independent experiments in collaboration with Dr. Gang Hu), FFN200 appears lower ($K_m = 13.74 \pm 2.66 \mu M$, mean $\pm$ SEM, $n = 3$ independent experiments), and AGH180 preliminarily has an intermediate $K_m$ at approximately 9 µM (preliminary results, $n = 1$).
**Figure 11:** Representative Michaelis-Menten plots of initial rates of VMAT2-specific uptake for FFN200 (left, $K_m = 13.74 \pm 2.66 \mu M$, mean $\pm$ SEM, n = 3 independent experiments) and AGH180, (right, $K_m \sim 9 \mu M$.) By comparison, the $K_m$ of FFN206 was previously determined to be $1.16 \pm 0.1 \mu M$, n = 5, using a similar method, and verified in Part II of this chapter.

While it was prohibitive for us to measure $V_{max}$ in these systems, direct comparisons of the specific uptake of each probe at a single concentration is sufficient to derive relative information about VMAT2-dependent uptake. At 5 $\mu M$ with a corresponding 15 minute treatment (**Figure 10**), FFN206 is above its $K_m$ and specific uptake is quite high, FFN200 is below its $K_m$ and total and specific uptakes are low, and AGH180 is near its $K_m$, and total uptake is greater than for FFN200. These results suggest that AGH180 may serve as an alternative, stronger VMAT2-dependent FFN compared to FFN200.

**AGH180 is Potentially a Useful FFN in Acute Mouse Brain Slice: Preliminary Results**

In collaboration with Paolomi Merchant, these probes have been evaluated in the dorsal striatum of acute mouse brain tissue. FFN206 has been found to be inappropriate for use in acute mouse brain slice, as we have noticed that substitution of the aniline of 7-aminocoumarins appears to reduce selectivity of
labeling in acute mouse brain slice by a yet unknown mechanism (Paolomi Merchant, Dr. Shu Li, Dr. Daniela Pereira; **Figure 12B, left**). This is true for a number of probes including FFN206, and this is despite favorable VMAT2 activity in secondary cell-based systems (**Figure 10B-D**, and Part III of this chapter). The behavior of FFN200 in mouse brain slice is currently being characterized in detail by Dr. Daniela Pereira, and FFN200 is found to colocalize to a high degree with TH-GFP (**Figure 12B, right**). AGH180 shows a similar or possibly higher degree of colocalization, with more defined labeling compared to the other probes (**Figure 12A**). AGH180 is also inhibited to a slightly higher degree by reserpine than FFN200 (~37% reduction in puncta for AGH180 compared to ~25% reduction for FFN200 in preliminary experiments, data not shown, Paolomi Merchant). If true, this would indicate that AGH180 is a more VMAT2-selective FFN, consistent with favorable uptake results observed in cell culture (**Figures 10 & 11**).
**Figure 12:** AGH180 and related FFNs labels dopaminergic presynaptic terminals in dorsal striatum of acute mouse brain slices. A) AGH180 (red) and TH-GFP (Green) puncta colocalize approximately 85% with respect to AGH180 in the dorsal striatum of acute mouse brain slice, when analyzed as previously reported.\(^{(35)}\) B) Side-by-side comparisons of FFN206, FFN200, and AGH180 labeling and colocalization with TH-GFP in the dorsal striatum of acute mouse brain slice. As above, each probe is shown in the pseudocolor red, TH-GFP is shown in green. Yellow indicates colocalization. AGH180 displays a colocalization similar to, if not better than, FFN200. Each image represents a 30-minute treatment of the slice with 10 µM probe, followed by a 30-minute wash period with artificial cerebral spinal fluid (ASCF). Images are courtesy of Paolomi Merchant.

**A Note Regarding 7-Aminocoumarin Labeling of Aminergic Neurons in the Mouse Brain**

If one assumes that these 7-aminocoumarins are indeed driven into aminergic neurons in brain tissue primarily by action of VMAT2, then the interplay between an FFN’s kinetic parameters at VMAT (\(K_m\) and \(V_{max}\)) and nonspecific uptake (or uptake/binding via secondary mechanisms) will define the labeling selectivity of
the probe. If VMAT2 uptake outcompetes background labeling, the FFN will accumulate to a greater extent in aminergic neurons than the surrounding, non-aminergic cells. After a brief wash to remove background extracellular and cytosolic FFN from the brain slice, the result is a fairly selective label of VMAT2-expressing neurons. While it is impossible for such an FFN exhibiting this behavior to selectively label one type of aminergic neuron over another, as each expresses VMAT2, it would be possible to observe areas in the brain where only one type of aminergic synapse is present, such as the dorsal striatum for dopaminergic synapses or the hippocampus for 5HT-ergic synapses.

FFN206 is one example of a series of 7-aminocoumarins, with substitutions on the aniline, which seems to become heavily accumulated in other cells in mouse brain slice despite excellent VMAT2 uptake in culture and excellent behavior in d. melanogaster whole-brain preparations (Sonders et al., manuscript in preparation). As shown above, AGH180 is a better VMAT2 substrate than FFN200 in culture, and is a cleaner label than FFN206 in the dorsal striatum of the mouse brain. As such, it could prove to be an important FFN for future studies.

**NG54: Revisiting a First-Generation Probe**

NG54 was synthesized and tested in chromaffin cells and acute mouse brain slice by Dr. Niko Gubernator. Although NG54 displayed a highly punctate signal in preliminary studies in the dorsal striatum of acute mouse brain slice, it was not pursued further, as it appeared to photobleach rapidly under imaging conditions used at that time. Additionally, it was found not to be clearly accumulated by
VMAT1 in mouse primary chromaffin cells.\textsuperscript{[48]} Because of the apparent punctate labeling in the dorsal striatum, we chose to include NG54 in our plasma membrane monoamine transporter screens, where it was found to be a substrate for DAT, NET, and SERT (Chapter 3). We then chose to investigate the behavior of NG54 at VMAT2.

![NG54](image)

**Figure 13:** Structure and fluorescence spectra of NG54. $\lambda_{\text{max}}(\text{excitation}) = 390$ nm, $\lambda_{\text{max}}(\text{emission}) = 422$ nm. Fluorescence excitation and emission were recorded slightly off maxima, at 446 nm and 380 nm respectively, as to observe clear peaks and overlap of the spectra.

When VMAT2-HEK cells and TetR-HEK (control) cells are treated with 20 µM NG54 for 30 minutes and imaged with an epifluorescence microscope, NG54 displays a punctate labeling pattern that is VMAT2-dependent (Figure 14), although there is considerable background uptake (Figure 14A) presumably due to lipophilicity of the compound (logP predicted $\sim 1.8$). As in the case of FFN206 above, a hypothetical transporter on the plasma membrane can not be ruled out. The difference in brightness between control cells and VMAT2-HEK cells was quantified by contrasting images to the same level using ImageJ, measuring the mean fluorescence intensity of each image, and normalizing each image to the number of cells in the frame. This was repeated for three images per condition, over
three independent experiments. The resulting normalized mean fluorescence intensities were plotted with the mean brightness of TetR-HEK = 1 (Figure 14C). Specifically, it was found that NG54 is 1.24 ± 0.02 times brighter in VMAT2-HEK cells than control cells. When taken together with the punctate label in VMAT2-HEK cells, these results suggest VMAT2-dependent accumulation (mean ± SD, n = 3 independent experiments).

![Figure 14](image)

**Figure 14:** NG54 uptake is VMAT2-dependent. A) TetR-HEK cells treated with NG54 (20 µM, 30 min) and B) VMAT2-HEK cells under the same conditions. C) Normalized mean fluorescence uptake per cell indicates that VMAT2-HEK cells preferentially accumulate NG54 by a factor of 1.24 ± 0.02 (mean ± SD, n=3 independent experiments, 3 images per condition per experiment contrasted to the same level). There is considerable background uptake in the empty vector-transfected cells, although the difference in labeling morphology is clear.

This punctate labeling can be inhibited by TBZ (Figure 15). Interestingly, the VMAT2-HEK cells are still quite bright in the presence of the TBZ inhibitor (Figure 14), possibly due to a secondary uptake mechanism as the VMAT2-HEK
cells are not isogenic with the control TetR-HEK cells (the VMAT2 transfection was selected under pressure of MPP+).

Figure 15: NG54 labeling morphology is altered by the VMAT2 inhibitor TBZ. A) Uninhibited 20 µM NG54 in VMAT2-HEK cells affords a punctate label consistent with previous VMAT2 substrates in the same cell line.\(^{(34,36)}\) B) 2 µM TBZ changes the morphology of the NG54 label. Inhibited cells appear far less punctate. C) Corresponding brightfield image of uninhibited and D) corresponding brightfield image of TBZ-inhibited conditions. E) Dr. Adam Henke and Yekaterina Kovalyova (an undergraduate student trained and advised by RJK) have found that the initial rates of NG54 uptake can be estimated from this system following a 30 minute period of washing with PBS, where selective retention of NG54 by VMAT2-expressing intracellular compartments allows for detection of vesicularized NG54 with minimal contributions from background cytosolic staining. \(K_m\) (estimated) = 12.5 ± 2.3 µM (mean ± SEM, 3 independent experiments). Retention of NG54 during
this period is not affected by 2 μM TBZ in the wash buffer. All images are representative of 3 independent experiments.

It is known that acridones undergo self-quenching at high concentrations;\(^{(49)}\) this could be the cause of the relative difference in brightness between conditions. These images could be underestimating the concentration of NG54 in VMAT-expressing compartments relative to the cytosolic concentration. This possible quenching effect is important for interpretation of results from the brain, as NG54 appears to be a pH-independent “flashing” FFN. For detailed information on the behavior of NG54 at DAT, NET, and SERT, and for representative images of uptake and release from the brain, see Chapter 3.
C. Conclusions and Outlook (Part III)

A new FFN, AGH180, has been characterized as a VMAT2 substrate comparable to FFN206 in VMAT2-HEK cells. Because of nonspecific staining in mouse brain slice, FFN206 is not an acceptable FFN despite superior VMAT2 uptake in cultured cells. FFN200, a poorer VMAT2 substrate in culture, appears far more selective in acute mouse brain tissue for dopaminergic terminals in the dorsal striatum, and is used in the Sulzer Lab as a “destaining” aminergic-selective FFN. Although the mechanism of FFN200 labeling appears VMAT2-dependent in brain slice, inhibition only partially reduces the number of labeled structures and brightness of FFN200 signal in the dorsal striatum, despite a moderate to high degree of colocalization with dopaminergic markers. AGH180, a regioisomer of FFN200, has been identified herein as an excellent VMAT substrate compared to FFN200 in cell culture. Moreover, preliminary results from acute mouse brain slice suggest that AGH180 behaves as a more VMAT2-dependent FFN in the dorsal striatum. Thus, AGH180 may serve as a more selective “destaining” FFN for imaging synaptic activity in the dorsal striatum and other areas containing aminergic synapses. Studies of AGH180 may also contribute to a more complete understanding the behavior of aminocoumarin-based FFNs in the mouse brain.

In this section, I have also identified substrate activity of NG54 at VMAT2. This probe has also been identified as a substrate for hDAT, hNET, and hSERT, and is currently being pursued as an FFN in brain tissue as a next-generation FFN capable of moving beyond dopaminergic activity (see Chapter 3 for a full discussion).
IV: Experimental

General Cell Culture

A HEK cell line stably expressing rVMAT2 (VMAT2-HEK) and an empty-vector transfected HEK cell line to serve as a control (TetR-HEK) were kindly provided by Professor Robert Edwards of the Department of Neurology at the University of California San Francisco (UCSF). Cells were grown on 10 cm polystyrene plates (Corning). Complete growth medium consisted of DMEM + Glutamax (Invitrogen) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂, and were subcultured approximately every 3 days, or before reaching 75% confluence.

Kₘ determinations of FFN206

To determine if the rate determining step of FFN206 uptake is dependent on the plasma membrane, an experiment using digitonin to permeabilize the membrane was adapted from Schuldiner, et al. Measurements were taken using a BioTek H1MF plate reader in bottom read mode, and a similar protocol to that described above (see Methods). First, Kₘ was measured on this platform under non-permeabilizing conditions to serve as an independent control. Cells stably transfected with VMAT2 (VMAT2-HEK) were seeded at a density of 3.0 x 10⁴ cells/well in white clear-bottom 96-well plates and allowed to proliferate in growth medium for ~2 days at 37 °C to reach confluence. On the day of experiment, the growth medium of confluent VMAT2-HEK cells was aspirated and the cells were
incubated with DTBZ (2 μM, 100 μL/well) or DMSO vehicle for 1 hour in experimental medium. Experimental medium (100 μL/well) containing either FFN206 or a cocktail of FFN206 and DTBZ (2 μM) was added and the cells were incubated for the required duration of time. The uptake was terminated with two rapid PBS washes (2x200 μL/well) and the cells were maintained in fresh PBS buffer (120 μL). The fluorescence uptake in each well was immediately recorded in a plate reader BioTek Synergy H1MF Hybrid Reader (3x3 and 5x5 area scan, bottom read mode) with excitation and emission wavelengths set at 369 nm and 464 nm, respectively. The specific uptake was defined as the overall fluorescence uptake (with DMSO vehicle) subtracted by that in the presence of DTBZ. The specific uptake rates were linear for concentrations of FFN206 up to 6 μM with a corresponding incubation time of 12 min. The initial uptake rates for each concentration were fit to the Michaelis-Menten equation using GraphPad Prism 5 software to give apparent \(K_m\) value for FFN206 uptake. Each independent experiment consisted of duplicate plates.

For experiments using digitonin, cells were treated as above with an additional pretreatment of digitonin (10 μM, 10 min) in DMEM (without phenol red) + 25 mM HEPES containing 1% BSA immediately before initiating uptake. After digitonin pretreatment, the permeabilization medium was removed, cells were washed (100 μL PBS), and FFN206 solutions in the same experimental medium (without digitonin) supplemented with 5 mM Na\(_2\)ATP were applied. Treatment times and wash protocols were otherwise the same as described above. Disruption of membrane integrity by digitonin was verified in an independent control using
trypan blue (0.4% in PBS, 10 minute treatment) to indicate that the majority of VMAT2-HEK cells permeabilized as above were labeled by the dye. The specific uptake rates of FFN206 in digitonin-treated cells were linear for concentrations up to 6 µM for a corresponding incubation time of 9 min.

**Determination of High-Throughput Screening (HTS) Parameters**

Data for the HTS assay were recorded on BioTek Synergy H1MF Hybrid Plate Reader using poly-D-lysine coated 96-well micro-assay plates. VMAT2-HEK cells were seeded at a density of 3.0 x 10^4 cells/well (by addition of 200 µL of a pre-diluted cell suspension) in white clear-bottom 96-well plates (allowed to attach the plate in the biosafety cabinet for ~20 min) and allowed to proliferate in growth medium for 50-54 h at 37 ºC to reach full confluence. On the day of experiment, the growth medium was aspirated and the cells were incubated for 30 min in experimental media with DTBZ (2 µM, 100 µL/well) or DMSO vehicle. A solution of FFN206 (2 µM) or a cocktail of FFN206 (2 µM) and DTBZ (2 µM) in the experimental medium (100 µL/well) were added and the cells were incubated for 1 h. The final concentration of FFN206 in each well was 1 µM, and the final concentration of DTBZ inhibitor was 2 µM. The layout of the plate consisted of 48 wells of positive control (2 µM DTBZ) and 48 wells of uninhibited negative control (DMSO vehicle). The uptake was terminated by washing with PBS (2x200 µL/well) and the cells were maintained in fresh PBS buffer (120 µL). The fluorescence uptake in each well was immediately recorded in bottom-read mode (using three different methods – single point, area scan (matrix 3x3) and area scan (matrix 5x5)) in the plate reader with
excitation and emission wavelengths set at 369 nm and 464 nm respectively. Z’ value, coefficient of variation (CV) and signal to basal ratio (S/B) were calculated for each plate run in duplicate per experiment. Presented are the arithmetic means of these values calculated from 5 independent experiments consisting of duplicate plates.

**Procedure for Kinetic Study of FFN200 and AGH180 Uptake in VMAT2-HEK Cells**

The apparent $K_m$ of FFN200 at VMAT2 was determined using a similar protocol to that employed above. After pre-treatment with experimental medium with and without TBZ (2 μM) or DMSO vehicle (0.02% v/v), the medium was aspirated and a cocktail containing the probe at indicated concentrations and TBZ (2 μM) or DMSO vehicle (0.02% v/v) was added (100 μL/well) and the cells were incubated for 12 minutes. Uptake was terminated with two PBS washes (200 μL/well) and the cells were treated with fresh PBS buffer (120 μL). The fluorescence uptake in each well was immediately recorded with a BioTek H1MF plate reader in bottom read mode with excitation and emission wavelengths set to 352 nm and 451 nm, respectively. The specific uptake was defined as the overall fluorescence uptake from uninhibited wells subtracted by that in the presence of TBZ. The specific uptake was linear for concentrations of FFN200 at 50 μM or less with a corresponding incubation time of 12 min. The initial rates of uptake for each concentration were fit to the Michaelis-Menten equation using GraphPad Prism 5 software to give apparent $K_m$ value for probe uptake by VMAT2. Each experiment
consisted of duplicate plates, and $K_m$ for FFN200 was calculated from the arithmetic mean of three independent experiments, and $K_m$ of AGH180 is from one preliminary experiment.

**Examination of FFNs in VMAT2-HEK Cells**

For fluorescence microscopy experiments, cells were plated on poly-D-lysine (Sigma-Aldrich, 0.1 mg/mL) coated six-well plates (Falcon) at a density of 100,000 cells/well and were incubated until confluence (4 days at 37°C in a humidified atmosphere containing 5% CO$_2$). The medium was then removed by aspiration and wells were carefully washed with PBS. Cells were then treated with 900µL of experimental medium (DMEM minus phenol red containing 25 mM HEPES (Invitrogen) with 1% FBS (Atlanta Biologicals)) for 1-3h with or without DTBZ or reserpine as inhibitors at the indicated concentrations. Cells were then treated with 100 µL of FFN, or a cocktail of FFN with inhibitor for final concentrations as indicated for the indicated time. Wells were aspirated, washed once with PBS (2 mL/well), and wells were filled with warm experimental medium. Brightfield and fluorescence images were acquired using a Leica DMI 4000B inverted epifluorescence microscope equipped with a Leica DFC 360 FX digital camera controlled through Leica LAS AF 6000E software. Bright field and fluorescence images were acquired sequentially (BF acquisition time = 37 ms). Fluorescence images were acquired using dichroic filter cubes to selectively excite and record emission wavelengths for each FFN probe. All fluorescence images of each respective fluorophore under different conditions were adjusted to the same
brightness and contrast level, respectively, using ImageJ (National Institutes of Health). For direct comparison of the brightness of FFN206, FFN200, and AGH180 in VMAT2-HEK cells, all images were contrasted to the same level optimal for FFN206, for direct comparison.

**Fluorescence Measurement of NG54**

Fluorescence measurements of NG54 were recorded using a BioTek H1MF plate reader/spectrofluorometer operated through Gen5 Data Collection and Analysis software. Emission/Excitation spectra were acquired by adding NG54 (1µL of 10 mM stock solution in DMSO) to 5mL of PBS pH = 7.4 (final probe concentration = 2 µM). The solution was added to a quartz cuvette and read using the plate reader/spectrofluorometer. Emission data were collected by exciting at 300 nm, and excitation data were collected by recording at 446 nm, each slightly off from the maxima, in order to highlight the overlapping excitation and emission spectra. Resulting data were normalized to the highest reading of each series, and plotted using Microsoft Excel.
V: References


Chapter III

Determination of FFNs as Fluorescent Substrates for the Plasma Membrane Monoamine Transporters DAT, NET, and SERT
I: Introduction

Within the FFN program, it has previously been established that VMAT2 can drive FFN uptake into monoaminergic synapses.\(^1\) We envisioned the possibility of selectively loading a fluorescent VMAT substrate into monoaminergic neurons via action of plasma membrane monoamine neurotransmitter transporters (MATs), akin to a false neurotransmitter (introduced in Chapter 1). Such a compound would be a more realistic fluorescent false neurotransmitter, capable of labeling monoaminergic neurons by action of the high affinity transporter MATs in addition to labeling synaptic vesicles by action of VMAT2. A MAT-active FFN would similarly provide information on VMAT2 function, vesicle fusion and quantal release of neurotransmitter, with the added possibly of observing monoamine reuptake (Figure 1). In order for a FFN to act as a true tracer of monoamine neurotransmitters in the brain, it must exhibit substrate behavior at the transporters involved in monoamine neurotransmission, including VMAT (as discussed in Chapter 2) and at least one of the MATs, specifically the dopamine transporter (DAT), the norepinephrine transporter (NET), or the serotonin transporter (SERT). Because MATs are expressed primarily by neurons that signal with their cognate neurotransmitter, we hypothesized that MAT activity could be exploited to selectively label a subset of monoaminergic neurons in the brain.\(^2\)
Figure 1: A.) Representation of a dopaminergic synapse in the dorsal striatum. B) Representation of a dual MAT/VMAT2 substrate FFN in a dopaminergic presynaptic release site. While FFNs were designed specifically as VMAT substrates, a large number were identified and it was hoped that the structural similarity to monoamine neurotransmitters would also lead to substrate activity of FFNs at the more discriminating MATs (gray ovals). Screening was undertaken to identify potential MAT substrate activity of several fluorescent probes.

Brief Introduction to Monoamine Transporter Expression, Pharmacology, and Contributions to Pathophysiology of Disease

The high affinity plasma membrane monoamine neurotransmitter transporters (MATs) of the solute carrier 6 family of transporters (SLC6) are known to regulate monoaminergic signaling via control of monoamine neurotransmitter reuptake from the extracellular milieu, including the synaptic cleft and extrasynaptic space\(^{2,3}\). These transporters include the dopamine transporter (DAT, SCL6A3), the norepinephrine transporter (NET, SLC6A2), and the serotonin transporter (SERT, SLC6A4), all of which are known to play central roles in synaptic function. As such, they serve as targets for recreational and therapeutic drugs\(^{4,5}\).
are involved in the mechanism of numerous diseases, and are attractive imaging targets in the brain. Each transporter is briefly introduced below, with a focus on function, expression, involvement in disease, and pharmacology. For a discussion of the life cycle and signaling of monoamine neurotransmitters, see Chapter 1.

**The Dopamine Transporter (DAT)**

While the DAT was understood to exist as the mechanism for dopamine clearance of the synapse for quite some time,\(^6\) it was first cloned and fully characterized in the early 1990s.\(^7\)-\(^9\) DAT is primarily responsible for translocating dopamine from the extracellular space into the cytosol, thus acting as a reuptake transporter, although it is also capable of carrying some select small molecules (Figure 2). Based on results from DAT knockout studies, it has been suggested that approximately 75-95% of the dopamine in synaptic vesicles of dopaminergic neurons has been recycled through DAT.\(^2,10,11\) DAT relies on the electrochemical potential across the plasma membrane established by Na+/K+ ATPase to drive the transport of monoamines.\(^2\) It has been shown that DAT binds two sodium ions and one chloride along with dopamine, and couples the inward, energetically favorable transport of sodium ions with the transport of dopamine or other organic substrates against their concentration gradient. DAT is widely expressed in dopaminergic neurons of the substantia nigra and ventral tegmental area, which project to and modulate signaling in the cortex and striatum,\(^12\) with its expression and activity tightly regulated by a number of signaling mechanisms.\(^13\)-\(^15\)
As dopamine signaling plays a central role in the modulation of behavior,\textsuperscript{(16)} DAT serves as a target for a myriad of recreational and pharmaceutical drugs.\textsuperscript{(4, 5, 17, 18)} A classic example is cocaine, which exerts its psychoactive and stimulatory effects by inhibiting dopamine reuptake through DAT, resulting in an increase in the synaptic concentration of DA. Additionally, methylphenidate is a widely prescribed drug for the treatment of attention deficit hyperactivity disorder (ADHD), and exerts its action through inhibition of DAT. Amphetamines are also substrates for DAT, and have been shown to induce reverse transport of cytosolic DA via DAT, thus increasing extracellular/synaptic concentrations of the neurotransmitter.\textsuperscript{(17)} DAT is also known to transport the neurotoxin MPP+, and DAT activity plays a key role in the mechanism of neurotoxicity of this compound.\textsuperscript{(19, 20)}

Changes in expression of DAT are characteristic of a number of neurological and psychiatric conditions. Dysregulation or altered function by an increase or decrease in expression or activity of DAT is characteristic of a number of psychiatric disorders, including attention deficit hyperactivity disorder (ADHD) and bipolar disorder.\textsuperscript{(21, 22)} DAT is lost along with dopamine neurons as a consequence of neurodegenerative diseases such as Parkinson’s disease (PD) and Huntington’s disease (HD). Because of its role in the etiologies of neurological diseases and its potential use as a marker for dopaminergic innervation, DAT is actively pursued as an imaging target, and a number of PET probes have been developed for this purpose.\textsuperscript{(23)}

While DAT has been widely studied, numerous questions remain unanswered about its role in disease and pharmacological treatment thereof, and it
is quite likely that a DAT-selective, fluorescence-based imaging agent could be useful in the study of dopaminergic neurotransmission in both healthy and diseased tissue.

**The Norepinephrine Transporter (NET)**

NET is expressed in noradrenergic neurons originating in the locus coeruleus, which project to and modulate signaling in many regions including the hippocampus and cortex.\(^{12,24}\) NET was cloned and fully characterized in the early 1990s.\(^{25}\) Like DAT, NET relies on the electrochemical gradient across the plasma membrane to transport monoamine substrates, although only one Na\(^+\) and one Cl\(^-\) ion are co-transported along with the organic substrates.

Currently, only one polymorphism of NET is known to cause disease, ALA457PRO in the transmembrane region TM9, which manifests in orthostatic intolerance and tachycardia.\(^{26,27}\) Epigenetic attenuation of NET expression through hypermethylation of the promoter region has been linked to panic disorder.\(^{28}\) While it has been hypothesized that dysregulation of NET is responsible for a number of psychiatric illnesses, a causal link has not been rigorously established,\(^{27}\) though NET remains a drug target owing to the importance of norepinephrine (NE) in mood and homeostasis.\(^{27}\) Development of a noradrenergic FFN could lead to methods facilitating observation of individual adrenergic inputs in real time as they modulate cortical or other neurons. Such a method would allow for a greater understanding of endogenous NE signaling in healthy brains as well as the role of NE in disease.
The Serotonin Transporter (SERT)

SERT is analogously important in the regulation of extracellular concentrations of serotonin (5HT). As with DAT and NET, SERT function also relies on the electrochemical potential of the plasma membrane, and like NET, co-transport one Na\(^+\) and one Cl\(^-\) ion along with the organic substrates. Numerous polymorphisms in SERT have been characterized, and many are linked to neuropsychiatric conditions, including obsessive compulsive disorder and severe depression.\(^{(29)}\)

The serotonin hypothesis of depression has led to development of serotonin reuptake inhibitors, and later, selective serotonin reuptake inhibitors (SSRIs). SSRIs are efficacious treatments for many depressed patients, although there are still variables in phenotype which give rise to treatment-resistant depression in some individuals (see Chapter 5 for a discussion of a possible compensatory 5HT transport mechanism in some individuals). As with DAT and NET, exogenous substrates can induce reverse transport through SERT.\(^{(5)}\) For example, MDMA exerts its empathogenetic effects by acting as a substrate for SERT and releasing 5HT into the synapse from cytosolic stores.\(^{(30)}\) Because of its role in depression and mood disorders, and the still poorly understood relationship between SERT expression, function, and resultant disease states,\(^{(31, 32)}\) development of an imaging agent to study serotonergic signaling on the synaptic level could reveal much about the pathophysiology of various mood disorders.
A. Endogenous Substrates:

- Dopamine
- (R)-Norepinephrine
- Serotonin
- (R)-Epinephrine
- Tyramine

B. Representative Exogenous Substrates:

- (+/-)-Amphetamine
- (+/-)-Methamphetamine
- MDMA
- Ephedrine
- Aminorex
- Phenmetrazine
- Kynuramine
- PAL-287
- MPP+

C. Fluorescent Exogenous Substrates:

- ASP+
- APP+
- Wilson 5 (likely)
- Wilson 6 (likely)

Figure 2: Some representative MAT substrates. A) Endogenous monoamines and some related metabolites are known to be transported by MATs. B) A selection of exogenous DAT, NET, and SERT substrates. Most are small aromatic ethylamines, with a few notable exceptions. PAL-287 inspired us to pursue larger, fluorescent substrates as FFNs. C) Some fluorescent substrates of MATs. None have been shown to be appropriate FFNs, although the recently reported compounds Wilson 5 and Wilson 6 may serve as future leads if shown to function as VMAT2 substrates.

Our Approach to the Design of Fluorescent Dual MAT/VMAT Substrates

While the substrate scopes of the MATs are slightly broader than their namesake neurotransmitters, it is understood that they are more discriminating than the intracellular vesicular monoamine transporter (VMAT2) as a mechanism of protection from toxins (see Chapter 2 for discussion). Substrate scopes of the three MATs overlap considerably, although there are some notable
divergences which lead us to consider the possibility of achieving FFN labeling selectivity not just for monoaminergic neurons over surrounding tissue, but for additional selectivity between classes of monoaminergic neurons.

In order for a substrate to be sufficiently fluorescent for imaging applications, the fluorophore must intrinsically be larger than a simple catecholamine or tryptamine, posing a challenge for molecular design. Several fluorescent substrates of MATs have been reported and studied, although none function as FFNs due to inadequate photophysical and biological properties (discussion and example study in Chapter 4). The fluorescent pyridinium analog of MPP+, 4-(4-dimethylaminostyryl)-N-methylpyridinium (ASP+), has been shown to act as a substrate of plasma membrane monoamine neurotransmitter transporters, demonstrating that activity of these transporters can be monitored using fluorescent substrates.\(^{(33)}\) ASP+ has been widely used to study MAT expression and pharmacology in a number of contexts,\(^{(39-41)}\) although this probe has slow transport kinetics at all MATs\(^{(39)}\) with especially poor uptake at SERT\(^{(33, 42)}\). APP+, a smaller pyridinium analog was recently introduced to overcome this, and we simultaneously became interested in studying this compound as a potential FFN (Chapter 4).\(^{(43)}\) Other larger pyridinium analogs have been used to study transport and binding in MATs,\(^{(44)}\) although due to poor substrate kinetics, high background uptake in cells through passive diffusion, and mitochondrial localization of the compounds, only APP+ was pursued as a FFN lead (Chapter 4).

Recent reports identified a naphthalene-based amphetamine analog PAL-287 as a DAT and SERT substrate.\(^{(35, 36)}\) This served as a proof of principle that
monoamine transporters could indeed accept larger, polycyclic aromatic, non-pyridinium substrates. Additionally, two fluorescent ethylamines have recently been identified to have likely MAT activity (Figure 2), although no VMAT activity has been examined.\(^{37}\)

Within our research group, it was discovered by Dr. Pamela Rodriguez of the Sames and Sulzer labs that FFN102 is a DAT substrate in acute mouse brain slice.\(^ {45}\) FFN102 has been fully characterized as a dopaminergic FFN, deriving its selectivity from DAT substrate activity. FFN102 is also a VMAT2 substrate and a ratiometric pH sensor;\(^ {46}\) undergoing an increase in brightness upon exocytotic release. Importantly, FFN102 served as a proof of principle that fluorescent probes could serve as dual substrates for a monoamine transporter and VMAT2, affording selective labeling of monoaminergic neurons compared to surrounding cells in the tissue while allowing the probe to act as a tracer of monoamine uptake and release.

As discussed in Chapter 1, our approach has largely been to incorporate a minimal recognition element into our molecular design by adding an aminoethyl group to small fluorophores. The vast majority of compounds screened here are novel compounds synthesized within the Sames Lab as potential VMAT substrates, and later as potential dual substrates (unpublished results).\(^ {46}\) Promising compounds identified from initial screening in heterologous cell-based systems (expressing hDAT, hNET, or hSERT) are thoroughly characterized in mouse coronal brain slice by members of the Sames and Sulzer groups.
II: Results and Discussion

Screening of Probes at hDAT, hNET, and hSERT

A structurally diverse library of fluorescent probes was screened for substrate activity at DAT, NET, and SERT using epifluorescence microscopy. Many of the screened compounds were chosen based on their known VMAT2 substrate activity (unpublished results)\(^{(46, 47)}\) as this parameter is essential for imaging quantal release of neurotransmitter in the brain\(^{(1, 45)}\). Other compounds were included based on suspected structural similarity to known substrates in order to examine the limits of substrate tolerance.

For DAT experiments, hDAT-EM4 and empty vector-transfected EM4 cells were provided by Dr. Mark Sonders and Professor Jonathan Javitch of the New York State Psychiatric Institute. For hSERT experiments, EGFP-hSERT Flp-in MDCK cells and control Flp-in MDCK cells were also provided by Dr. Sonders and Professor Javitch. Additionally, hSERT-HEK and hNET-HEK cells were provided by Professor Brian Roth of The University of North Carolina, Chapel Hill. In each experiment, the known MAT substrate ASP+, or later, APP+, was used as a control to validate transporter expression and activity (Figure 3).
Figure 3: A pyridinium-based MAT substrate was used as a positive control for transporter activity and for inhibitor efficacy in each independent experiment during this screen. Shown here is a representative image of APP+ (2 μM) uptake in 10 minutes by A) hNET, showing a high degree of transporter-specific labeling compared to B) uptake in control HEK293T cells, C) uptake in the presence of nomifensine (2 μM, NET/DAT inhibitor). D) Nomifensine (2 μM) had no effect on the uptake of APP+ by control HEK293T cells. While APP+ is a substrate for DAT, NET, and SERT, it is not appropriate for use as a FFN in our systems (detailed in Chapter 4). Images are representative of control results acquired for validation of hDAT, hNET, and hSERT expression and function in screening experiments.

Each experimental compound was screened initially at 20 μM concentration in transporter-transfected cells and directly compared to transporter-independent uptake in control cells. If substrate activity was suspected based on these results, further evaluation was conducted including inhibition by nomifensine (2 μM) for DAT and NET, or imipramine (2 μM) for SERT. Each probe identified as a substrate here was found to accumulate in a transporter-dependent and inhibitor-sensitive manner in at least three independent experiments. This screen was divided between myself and Dr. Adam Henke, and the results are shown in Table 1 (7-hydroxycoumarins) and Table 2 (7-aminocoumarins and other structures). Results from inhibition experiments conducted with identified hits are detailed below as
confirmation of substrate activity of the indicated probes. Each uptake and inhibition result was confirmed in at least three independent experiments.

Table 1: 7-Hydroxycoumarins Screened for Substrate Activity at MATs

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<th>hNET</th>
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‡ = Compounds have been previously identified as VMAT2 substrates by our lab.
* = Minimal but discernable transporter-dependent labeling; possibly a weak substrate.
** = Transporter-dependent labeling, clear inhibitor-sensitive uptake.
*** = Strong substrate, inhibited cleanly, $K_m$ determined (see below).
X = no transporter-dependent accumulation
Table 2: 7-Aminocoumarins and Other Probes Screened for Substrate Activity at MATs

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‡ = Compounds have been previously identified as VMAT2 substrates by our lab.
* = Minimal but discernable transporter-dependent labeling; possibly a weak substrate.
*** = Strong substrate, inhibited cleanly, $K_m$ determined (see below).
X = no transporter-dependent accumulation

Identification of 3-Aminoethyl-7-hydroxycoumarins as Substrates for NET

After many discouraging results from hDAT and hSERT, we were surprised to discover that two of our 7-hydroxycoumarins appeared to be substrates of hNET (Figures 4,5). Initially, AGH093 was found to display transporter-dependent uptake into hNET-HEK cells. Full inhibition studies were conducted, and representative results are shown in Figure 4. Treatment of hNET-HEK and
HEK293T cells with 20 µM AGH093 indicated transporter dependent uptake, as evidenced by bright labeling of hNET-HEK cells (Figure 4A), and a relatively lower label in control cells (Figure 4B). Pretreatment of each cell type with nomifensine (2 µM) followed by co-treatment with AGH093 (20 µM) and nomifensine (2 µM) indicates that this label is sensitive to NET inhibition, with no effect of the inhibitor on control cells (Figure 4C,D). Further, the morphology of the label is consistent with a general, cytosolic label rather than a membrane label, where the junctions between cells would be bright.

**Figure 4:** AGH093 is a substrate for hNET. A) hNET accumulates AGH093 (20 µM, 60 min) in transfected HEK cells. B) Nonspecific uptake accounts for a lower amount of AGH093 staining in control HEK293T cells. C) Nomifensine (2 µM) abolishes AGH093 uptake in hNET-HEK cells, and D) has no effect on control cells in the presence of the probe. The labeling pattern in uninhibited hNET-expressing cells is consistent with a cytosolic label, suggesting transport of the probe rather than simple interaction with the transporter on the plasma membrane. Images are representative of 3 independent experiments.

FFN201 was almost left out of the preliminary screening, as it has previously been shown to be a VMAT substrate, but quite dim in cells expressing VMAT2, as
FFN201 lacks a halogen substitution on the 6- or 8-position. This halogen acts as a pKₐ modulator by withdrawing electron density from the O-H bond of the phenol through the sigma framework of the arene, resulting in a lower pKₐ of the phenolic proton, and a concomitantly higher population of the brighter phenolate in the cytosol (pH = 7.4) or the acidic vesicles (pH ~ 5.5 - 6). When FFN201 was examined in hNET-HEK cells and HEK293T cells by epifluorescence microscopy, it was found that cells expressing hNET accumulated the probe selectively over non-transfected HEK cells. Detailed inhibition studies confirmed this uptake and showed that it is indeed sensitive to nomifensine (2 µM) (Figure 5 A,B), similar to the behavior of AGH093 (Figure 5 C,D).

![Figure 5: FFN201 is also a substrate for hNET. A) hNET accumulates FFN201 (20 µM, 60 min) in transfected HEK cells. B) Nonspecific uptake accounts for a considerably lower amount of FFN201 staining in control HEK293T cells. C) Nomifensine (2 µM) abolishes FFN201 uptake in hNET-HEK cells, and D) has no effect on control cells in the presence of the probe. The labeling pattern in uninhibited hNET-expressing cells is consistent with a cytosolic label, suggesting transport of the probe rather than simple interaction with the transporter on the plasma membrane. Images are representative of 3 independent experiments.](image-url)
This is not surprising considering the relatively similar steric properties of AGH093 and FFN201, as an sp² hybridized C-F bond is only marginally longer than that of an sp² hybridized C-H bond. What is most surprising is the observation that FFN201 is consistently brighter in hNET-HEK cells (Figure 6), despite the fact that it is a dimmer probe at cytosolic pH.\(^{46}\) It should also be noted that the structurally-related compounds FFN101 and FFN102 are not substrates, revealing the limited tolerance of hNET for the location of the ethylamine with respect to the coumarin ring.

![Figure 6](ImageJ, NIH)

**Figure 6**: Direct comparison of select 7-hydroxycoumarins of similar structure reveals substrate preferences of hNET. All images are from the same experiment (20 µM probe, 60 min treatment) and adjusted to the same levels of brightness (ImageJ, NIH) to highlight similar labeling of hNET-HEK and HEK293 by FFN202, thus highlighting increasing brightness relative to background uptake as the steric bulk at position 6 is reduced (Cl < F < H) and no uptake by 4-aminoethyl coumarins.

Amphetamine (AMPH) is known to induce reverse transport of MATs, including NET. To further confirm substrate activity of these probes, hNET-HEK cells pre-loaded with FFN201 (20 µM, 1 hour), followed by treatment with amphetamine (1 µM, 10 min) or MeOH vehicle (0.03% v/v) in experimental medium. Images taken immediately after wells were washed show attenuation of probe signal relative to
images recorded immediately after uptake is terminated, with a greater degree of destaining in cells treated with 1 µM AMPH (Figure 7). The decrease in signal common to both conditions can be ascribed to washout via passive diffusion across the plasma membrane over the ten-minute wash period or through leaking via basal reverse transport through NET. The greater amount of destaining in the presence of AMPH is likely caused by AMPH-induced reverse transport through NET, further suggesting substrate activity of FFN201.

![MeOH (Vehicle) and 1µM AMPH](image)

**Figure 7:** FFN201 labeling of hNET-HEK cells (20 µM, 30 min) can be considerably reduced by treatment with A) amphetamine (AMPH, 1 µM, 10 minutes) compared to B) vehicle control (MeOH 0.03%). This result is preliminary and should be repeated in order to be conclusive, but it is suggestive of reverse transport of a cytosolic population of probe. This would imply true substrate activity rather than simple interaction with the transporter. Representative images from one preliminary experiment.

**Determination of K_m for FFN201 and AGH093 at hNET**

In order to provide a quantitative measure of the interactions of FFN201 and AGH093 with hNET, cellular uptake kinetics were measured fluorometrically. Initial rates of NET-dependent uptake of FFN201 and AGH093 were observed and compared using hNET-HEK cells grown on 96-well plates. It was found that specific uptake of each probe was linear within 12 minutes. Initial rates of specific uptake
were determined by measuring the total fluorescence of different concentrations of both AGH093 and FFN201 after 12 minutes of treatment in 8 uninhibited wells (treated with a DMSO vehicle control, 0.02% v/v), and by subtracting the background fluorescence of the same concentration of probe in the presence of nomifensine (2 µM, 8 inhibited wells). Fitting the resulting plots to the Michaelis-Menten equation afforded an interesting result. The $K_m$ of FFN201 at hNET was determined to be $5.0 \pm 0.3 \, \mu M$ (mean ± SEM, 3 independent experiments, two plates per experiment). AGH093 was found to have a considerably lower affinity for the transporter, as I was unable to saturate the transporter with a reasonable concentration of the compound ($K_m > 60 \, \mu M$ in each of 2 independent experiments, Figure 8). Additionally, FFN201 consistently displayed a higher specific uptake compared to AGH093, consistent with the images in Figure 6.

**Figure 8:** Initial rates of specific uptake of FFN201 ($K_m = 5.0 \pm 0.3 \, \mu M$, mean ± SEM; left, n=3 with Adam Henke) and AGH093 ($K_m > 60 \, \mu M$, right, n=2). It was not found to be possible to saturate hNET with reasonable concentrations of AGH093, and the effort was suspended in order to economize material for further studies.

This result is surprising, since the sterics of FFN201 and AGH093 are quite similar. Two possible explanations exist for this disparity. First, the slightly increased steric bulk of the fluoride substitution in the 6-position of AGH093 may
contribute to the lower apparent affinity for the transporter. Second, if the substrate is in fact the protonated phenol rather than the phenolate, the effective concentration will be lower than that of FFN201 in uptake medium (pH = 7.4). This is because the pKₐ of AGH093 is significantly lower than the pKₐ of FFN201 (pKₐ AGH093 = 6.2, pKₐ FFN201 = 8.1, Dr. Adam Henke and Dr. Minhee Lee, respectively). Preliminary experiments suggest that there is a pH-dependent difference in uptake behavior of these two compounds (data not shown), although more experimental evidence would be needed to test this hypothesis.

**Preliminary Results from the Mouse Cortex**

Matt Dunn has conducted preliminary two-photon microscopy experiments using the identified hNET substrates in the barrel cortex of acute mouse brain tissue. The barrel cortex is innervated by noradrenergic projections from the locus coeruleus (LC),⁴⁸ and may serve as a useful experimental system for characterizing the behavior of NET-FFNs.
Figure 9: Images of FFN202, AGH093, and FFN202 (10 µM, 30 min) in the barrel cortex of a mouse coronal brain slice. All images are z-projections of 10 images taken using a two-photon microscope. A) FFN202 appears to sparsely label axonal structures (red arrows). The large, bright structures are blood vessels (blue arrow). B) AGH093 brightly labels axonal structures to a greater extent than FFN202 in the same area of the brain. C) FFN201 labels structures with intermediate density and lower brightness than AGH093. D) The AGH093 label can be inhibited by nomifensine (1 µM). Representative images are courtesy of Matt Dunn.

When an acute mouse brain slice comprising the barrel cortex is treated with hNET substrate FFNs (10 µM perfusion, 30 min), some interesting results arise. Each FFN appears to label axonal structures to different degrees, depending on the probe (Figure 9). It should be noted that the label of FFN202 (Figure 9A) may result from dopaminergic projections to the cortex, as FFN202 is a known mDAT
substrate (unpublished results, Matt Dunn). AGH093 and FFN201 labeled axonal structures more densely, and these structures are thought to represent noradrenergic innervation. While the morphology of labeled structures is consistent with noradrenergic axons and presynaptic elements, we are in the process of developing a positive control for noradrenergic innervations in this region of the brain, as the TH-GFP signal is too weak to visualize even with multiphoton fluorescence microscopy. These preliminary results support further study of FFN201 and AGH093 in the brain, and suggest that they may represent noradrenergic FFNs. Collaboration is currently emerging with Prof. Randy Bruno (CUMC) to develop an in vivo imaging experiment for noradrenergic modulation of cortical neurons, using either FFN201 or AGH093 as a tracer.

One interesting discrepancy to note is that FFN201, while it appears the better substrate for hNET in transfected HEK cells, it is considerably dimmer in the mouse brain. If indeed these labeled structures are noradrenergic, as suspected, this disparity could have a few causes. First, it is possible that there is a species- or system-dependent variation in transporter function that is responsible for different substrate tolerances ($K_m$, $V_{max}$). Second, these compounds are ratiometric pH sensors with different pK$_a$ values (see above). FFN201 will consequently be less bright at environments of lower pH, including synaptic vesicles. In fact, upon release, FFN201 produces a bright “flash” despite its higher pK$_a$, suggesting that it may be well vesicularized (preliminary results from Matt Dunn, not shown). The Sames lab is currently pursuing comparative studies of hNET and mNET (mouse NET) in HEK cells, to determine if the cause of such disparity is indeed the slightly
different sequence homology of the transporter between species, or if it is caused by the experimental system itself (acute brain slice versus cultured HEK cells).

**Identification of AGH180 as a Possible Substrate for NET**

From the screen ([Table 2](#)) it was also found that two of the 7-aminocoumarins, FFN200 and AGH180, displayed a small transporter-dependent increase in fluorescence in hNET-HEK cells compared to background uptake in control HEK293T cells. Detailed inhibition results seem to suggest that there is indeed a small amount of nomifensine-sensitive uptake of the probes ([Figure 10](#) for a representative example, representative of 3 independent experiments). This amount of labeling is minimal, however. It will be important to consider this result in the context of mouse brain slice or other advanced experimental systems, although the utility of these compounds in cell culture is minimal at best.

![Figure 10:](#) AGH180 is a possible weak substrate for hNET. A) hNET accumulates AGH180 (20 μM, 60 min) in transfected HEK cells. B) Nonspecific uptake accounts for a considerably lower amount of AGH180 staining in control HEK293T cells. C) Nomifensine (2 μM) decreases AGH180 uptake in hNET-HEK cells, and D) has no
effect on control cells in the presence of the probe. FFN200 afforded similar results. Representative images from 3 independent experiments.

**NG54: A Substrate for hDAT, hNET, and hSERT, and a New Lead for Future FFNs**

NG54 was an early-generation FFN lead originally synthesized and studied by Dr. Niko Gubernator of the Sames laboratory. NG54 was set aside after initial studies in acute mouse brain slice by Dr. Niko Gubernator revealed a high degree of photobleaching under the imaging conditions used at the time. However, it was noted that NG54 displayed a highly punctate label in the dorsal striatum suggestive of dopaminergic morphology. Additionally, uptake studies in VMAT1-expressing primary mouse adrenal chromaffin cells showed little difference in uptake between reserpine-inhibited and uninhibited conditions.\(^{49}\) As part of our screening in hDAT/hNET/hSERT cells, we included this compound because of the highly punctate label in the dorsal striatum that had been observed years prior. NG54 was first identified as a substrate for hNET. Inhibition studies similar to those conducted above confirmed this result (**Figure 11**). This positive result was surprising but not inconceivable, as the extended aromatic system is reminiscent of the DAT/SERT substrate PAL-287,\(^{35}\) as well as the likely MAT substrates Wilson 5 and Wilson 6 (**Figure 2**).\(^{37}\)
**Figure 11:** NG54 is a fluorescent substrate for hNET. A) hNET accumulates NG54 (2.5 µM, 60 min) in transfected HEK cells. B) Nonspecific uptake accounts for a considerably lower amount of NG54 staining in control HEK293T cells. C) Nomifensine (2µM) abolishes NG54 uptake in hNET-HEK cells, and D) nomifensine (2µM) has no effect on control cells in the presence of the probe. Representative images from 3 independent experiments.

**Figure 12:** NG54 is a fluorescent substrate for hDAT. A) hDAT accumulates NG54 (10 µM, 60 minutes) in transfected HEK cells. B) Nonspecific uptake accounts for a considerably lower amount of NG54 staining in control EM4 cells. C) Nomifensine (2µM) abolishes NG54 uptake in hDAT-HEK cells, and D) nomifensine (2µM) has no effect on control cells in the presence of the probe. Images are courtesy of Adam Henke. Representative images from 3 independent experiments.
NG54 has also been demonstrated to be a substrate for hDAT (Figure 12) and hSERT (Figure 13). In each case, the compound labeled cells in a transporter-dependent and inhibitor-sensitive manner. Dr. Adam Henke has measured the $K_m$ of NG54 uptake at each transporter, and it was found to have reasonable $K_m$s for the MATs ($K_m$ hDAT = $6.3 \pm 0.7$, $K_m$ hNET = $7.9 \pm 3.6$, and $K_m$ hSERT = $3.84 \pm 1.0$; mean $\pm$ SEM from 3 independent experiments). I have also found NG54 to be a fluorescent substrate for VMAT2 (Chapter 2). Thus, NG54 will serve as a foundational lead for further structure-activity relationships designed to examine the limits of substrate tolerance at each transporter. Preliminary results from a small library of analogs suggest that functionalization of the acridone core can lead to differential activities at the various MATs while retaining VMAT2 activity (data not shown). Along with yielding information regarding the substrate tolerances of each transporter, this study may furnish FFN probes selective for each of the MATs.

**Figure 13:** NG54 is a fluorescent substrate for hSERT. A) A fluorescent SERT construct (EGFP-hSERT) accumulates NG54 (30 $\mu$M, 60 minutes) in transfected EGFP-hSERT MDCK cells. B) Imipramine (2 $\mu$M, SERT inhibitor) abolishes NG54 uptake in EGPF-hSERT MDCK cells. In these images, the pseudocolor green coronal signal corresponds to EGFP-hSERT, where the vast majority of the signal is on the membrane. The pseudocolor purple corresponds to NG54. Representative images from 3 independent experiments. Also confirmed twice in hSERT-HEK by Dr. Adam Henke (not shown).
NG54 is a Fluorescent False Neurotransmitter in the Mouse Brain

NG54 is currently being characterized as a FFN in mouse coronal brain slice by Matt Dunn and Paolomi Merchant, colleagues in the Sames group. NG54 uptake has been studied in the dorsal striatum, where nigrostriatal dopaminergic neurons project and modulate cortical and other inputs. Preliminary experiments show a very strong degree of colocalization with signal from TH-GFP (where GFP expression driven by the promoter for tyrosine hydroxylase), a control for aminergic innervation (≈90% colocalization, preliminary results from Paolomi Merchant, Figure 14 A-C). The observed high level of anatomical coincidence between NG54 and the TH-GFP signals suggests dopaminergic labeling by NG54. This labeling is also sensitive to nomifensine (1 μM, data not shown, Matt Dunn). NG54 (10 μM) also appears to label dopaminergic cell bodies in the substantia nigra (Figure 14 D,E). These results indicate that NG54 is indeed a dopaminergic label in areas of dopaminergic innervation, supporting the hypotheses that NG54 is a DAT substrate and that positive results gathered from cell culture-based screening may translate into functional dual MAT/VMAT substrates FFN in brain tissue.
**Figure 14:** NG54 colocalizes to a high degree ~90% with a marker for dopaminergic neurons. A) NG54 affords a punctate label in the dorsal striatum of acute mouse brain tissue (10 μM, 30 minute treatment, 15 minute wash with artificial cerebral spinal fluid (ACSF)). B) TH-GFP is a marker of dopamine neurons (GFP expression is driven by the promoter for tyrosine hydroxylase). C) Overlay of NG54 signal with that of TH-GFP reveals a strong colocalization (~90%, preliminary results). D) In the substantia nigra, NG54 (10 μM) also labels cell bodies. E) Examination of TH-positive dopamine neurons reveals that NG54 labels dopaminergic cell bodies in a manner similar in to FFN102,(45) a confirmed dopaminergic FFN. Representative images are courtesy of Paolomi Merchant (A-C), and Matt Dunn (D,E).

It is known that high concentrations of potassium can induce neuronal depolarization and subsequent exocytotic release of neurotransmitter from presynaptic elements; destaining by KCl is a requisite behavior of FFNs. Indeed, upon application of KCl (80 μM) to a coronal mouse brain slice comprising the dorsal striatum that has been pre-loaded with 10 μM NG54 (30 minute NG54 treatment), this probe is released similar to reported FFNs (Figure 15). Upon treatment with KCl, there is a noticeable increase in the total signal background signal of the images (Figure 15B) as the punctate signal is lost. As acridones are known to experience a small degree of self-quenching,(51) NG54 is currently being
investigated as a potential “flashing” FFN that derives its release readout from a distinctly different process from the hydroxycoumarins.\(^{52}\) NG54 may be a new “unquenching” FFN, capable of signaling release from presynaptic stores by diffusing from high concentration in the synaptic vesicles where the compound quenches its fluorescence, to lower concentrations in the extracellular space where the compound fluoresces more brightly.

**Figure 15:** NG54 is released by action of KCl A.) NG54 labels punctate structures in the dorsal striatum (10 µM, 30 min). B) Action of KCl (80 µM, 3 minutes) on the same field of view induces release of NG54 in a manner similar to other confirmed FFNs.\(^{45}\) Images are courtesy of Matt Dunn.

**A Note About Disparities Between Experimental Systems**

It is important to note that while cell-based experiments have proven useful for identification of FFNs that are functional in the brain, there is an important inconsistency in our data in the case of DAT. In the mouse brain slice, FFN102 and FFN202 (see **Table 1** for structures) are substrates for DAT, despite the fact that neither is a substrate in cultured cells heterologously overexpressing hDAT. The hDAT cell system seems to work for other probes, as the fluorescent pyridinium APP+ and NG54 are both substrates in our hDAT-EM4 cells. There are a few
possible causes of this disparity concerning FFN102 and 202. First, the sequence homology differences between the human and mouse transporters may give rise to dissimilar substrate tolerances. Although the homology between species is quite high, it is possible that minor differences in transporter sequence can have noticeable effects on the extrema of the transporter’s substrate tolerance. Some functional differences in DAT transport between species have previously been characterized,\(^{(53)}\) although the pharmacological profile of inhibitors has been shown to be similar between hDAT and mDAT.\(^{(54)}\) Another possible explanation is that DAT function and substrate scope are somewhat dependent on the context in which it is expressed, ie: when overexpressed in HEK cells compared to endogenous expression in the native environment of brain tissue. In order to address this important discrepancy, we will transfec439t HEK293T cells with mDAT in order to compare substrate activity of probes between hDAT and mDAT. If no difference is observed, this would suggest that system-specific differences in transporter function give rise to the different apparent substrate scope. If FFN102 and 202 act as substrates in mDAT-transfected HEK cells, then it is quite likely that species-specific differences in transporter sequence are responsible.

A similar inconsistency, albeit not as blatant, may exist in the case of hNET (above). If preliminary studies from the brain confirm that the labeled structures in the barrel cortex (\textbf{Figure 9}) are indeed noradrenergic axons and presynaptic elements, and the opposite substrate activity in brain slice as compared to the activity in cell culture is verified for AGH093 and FFN201, we will undertake a similar study with mNET.
III: Conclusions and Outlook

As mentioned above, the discovery of FFN102 as a mDAT substrate in mouse coronal brain slice led to the development of a new method for the selective imaging of dopaminergic signaling at the synaptic level. The identification of an FFN active at or selective for SERT or NET would facilitate the study of serotonergic and noradrenergic neurons, respectively, in a similar manner. After screening a number of small, fluorescent, aminoethyl-functionalized fluorescent probes for specific transporter-dependent uptake in epifluorescence microscopy experiments, it was determined that a select few of the previously-developed and new FFNs are indeed substrates of MATs.

With the caveat that the human dopamine transporter, when expressed heterologously in HEK cells, seems more discriminating than its mouse counterpart in brain slice preparations, we can make some preliminary conclusions about the extent of the substrate scopes of these transporters. Interestingly, the large acridone NG54 is tolerated as a substrate for each transporter. Along with the putative substrates from the Wilson lab discussed above, this suggests that MATs are indeed capable of transporting larger, non-pyridinium based substrates. Additionally, the 3-aminoethyl-7-hydroxycoumarins AGH093 and FFN201 behave as selective hNET substrates in cultured HEK cells, supporting the notion that DAT is more discriminating than NET.

These larger, fluorescent substrates push the functional limits of the MATs. As environmental exposure to neurotoxins is hypothesized to be involved in the development of some forms of idiopathic parkinsonism, this expanded
understanding of the substrate scope may prove useful. Additionally, because no crystal structure has been solved for any of these transporters, any pharmacological data indicating substrate scope may prove helpful in the design of pharmaceutical agents.

AGH093 and FFN201 represent a new development within the FFN program, as they are pH-sensitive NET/VMAT FFNs. Both compounds have demonstrated substrate activity at VMAT2, and both are currently being investigated in noradrenergic systems, including the cortex of mouse brain slice. Preliminary results suggest that they function as “flashing FFNs,” as expected. We are initiating a collaboration with Professor Randy Bruno (CUMC) for the purpose of in vivo investigation of aminergic signaling in the cortex of living, behaving mice using AGH093 (or FFN201) as an optical tracer with sufficient imaging properties to resolve neurotransmitter release at individual presynaptic terminals. Since noradrenergic (and dopaminergic) inputs are known to modulate cortical signaling networks, these compounds could lead to a breakthrough discovery of how individual synaptic inputs or collections of synaptic inputs modulate these cortical neurons. Ultimately, such a program could be used in preliminary, animal based experiments relating to the Brain Activity Map Project (introduced in Chapter 1). Since noradrenergic neurons also innervate the periphery, these probes may play additional roles in the investigation of NE signaling in the peripheral nervous system as well.

NG54 was found to be a substrate for hDAT, hNET, and hSERT, with reasonable Michaelis constants at each (K_m apparent < 10 µM), and is currently
under investigation in the lab of Professor Scott Thompson (UMD) as a potential serotonergic FFN lead. Because NG54 is a substrate for each of the MATs, derivatization of this acridone core could provide a new toolkit of FFNs selective for subsets of monoaminergic neurons (as discussed above). The Sames group is currently undertaking the synthesis and evaluation of a series of derivatized aminoethyl-acridones for the purpose of identifying selective FFNs for dopaminergic, noradrenergic, or serotonergic synapses based on differential substrate activity at the respective plasma membrane transporters.
IV: Experimental

Cell Culture

For hDAT-EM4 Experiments:

An EM4 cell line stably expressing hDAT (hDAT-EM4) and an empty-vector transfected EM4 cell line to serve as a control were kindly provided by Drs. Jonathan Javitch and Mark Sonders of the Department of Psychiatry at Columbia University Medical Center. Cells were grown on 10 cm culture plates (corning) in DMEM + GlutaMAX (Invitrogen #10569) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

For hSERT-HEK and hNET-HEK Experiments:

HEK293 cell lines stably expressing hNET or hSERT were obtained from the laboratory of Professor Bryan Roth (University of North Carolina at Chapel Hill). hNET-HEK and hSERT-HEK cells were treated as above, and maintained in the complete growth medium above with additional 500 µg/mL G418 (Calbiochem) to maintain the transfection. HET293T cells were grown in parallel as untransfected controls for background uptake.

For EGFP-hSERT Experiments:

MDCK cells expressing EGFP-hSERT under the Flp-in T-REx inducible expression system were treated as the HEK cells above, and maintained in the same
conditioned medium containing additional 150 µg/mL hygromycin B to maintain the transfection.

**Screening of Probes at Neurotransmitter Transporters**

For epifluorescence microscopy experiments, cells expressing either hDAT, hNET, or hSERT were plated on poly-D-lysine (Sigma-Aldrich, 0.1 mg/mL) coated six-well plates (Falcon) at a density of ~100,000 cells/well and were incubated until confluence (3-4 days at 37°C in a humidified atmosphere containing 5% CO₂). For Flp-TetR MDCK cells expressing EGFP-hSERT or an empty vector, expression of the transporter was induced with 2 µg/mL tetracycline in growth medium 24 h prior to the experiment. On the day of the experiment, complete growth medium was removed by aspiration and wells were carefully washed with PBS (1 mL/well). Cells were then treated with 900µL of experimental medium (DMEM minus phenol red containing 25 mM HEPES (Invitrogen) with 1% FBS (Atlanta Biologicals)), for 1-3 h. 100 µL of 200 µL probe solutions were added to each well, for a final concentration of 20 µM probe. Cells were treated for approximately 30 minutes with experimental probes at 37°C before uptake medium was removed by aspiration, wells were carefully washed with PBS. Cells were maintained in 1 mL warm experimental medium. Images were taken using a Leica DMI 4000B inverted epifluorescence microscope equipped with a Leica DFC 360 FX digital camera controlled through Leica LAS AF 6000E software. Bright field and fluorescence images were acquired sequentially (BF acquisition time = 37 ms). Fluorescence images were acquired using filter cubes (ex = 350 ± 25 nm, em = 450 ± 25 nm, for coumarin probes; ex =
440 ± 25 nm, em = 550 ± 25 nm for benzofurazan probes (JAM127, JAM138, JAM157). All images of each probe in transporter-transfected and control cells were adjusted to the same brightness and contrast level using ImageJ (National Institutes of Health).

**Pharmacological Inhibition of Suspected MAT Substrates**

For inhibition studies of suspected substrates, cells were plated as above. On the day of the experiment, cells were washed as described and pretreated with 900 µL experimental medium or experimental medium containing inhibitor (2 µM nomifensine for DAT and NET, and 2 µM imipramine for SERT). After the preincubation period, cells were treated with either 100 µL of 10X probe in experimental medium or the same with additional inhibitor 2 µM. Treatment times were the same, and images were acquired as described above.

**Determination of Kinetic Parameters for FFNs**

Initial rates experiments were performed essentially as described in Chapter 2. hNET-HEK cells were plated onto 96-well fluorescence microplates (Falcon) at a density of ~45,000 cells/well. Cells were incubated for 2 days until confluent. Specific fluorescence uptake was found to be linear for AGH093 and FFN201 at hNET for at least 12 minutes, and specific uptake at each concentration was calculated by subtracting background uptake in wells inhibited with 2 µM nomifensine from uninhibited uptake values. Data were fit to the Michaelis-Menten equation using GraphPad.
Studies of FFNs in Acute Mouse Brain Slice

These studies were based on protocols developed and reported recently by Dr. Pamela Rodriguez. These protocols will be discussed in detail in future reports as well as in the dissertations of Matthew R. Dunn and Paolomi Merchant (doctoral graduate students in the Sames Laboratory).
V: References


induced downregulation of the serotonin transporter than by acute blockade of this transporter, *The Journal of Neuroscience* 22, 6766-6772.


A Small Fluorescent Analog of the Neurotoxin MPP+ is a Marker for Catecholamine Neurons in the Brain, but is Not a Fluorescent False Neurotransmitter\(^{(1)}\)
I: Introduction

MPP+, Parkinsonism, and the Dopamine Transporter

In 1947, Hoffman-LaRoche introduced desmethylprodine, or 1-Methyl-4-phenyl-4-propionoxypiperidine (MPPP), a synthetic opioid, as a potential alternative to morphine.\(^2,^3\) It was found that MPPP was a \(\mu\)-opioid receptor agonist with approximately 70\% the analgesic efficacy of morphine, although MPPP was never introduced as a clinical drug. MPPP was largely forgotten until a chemistry graduate student in 1977 synthesized a batch of MPPP for recreational purposes, but took a synthetic shortcut which resulted in the production of a significant amount of an elimination side product, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as a major impurity (Figure 1).\(^4\) Within days he began experiencing symptoms consistent with severe idiopathic parkinsonism. This case represented a lone medical anomaly until a group of “frozen addicts” suffering from severe parkinsonism appeared a few years later, each appearing to have injected an amount of impure MPPP, containing primarily MPTP.\(^5\) It was discovered that the MPTP impurity was responsible for this parkinsonism, as MPTP readily crosses the blood brain barrier, where it is metabolized by monoamine oxidase-B (MAOB) to 1-methyl-4-phenylpyridinium (MPP+).\(^6,^7\)
Figure 1: Attention to detail can mean the difference between life and death (of nigrostriatal dopamine neurons). A synthetic shortcut in the preparation of MPPP, a synthetic opioid, leads to generation of MPTP, a side product with significant neurotoxic activity.

MPP+ is a substrate for high affinity monoamine neurotransmitter transporters DAT,(8, 9) NET,(10) and SERT.(11) For reasons still not entirely clear, MPP+ selectively destroys dopaminergic neurons in the substantia nigra. Once inside the cell, MPP+ is driven across the mitochondrial membrane in a manner dependent on the mitochondrial potential (Figure 2).(6, 8) MPP+ then interferes with the electron transport chain by inhibiting Complex I, leading to apoptosis and ultimate parkinsonian pathophysiology. MPP+ is also a VMAT2 substrate, and VMAT2 activity is known to rescue neurons and secondary cells from MPP+ toxicity, presumably by sequestering an intracellular population of the toxin.(13-16) The nature of MPP+ toxicity is complex, as MPP+ has been shown to inhibit tyrosine hydroxylase, and evidence exists that toxicity may be associated in part with reactive oxygen species (ROS) resulting from high concentrations of dopamine that have been redistributed to the cytosol from vesicular stores.(12)
MPTP and MPP+ have been used as tools to generate parkinsonism in animal models,\(^7,\,17\) and radiolabeled MPP+ is also a common tool used in the study of monoamine and cation transporters (see Chapter 5).

**Figure 2:** A metabolite of MPTP induces parkinsonism by selectively killing nigrostriatal dopamine neurons. A.) Circulating MPTP is lipophilic and readily crosses the blood brain barrier (red line), where it is converted by action of monoamine oxidase B (MAO-B) to MPP+ in astroglia. B.) MPP+ is a substrate for high affinity monoamine transporters including DAT. Once inside dopaminergic neurons, MPP+ is known to: 1 – become localized to mitochondria by action of the electrochemical potential across the mitochondrial membrane and lead to apoptosis, and 2 – become vesicularized by action of VMAT2 (blue ovals). The interplay between these two competing processes is important for MPP+ toxicity and protection therefrom, and we became curious about whether VMAT2-dependent sequestration (situation 2) of a fluorescent MPP+ analog would be sufficient to exploit for imaging purposes.

**Fluorescent Analogs of MPP+ Have Been Used to Study Plasma Membrane Monoamine Transporters (MATs)**

Pyridiniums have been used for decades in the study of mitochondrial membrane potential, owing to the permanent positive charge of the quaternary ammonium moiety.\(^18,\,19\) It was also found that a fluorescent styryl pyridinium analog of MPP+, 4-(4-(dimethylamino)styryl)-1-methylpyridinium (ASP+), is a substrate for a family of widely expressed, low affinity, polyspecific solute carriers
known as the Organic Cation Transporters (OCTs; for a full discussion see Ch5).\(^{[20-22]}\)

ASP+ has been used as a fluorescent substrate to study and image OCT expression, activity, and pharmacological properties in transfected cells and tissues.\(^{[21, 23, 24]}\)

In a groundbreaking 2003 report, DeFelice et al demonstrated ASP+ substrate activity at plasma membrane monoamine transporters DAT, NET, and SERT (Figure 3).\(^{[25]}\) This represented the first conclusive report of a fluorescent substrate for any of the high affinity monoamine transporters (MATs), proving that these MATs are capable of accepting substrates significantly larger than endogenous monoamines or MPP+. It was found that ASP+ fluoresces brightly when bound to the transporter or intracellular compartments including mitochondria, but is quenched in aqueous environments via the twisted intramolecular charge transfer (TICT) mechanism.\(^{[26]}\) Since the rate constant of transport is slow and since ASP+ has a moderate affinity for the MATs, there is a considerable steady-state population of the compound bound to the transporter in the hydrophobic transmembrane region, where ASP+ fluoresces brightly. Because of this property, it was possible for the authors to spectroscopically separate ASP+ binding from transport, and to study the substrate stoichiometry and ion dependence of both processes.\(^{[25, 27]}\)

It was shown that DAT, NET, and SERT can bind and accumulate ASP+, although uptake kinetics are slow and ASP+ is a comparably poor substrate for SERT. In 2010, Wilson reported a detailed study consisting of a number of ASP+ analogs, which further elucidated the scope of pyridinium interactions and transport through NET, and found a relationship between the photophysical properties of transporter-bound pyridiniums and the geometry of binding.\(^{[28]}\)
Because of the slow catalytic turnover ($V_{\text{max}}$) and relatively high background uptake of these styryl pyridiniums (likely related to their high lipophilicity),\(^{25,27}\) ASP+ and related styryl pyridiniums are poor candidates for development of FFNs. Despite these drawbacks, however, ASP+ has been used to image implanted glioma cells in brain tissue because of its favorable uptake kinetics at OCT3, which is heavily overexpressed in gliomas.\(^{23}\)

These reports encouraged us to consider how smaller, less lipophilic analogs of MPP+ would behave in brain tissue. To explore the possibility that a fluorescent MPP+ analog may serve as a lead for FFNs, we considered a fluorescent analog with the strongly electron donating dimethylamino-group in the 4’-position of a biaryl pyridinium, 4-(4-dimethylamino)phenyl-1-methylpyridinium (APP+) (Figure 3).

**Figure 3:** MPP+ and useful fluorescent derivatives. (A) Structure of the monoaminergic neurotoxin MPP+, which served as a lead for these studies. (B) Addition of a strongly electron-donating group (EDG) to the 4’ position of MPP+ generates APP+, a small, fluorescent analog. (C) Stilbazolium dyes were first shown to act as fluorescent substrates of MATs.
**APP+ is a Fluorescent Substrate for DAT, NET, SERT, and VMAT – A FFN Lead?**

We became curious to test whether a smaller analog of MPP+ would serve as a superior substrate for DAT, NET, SERT, and VMAT, and whether it could serve as a lead for FFNs. While MPP+ is known to interact with mitochondria, it is unclear whether accumulation by VMAT would outcompete mitochondrial binding (**Figure 4**).

**Figure 4:** Overview of a representative dopaminergic synapse and labeling by FFNs and APP+. (A) Dopamine is synthesized in the DA neuron and packaged into synaptic vesicles by the action of VMAT2 (blue ovals), released upon arrival of an action potential, and is transported in large part back into the presynaptic neuron via DAT. MSN = medium spiny neuron, D2 = dopamine receptor D2. (B) FFNs such as FFN102 are actively transported into dopaminergic axonal structures and stain both the cytosol and the synaptic vesicles. (C) APP+ is transported via the same plasma membrane transporters, and we examined the labeling characteristics of APP+ with respect to potential FFN behavior, specifically whether a high degree of VMAT-driven sequestration can overcome the bright mitochondrial population of APP+. 

APP+ was first synthesized as an antimicrobial pyridinium dye\(^{(29)}\) and has been used in the study of nonlinear optical systems and fundamental photophysical chemistry.\(^{(26,30-33)}\) During our initial studies with APP+, a number of relevant reports were published describing the molecule as a MAT substrate. It was shown that
APP+ is in fact a fluorescent substrate for DAT, NET, and SERT,\(^{34,35}\) and APP+ is marketed by Molecular Devices as the fluorescent substrate in a kit designed for high-throughput screening of DAT, NET, and SERT inhibitors or potentiators.\(^{36,37}\) Some researchers are attempting to translate this kit into other systems without reported knowledge of the structure or properties of the dye.\(^{38,39}\)

Encouraged by initial studies in our lab (see results below) as well as these contemporaneous reports of APP+ as a substrate for DAT, NET, SERT, and VMAT, we began using APP+ as a positive control in our MAT uptake studies (Chapter 3), and decided to pursue studies of APP+ as a potential lead for Fluorescent False Neurotransmitters (Figure 4; see Chapter 1 for full discussion of FFNs). Described below are the synthesis, photophysical studies, secondary cell-based studies, and advanced characterization of APP+ in acute mouse brain slice preparations. If APP+ behaved as an FFN in the brain, a small library of analogs would be generated in search of a selective pyridinium-based FFN for dopaminergic, serotonergic, or noradrenergic neurons in brain tissue with optimal FFN-like behavior. In this chapter, the behavior of APP+ in brain tissue is compared to FFN102,\(^{40}\) and explanations for the observed behavior of APP+ are extrapolated from photophysical and cell-based studies.
II: Results and Discussion

Synthesis and Photophysical Evaluation of APP+

The structure and synthesis of APP+ iodide has previously been reported.\(^{(34, 41, 42)}\) Our synthesis of APP+ is outlined in Scheme 1. APP+ was prepared via a Suzuki cross coupling of 4-bromopyridine with 4-(dimethylamino)phenylboronic acid pinnacol ester to afford intermediate 1. Alkylation with iodomethane afforded APP+ iodide as a bright orange powder.

![Scheme 1: Synthesis of APP+ iodide.](image)

It was found that APP+ is brightly fluorescent in hydrophobic environments such as chloroform, and displays both inverse solvatochromism (blueshift) and fluorescence quenching with increasing solvent polarity (Figure 5). Specifically, the fluorescence of APP+ is approximately 10% as bright in aqueous PBS compared to chloroform, and slightly blueshifted in polar solvents. Excitation maxima were found to be 417 nm in PBS and 436 nm chloroform. Emission maxima are 502 nm in PBS and 506 in chloroform when excited at the corresponding excitation maxima.
Figure 5. Relevant photophysical properties of APP+. A. UV-Vis spectra of APP+ (20 μM) in different solvents illustrating the blueshift of the absorbance maximum with increasing solvent polarity, characteristic of pyridiniums (λ\text{max}_{PBS} = 416 nm, λ\text{max}_{DMSO} = 419 nm, λ\text{max}_{ethanol} = 424 nm, λ\text{max}_{CHCl_3} = 439 nm). (B) Emission and excitation profiles of APP+ (2 μM) in phosphate buffered saline (PBS), pH=7.4 (λ\text{ex}_{PBS} = 417 nm, λ\text{em}_{PBS} = 501 nm), and chloroform (λ\text{ex}_{CHCl_3} = 436 nm, λ\text{em}_{CHCl_3} = 506 nm), illustrating the strong solvent effects to which APP+ is subject. Spectra were measured at the corresponding emission/excitation maxima for APP+ in the respective solutions. Importantly, APP+ is approximately ten times more fluorescent in nonpolar environments than in aqueous buffer.

This fluorescence quenching is due to the well-known twisted intramolecular charge transfer (TICT) mechanism, in which an excited electron decays in a nonradiative manner to a triplet state.\textsuperscript{26, 33} This decay mechanism becomes favorable in polar environments or when the dihedral angle between the two arenes in excited states of biaryl systems becomes large, either due to a solvent effect or geometric restriction.\textsuperscript{26, 33} The resultant fluorescence quenching in aqueous environments is important for interpretation of results later in this chapter.

Cell-Based Results

APP+ was tested in human DAT-expressing EM4 cells (hDATEM4) for transporter-dependent accumulation, as well as for intracellular localization of compound. APP+ displayed a bright, punctate staining pattern consistent with recent reports from hSERT-expressing HEK cells.\textsuperscript{35} Cotreatment with APP+ and
Mitotracker Deep Red (Invitrogen) revealed a high degree of mitochondrial label, consistent with previously reported results.\(^{35}\) Both hDAT-transfected and empty vector-transfected EM4 cells were co-exposed for 10 minutes with Mitotracker Deep Red (20 nM) and APP+ (2μM). Each probe was imaged separately by using the appropriate optical filters with no cross-channel signal contamination. Both cell types were similarly stained by the Mitotracker dye (Figure 6C), while APP+ accumulated rapidly in hDAT-transfected EM4 cells. The empty vector-transfected EM4 cells afforded no significant uptake of APP+ under the same conditions (Figure 6B). A high degree of colocalization of APP+ and Mitotracker in hDAT-transfected cells (Figure 6D) confirms that APP+ labels mitochondria in these cells, which is consistent with a previous report using a SERT-expressing cell line, and is ascribed to its permanent positive charge. The quenched fluorescence in aqueous growth media is advantageous for cell culture assays, as continuous measurement or imaging is possible in the presence of APP+ dye with no need for washing or additional quenching agents.

**Figure 6:** APP+ becomes localized in mitochondria of EM4 cells expressing hDAT. Shown are epifluorescence microscopy images of APP+ (2μM, 10 min incubation) in (A) hDAT-transfected EM4 cells, and (B) empty vector-transfected EM4 cells. (C)
hDAT-transfected EM4 cells were also co-incubated with Mitotracker Deep Red (20 nM, 10 min). (D) Overlay of APP+ and Mitotracker images shows strong co-localization, indicating mitochondrial concentration of APP+. Scale bar = 15 μm.

APP+ was next tested in VMAT2-HEK cells (described in Chapter 2)\(^{(16)}\) to examine whether substrate activity at VMAT2 could overcome mitochondrial accumulation, and if accumulation in VMAT2-expressing acidic compartments was strong enough to overcome fluorescence quenching in the aqueous lumen. In a series of experiments comparing the APP+ staining pattern to Mitotracker Deep Red (control for mitochondrial staining), an interesting result was observed. Consistently, VMAT2-HEK cells treated with APP+ (2μM, 60 min) were significantly less bright and less punctate than null-transfected control HEK cells (TetR-HEK) (Figure 7 A,D). Note that a longer incubation time is required for ASP+ to enter the cells in the absence of MATs. TetR-HEK cells treated with APP+ displayed a highly mitochondrial staining morphology, overlaying again to a great degree with Mitotracker, although this was significantly diminished in VMAT2-HEK cells (Figure 7). The difference in staining does not likely arise from a difference in mitochondrial potential between each cell type, as Mitotracker labeled VMAT2-HEK cells similarly (Figure 7 B,E). Based on these results as well as reports of VMAT rescuing cells from MPP+ toxicity and the fact that these cells were cloned under pressure from MPP+, a hypothesis was developed. It is plausible that the concentration of cytosolic APP+ is low when there is not a high affinity transporter on the plasma membrane surface (as concurrently shown)\(^{(39)}\) and this low concentration is sequestered by VMAT2, affording a vesicularized concentration of
APP+ that is insufficient to overcome fluorescence quenching in the aqueous lumen. VMAT2 binding could also outcompete the electrochemically-driven mitochondrial binding. In order to further examine this hypothesis, VMAT2 inhibition studies were conducted.

**Figure 7:** The APP+ label appears to be reduced by VMAT2 in HEK cells. A) In control TetR-HEK cells, APP+ (2 μM, 60 min) consistently appears bright and punctate. B) Mitotracker (50 nM, 10 min) reveals the spatial location of mitochondria, and C) overlay of the two shows a high degree of colocalization. D) VMAT2-HEK cells are routinely less bright with fewer puncta. E) Mitotracker labeling shows that this lower apparent level of staining is independent of mitochondrial potential, and F) overlay of APP+ and Mitotracker signal in VMAT2-HEK cells demonstrates the lower overall mitochondrial labeling by APP+. While the overlay is obfuscated by the mitotracker label, it is apparent that the APP+ signal is greatly diminished compared to HEK control cells (top row).

However, treatment with VMAT2-inhibitors (2μM DTBZ or 1μM reserpine) revealed no consistent or reproducible increase in fluorescence of the cells (**Figure 8**). Several explanations for this result are possible. It is possible that there is simply not enough APP+ crossing the plasma membrane through passive diffusion.
to make a discernable difference, as the changes in fluorescence observed here are minor. It is also likely that the process that selected for VMAT2-expressing HEK cells has also upregulated an efflux transporter in the original clone or the cells evolved some similar clearance mechanism, which is capable of clearing the cell of small pyridinium compounds. The latter explanation is likely as inhibition of VMAT2 with reserpine (1μM) or DTBZ (2μM) did not reproducibly or consistently within each experiment lead to restoration of mitochondrial staining by APP+.

While these results may be suggestive that APP+ is a substrate for VMAT and that its fluorescence can be quenched in aqueous compartments, they are inconclusive, as inhibition of VMAT2 does not restore the mitochondrial label.

**Figure 8:** The effect of reserpine on APP+ labeling of VMAT2-HEK cells. A) APP+ in control TetR-HEK cells (2μM, 60 min) shows mitochondrial labeling as above (Figure 7). B) VMAT2-HEK cells treated under the same conditions are less punctate, also as above. C) Inhibition of VMAT2 with reserpine (1μM) does not restore strong APP+ staining of mitochondria in VMAT2-HEK cells.

From these results, it was still unclear whether action of a high affinity transporter on the plasma membrane such as DAT, NET, or SERT could provide sufficient cytosolic concentrations of APP+ to visualize a vesicular label, and it was still not completely clear whether APP+ was a VMAT2 substrate. Fortunately as we were conducting these experiments, it was reported that APP+ was a substrate for
VMAT2 (in the context of the Molecular Devices Neurotransmitter Transporter Uptake Assay Kit).\(^{(39)}\) Although our VMAT results were not conclusive, evidence from a DAT and VMAT-overexpressing system revealed staining of both mitochondria and VMAT2-expressing compartments,\(^{(39)}\) so we decided to pursue preliminary labeling and release studies in acute mouse brain slice with the goal of describing the behavior of APP+ in aminergic neurons in acute mouse brain slices.

**APP+ is a Marker for Catecholamine Neurons in Brain Tissue**

Curiosity about the behavior of APP+ in brain tissue led me to collaborate with Matt Dunn, a graduate student in the Sames Lab who works routinely with FFNs in acute mouse brain slice. Together, we developed a number of experiments to rigorously test the behavior of APP+ in catecholaminergic innervation in the brain, and Matt performed all microscopy in the brain.

It is important to note that the focus of the remainder of this chapter is on catecholaminergic neurons in the brain (DA-ergic and NE-ergic) because of the availability of a positive control for staining, specifically green fluorescent protein (GFP) expressed under control of the promoter of tyrosine hydroxylase (TH-GFP).\(^{(43)}\) It is quite likely that APP+ has similar behavior at 5HT neurons in the brain, although the lack of an adequate control at the time of our experiments caused us to focus on catecholaminergic innervation.

Previous studies investigating APP+ uptake by cells have been limited to cultured cells transfected with plasma membrane monoamine transporters,\(^{(34-36)}\) cultured cells endogenously expressing SERT,\(^{(44)}\) primary peripheral tissue expressing NET,\(^{(38)}\) or platelets and lymphocytes.\(^{(45)}\) We were motivated to examine
the behavior of APP+ in brain tissue, and we began by examining labeling characteristics of APP+ in acute mouse brain slices containing the midbrain region, where catecholaminergic cell bodies occur. The labeling selectivity of cell bodies was determined by co-localization of the APP+ and GFP signals in brain tissue obtained from mice expressing GFP under the control of tyrosine hydroxylase (TH) promoter (TH-GFP mice) via two-photon microscopy (Figure 9). For dopaminergic cell bodies, we examined the substantia nigra pars compacta region (SN) and the ventral tegmental area (VTA), the anatomical areas where the neuronal cell bodies of the nigrostriatal and mesolimbic dopaminergic systems, respectively, reside. Acute midbrain slices from TH-GFP transgenic mice were perfused for 30 minutes with oxygenated artificial cerebral-spinal fluid (ACSF) containing 500 nM of APP+, followed by washing for 10 min with oxygenated ACSF. Staining patterns and colocalization were determined by imaging individual slices sequentially at the excitation and emission wavelengths for APP+ and GFP (APP+: λ_{ex} = 800 nm, λ_{em} = 435-485 nm; GFP: λ_{ex} = 950 nm, λ_{em} = 500-550 nm). Excitation and emission wavelengths were chosen to minimize signal crosstalk between APP+ and GFP channels. Labeled cell bodies were defined as areas with mean fluorescence intensity greater than two standard deviations above background signal, with size and shape consistent with morphological parameters of dopamine neuronal soma. Colocalization was determined by assessing the number of cell bodies in each region where GFP signal and APP+ signal were both present at least two standard deviations above their respective backgrounds.
Every cell body labeled by APP+ in these regions was found to contain the GFP signal, while 76% of TH-GFP positive cells in SN/VTA contained APP+ signal (84/110 cells, n = 6, Figure 9A). Further, the DA neuronal cell body labeling was greatly reduced by treatment of the slice with the DAT inhibitor nomifensine (7%, 3/43 cells, n = 3, Figure 9B), confirming that APP+ uptake by DA neuronal soma is DAT dependent. The higher magnification image shows a heterogeneous perinuclear staining (Figure 9D) similar to that observed in hDAT-EM4 cells (Figure 6), suggesting mitochondrial staining. These results indicate that APP+ selectively labels DA neuronal cell bodies versus other neurons in the area, which appear as dark unstained regions (Figure 9A).

To obtain the level of soma labeling specified above, incubation of the slice with 500 nM APP+ for 30 minutes was required. Under these conditions, a high level of punctate staining was observed, which was not inhibited by nomifensine in SN/VTA (Figure 9A,B), and was also present in brain areas outside of SN/VTA (Figure 9E). These results indicate that the majority of the background staining is not related to DA neuronal structures such as the dendrites.

We also investigated labeling of noradrenergic cell bodies in the locus coeruleus (LC) where the majority of noradrenergic cells reside (Figure 9C). APP+ selectively labeled these neurons; all examined APP+ labeled cells contained the GFP signal and 59% of TH-GFP positive cells in LC were also labeled with APP+ (74/126 GFP positive cells, n = 3). We found that for analysis of APP+ uptake by noradrenergic neurons in LC, young mice (<30 d postnatal) were required, as the staining was dim and inconsistent in older mice (data not shown). This finding is
consistent with reported down-regulation of NET protein expression in cell bodies of noradrenergic neurons in the LC of older mammals, including mice.\(^{46,47}\) As in the SN/VTA region, APP+ provides a high level of punctate background staining in LC.

Our studies demonstrate that APP+ labels catecholamine neurons in the indicated brain areas in a DAT/NET dependent manner. Despite the fair degree of unidentified punctate staining, the catecholamine neuronal cell bodies could be readily identified in acute unfixed brain slice by simple perfusion of the tissue with the APP+ dye.
Figure 9: APP+ labels catecholaminergic neuronal cell bodies in acute mouse midbrain slices. TH-positive cell bodies (TH-GFP in green) in the (A) DAT-expressing ventral tegmental area and substantia nigra (VTA/SN), and (C) NET-expressing locus coerulesus (LC) accumulate APP+ (in red) in a selective manner relative to other cell bodies (black holes) after perfusion of APP+ (500 nM) for 30 min. (B) Accumulation of APP+ into cell bodies in the SN/VTA can be substantially inhibited if the slice is pre-treated with a DAT inhibitor (nomifensine, 1 µM; scale bars = 20 µm). (D) A closer view of an APP+ labeled cell body from SN/VTA illustrates a perinuclear, punctate staining pattern similar to what is seen in hDAT-transfected EM4 cells, suggesting mitochondrial staining (TH-GFP in green, APP+ in red; scale bar = 20 µm). (E) No staining of cell bodies was observed in the primary visual cortex of the same slice (scale bar = 20 µm). Images are from collaboration with Matt Dunn.
**APP+ Labels Dopaminergic Structures in the Dorsal Striatum**

The dorsal striatum is heavily innervated by the dopamine neurons originating in SN (see above). Among other functions, dopamine in the dorsal striatum modulates excitatory inputs from the cortex and other brain areas and plays crucial roles in sensory-motor coordination and habit formation. Coronal mouse brain slices comprising the dorsal striatum were loaded with APP+ using the experimental conditions described above, except that a lower concentration of APP+ (100 nM) for only 15 min was sufficient to provide bright punctate staining (Figure 10A). The overall pattern of APP+ staining is similar to that of GFP in TH-GFP mice. Quantitative comparison showed a good degree of colocalization: $83.4 \pm 6.9\%$ (mean ± SD, n=3) of APP+ puncta contained the GFP signal (Figure 10A-C). We observed a small degree of APP+ signal contamination in the GFP channel, enough for $10.1 \pm 5.7\%$ of the brightest APP+ labeled puncta to be picked up in the GFP channel of GFP negative brain slices (mean ± SD, n = 3). To provide another quantitative colocalization measure in complete absence of a signal crosstalk, we also examined colocalization of APP+ and FFN102 in wild type mice. As discussed above, FFN102 is to date the most selective FFN probe for dopamine neurons, featuring strong DAT dependence and ~90% colocalization with TH-GFP signal in dorsal striatum, and thus may serve as a reliable reference signal. We found that $74.1 \pm 6.9 \%$ (mean ± SD, n = 3) of APP+ puncta were FFN102 positive (Figure 10D-F). These results indicate that APP+ labels fine axonal processes of dopamine neurons in the dorsal striatum with selectivity >70% as defined by colocalization to two reference signals.
**Figure 10:** APP+ colocalizes with dopaminergic markers in the dorsal striatum. (A) Signal from GFP expressed under control of the TH promoter. (B) 100 nM APP+ perfusion for 15 min creates a punctate staining pattern in dorsal striatal acute slices. (C) APP+ colocalizes well with TH-GFP (83.4 ± 6.9%, mean ± SD, n = 3). Because of a small amount of crosstalk between APP+ and GFP channels, we confirmed dopaminergic labeling with another marker, FFN102, which has previously been shown to label dopaminergic terminals in the dorsal striatum. (D) FFN102 staining in the same frame. (E) APP+ staining when loaded under the same conditions as above in the presence of FFN102. (F) Overlay of the two channels shows good colocalization of puncta (74.1 ± 6.9%, mean ± SD, n = 3), confirming APP+ as a marker for dopaminergic innervations in the dorsal striatum. Scale bars = 5 µm. Images are from collaboration with Matt Dunn.

We next examined whether the good selectivity of APP+ for DA axonal processes was DAT dependent. Preincubation of the slice with nomifensine (1 µM, 15 min), followed by a 15 min coincubation of nomifensine (1 µM) and APP+ (100 nM) under continuous perfusion in oxygenated ACSF reduced the number of APP+ labeled puncta per image by 2.3 fold, from 147.8 ± 1.4 to 61.4 ± 10.0 (mean ± SD, n =
Figure 11C). Further, the staining pattern of the dorsal striatum was dramatically different under the DAT-inhibition conditions compared to that of control slices; the remaining puncta were more heterogeneous in size and brightness, owing to formation of large bright puncta (Figure 11A,B). These findings suggest that additional transporter systems are present in the dorsal striatum that may facilitate APP+ uptake when DAT is inhibited.

**Figure 11**: APP+ uptake into dorsal striatum is inhibited with the DAT blocker nomifensine. (A) Nomifensine significantly reduces the mean number of puncta per frame from 147.8 ± 1.4 to 61.4 ± 10.0 (mean ± SD, significance determined by t-test, p < 0.001, n = 3). (B) A representative image of APP+ staining in mouse dorsal striatum (100 nM, 15 min perfusion). (C) Pretreatment with nomifensine (1 µM, 15 min) followed by cotreatment with APP+ (100 nM, 15 min perfusion) significantly
reduces the number of stained puncta while changing the appearance of remaining structures. Scale bars = 5 µm. Images are from collaboration with Matt Dunn.

Indeed, inhibition of DAT with nomifensine afforded a dramatically different staining pattern with a smaller number of puncta (2.3 fold) and greater relative proportion of large punctate structures (>2 µm). Although the inhibition experiment supports the importance of DAT for uptake of APP+ and formation of the fine punctate staining pattern (which is similar to that of TH-GFP and FFN102 signal), it also suggests the presence of other transporter systems capable of transporting APP+ into different cellular structures. Consistent with this hypothesis are the observations in the midbrain (SN/VTA and LC), where in addition to cell bodies of catecholamine neurons other structures were labeled throughout the tissue surrounding the neurons. The staining of these unidentified punctate structures was not inhibited by nomifensine and was also present in the areas that do not contain catecholamine neurons. Relatedly, It has been reported that the endocytic dye FM4-64 was taken up by cortical astrocytes via store-operated Ca$^{2+}$ entry (SOCE) channels.(49) It was also suggested by the authors of this paper that the background staining in the brain slice (observed with FM4-64) might be due to astrocytic uptake occurring via these SOCE channels. As FM4-64 is considerably larger than APP+, it is possible that APP+ is accumulated by a similar process. The organic cation transporters such as OCT3, which is widely expressed in the brain and shown to transport MPP+,(50, 51) may also contribute to the extra-catecholaminergic staining (See Chapter 5 for a thorough discussion of APP+ at Uptake2 transporters). Finally,
APP+ is also a substrate for SERT\textsuperscript{34,35} so it is likely that serotonergic structures are labeled.

**APP+ is not a Fluorescent False Neurotransmitter**

Encouraged by the highly catecholaminergic label afforded by perfusion of acute mouse brain slice with sub-micromolar concentrations of APP+, we set out to apply previously developed FFN releasing techniques to APP+. If APP+ could be released, through exocytosis upon impulse by high concentrations of KCl or with direct electrical stimulation, it may serve as a functional “destaining” FFN specific to aminergic neurons.

It is known that neuronal depolarization and subsequent exocytotic release of neurotransmitters can be induced in vitro by high potassium concentrations. We have previously shown that FFN102-loaded presynaptic terminals in dorsal striatum were completely destained through the action of 40 mM KCl\textsuperscript{40} With APP+, however, we found that KCl only partially destained terminal fields compared to ACSF-treated control (Fig. 12). The analysis was accomplished by measuring mean fluorescence intensity of a field of background-subtracted puncta, before and during KCl treatment, and comparing the results to those obtained with an untreated control. It is important to note that individual puncta could not be tracked throughout the course of this experiment due to the high degree of slice deformation upon KCl stimulation. We found that a mean APP+ signal collected from all labeled structures is reduced by 15.3 ± 2.5 % after 8 minutes of KCl treatment (mean ± SD, n = 3; (t-test, p < 0.05 for t = 1-3 min, p < 0.01 for t = 4-8 min). These
results show that under the conditions of prolonged depolarization and exocytosis, APP+ is released from presynaptic terminals in a small but significant manner. This is consistent with previously reported release of MPP+ under similar depolarization conditions.\(^{52}\) We were not able to observe the released APP+ in the extracellular space with two-photon microscopy, most likely due to quenching of APP+ fluorescence in aqueous media (Figure 12). Due to the high degree of slice deformation upon KCl treatment, we were unable to track individual puncta and could not discern whether the loss of signal was due to a loss of a small amount of the label from each of the puncta or from a complete destaining of a subset of puncta (active synaptic zones vs. areas dense with mitochondria). To address this, we set out to pursue release studies using a milder and more physiologically relevant direct electrical stimulation of the dorsal striatum.

**Figure 12:** Treatment of APP+ loaded dopaminergic axonal structures with KCl leads to a small but significant degree of destaining. (A) Dorsal striatal slices were loaded with APP+ (100 nM) and treated with a KCl (40 mM) to induce depolarization and exocytosis. Images were collected every 60 seconds. APP+
destaining was greatest at 8 min after application of KCl. Release was statistically significant after 1 min of KCl treatment compared to a control imaged without KCl (t-test, p < 0.05 from t = 1-3 min, p < 0.01 from t = 4-8 min). (B) Representative image of APP+ signal at t = 0 min. (C) Representative image of APP+ signal at t = 8 min. Scale bars = 5 μm. Images are from collaboration with Matt Dunn.

Local electrical stimulation (application of electrical current) via a bipolar electrode induces neuronal depolarization and exocytosis, enables the control over the frequency and number of pulses applied to the brain region of interest, and is a less drastic means of inducing exocytotic release of vesicularized neurotransmitter or false neurotransmitter. Electrical stimulation leads to far less slice deformation and movement in comparison to KCl perfusion, and thus allows for measuring release kinetics of individual puncta. To study the effects of electrical stimulation on APP+ in the dorsal striatum, we employed a method used routinely within our laboratories for determining kinetics of electrically-stimulated FFN release, which involved imaging z-stacks at 15 second intervals. Under these conditions, photobleaching was a concern. It became apparent that photobleaching would obscure exocytotic destaining of puncta, as 73 ± 25% of APP+ signal was lost after application of the imaging sequence (controlled to slices imaged only at the start and end of the experiment; mean ± SD). We thus sampled a greater number of z sections throughout the experiment while exciting each area fewer times (once per minute), which effectively eliminated APP+ photobleaching and allowed us to track puncta over 6 min of continuous electrical stimulation. This duration of 10 Hz stimulation was sufficient to observe the destaining of FFN102 from DA presynaptic terminals, or the destaining of endocytic dye FM1-43 from excitatory inputs in the
striatum. Under these conditions, there was no significant decrease in the number of APP+ puncta (ANOVA, p > 0.05, n = 3) (Figure 13A). Relative to the number of puncta present at t = 0, 94.6 ± 11.8% of puncta were present after 6 minutes in control unstimulated slices, and 86.7 ± 9.7% of puncta remained after 6 min of 10 Hz electrical stimulation (mean ± SD, n=3). Similarly, there was no statistical difference in puncta intensity at the final time point (unstimulated control intensity after 6 min = 89.6 ± 9.2%; 10 Hz stimulation = 91.6 ± 3.1%; mean ± SD, n = 3, ANOVA, p > 0.05) (Fig. 13B). These data show that few if any APP+ puncta underwent complete destaining and that an ensemble of puncta (100’s per image) did not undergo significant destaining under conditions of local electrical stimulation. We thus conclude that the binding of APP+ to mitochondria and other cellular compartments within the DA synaptic terminals and axonal processes creates a non-releasable fluorescent background signal that overwhelms the releasable signal and thus limits the dynamic range of the APP+ destaining measurement.

These results indicate that only a small portion of the fluorescence signal of APP+ originates from the releasable synaptic vesicular pool, while the majority of the APP+ signal is derived from an intracellular mixture of labeled non-exocytotic compartments and cellular structures including mitochondria. Since APP+ is quenched in aqueous environments, these results from acute mouse brain slice preparations indicate that APP+ is not a sufficiently strong VMAT2 substrate to become entirely sequestered in synaptic vesicles. Our findings appear similar to those obtained with the antihypotensive agent amezinium (4-amino-6-methoxy-1-
phenyl-pyridazinium salt) reported by others.\textsuperscript{54} This pyridinium analog of similar size and shape to APP+ was taken up by noradrenergic terminals via NET in the rat occipital cortex (which receives noradrenergic inputs from the LC), although only ~1\% of the total \([^3\text{H}]-\text{amezinium}\) tissue content was released on electrical stimulation of the brain slice. These results indicate that only a small portion of amezinium is taken up by synaptic vesicles and thus releasable by exocytosis.

Our data indicate that APP+ is not a promising candidate for use in kinetic measurements of individual presynaptic terminals and thus is not a promising lead for development of catecholaminergic FFNs. However, the structural and behavioral similarities of APP+ to the mitochondrial potential probe 2-(4-(dimethylamino)styryl)-1-methylpyridiniumiodide DASPMI\textsuperscript{19} may facilitate the selective study of mitochondrial function of catecholamine neurons in brain tissue.
Figure 13: Effect of local 10 Hz electrical stimulation on the number and intensity of structures stained by APP+ over time in the dorsal stratum. Stimulation starts at the timepoint 0 and continues for 6 min, during which time no significant change in (A) relative number of puncta or (B) relative mean punctate intensity could be detected. 10 µm thick z-stacks were imaged at each time point to correct for changes in the z-plane that might be occurring over time. These stacks were imaged at 1 min intervals to minimize photobleaching of APP+. Included are representative images before (C) and after (D) 6 min application of 10 Hz electrical stimulation. Images are from collaboration with Matt Dunn.

Acute Toxicity Does Not Perturb DAT Activity Within 1 hour

To study whether acute toxicity has a significant effect on the ability of dopaminergic somata in the SN to load FFN102 after preloading with APP+. After a
30 minute loading of 500nM APP+ followed by a 15 minute wash (45 minute total treatment), APP+-labeled somata of the SN/VTA were capable of loading FFN102 under previously-reported conditions\(^{(40)}\) with imperceptible effects on FFN102 loading (Figure 14, n = 2 independent experiments). While APP+ is a likely neurotoxin similar to MPP+, this result suggests that acute toxicity is insufficient to perturb DAT activity. This also suggests that APP+ may have utility as a labeling control for catecholamine neurons, as the neurons are still competent to carry out basic functions in the presence of the APP+ label within at least an hour of initial treatment. This also suggests that toxicity is not directly responsible for the non-releasable APP+ labeling characteristics observed above.

**Figure 14:** Application of APP+ to SN/VTA regions of the midbrain has no perceptible effect on subsequent FFN102 labeling. SN/VTA slices were loaded with APP+ (500 nM, 30 min, washed for 15 min, and treated with FFN102 as described previously.\(^{(40)}\) No changes in the uptake of FFN102 were noted. This suggests that non-TH-GFP positive staining caused by this concentration of APP+ in the midbrain is not likely an effect of toxicity of APP+ in the time frame of the experiment. Scale bar = 20 µm. Images are from collaboration with Matt Dunn.
III: Conclusions and Outlook

In this study, we investigated the behavior of APP+, a small, fluorescent analog of MPP+, specifically for the purpose of examining pyridiniums as potential FFNs. Intrigued by the known substrate activity of APP+ at plasma membrane transporters DAT, NET, and SERT, (and later VMAT) we were curious to test APP+ staining characteristics in selected areas of the acute mouse brain tissue, with results from photophysical investigations and secondary-cell based systems providing rationale for the explanation of APP+ behavior in brain tissue.

As a DAT and NET substrate, APP+ labels catecholamine neuronal cell bodies with high selectivity in the relevant midbrain regions, namely SN/VTA and LC. Although the dendritic processes are largely obscured by the punctate staining that is DAT or NET-independent, the somata of catecholamine neurons can readily be identified. Thus, APP+ can be used as a marker of catecholamine neurons in the absence of additional fluorescent markers such as the GFP signal in TH-GFP mice. APP+ also enables examination of the DAT and NET function in these neurons in the native context of the living brain tissue. Because we lacked a reliable positive control for serotonergic innervation, we were unable to investigate serotonergic labeling, although reported substrate activity of APP+ at SERT suggests that it would behave similarly in regions of the brain innervated with serotonergic neurons. Since APP+ labels mitochondria inside the cells, it may therefore be envisioned that if the mitochondrial accumulation of the dye is dependent on the mitochondrial membrane potential, APP+ may serve as a catecholamine-neuron selective reporter of mitochondrial function.
We have also shown that APP+ serves as a marker of dopaminergic axonal processes and presynaptic terminals in the dorsal striatum (75-80% colocalization with GFP in TH-GFP mice and FFN102 in wild type mice) that is largely stable to exocytotic conditions. It was found that only a small degree of signal destaining occurs (<15%) when depolarization is induced by KCl or electrical stimulation. This can be attributed to low uptake of APP+ by synaptic vesicles and/or quenching of the fluorescence signal in the vesicular lumen relative to a bright, mitochondrial or protein-bound population. These results are consistent with results from secondary cells expressing VMAT2, and suggest that APP+ is not readily adaptable for quantitative imaging of exocytosis and neurotransmitter secretion under these conditions, and thus is not well suited for the development of new FFNs.
IV: Experimental

A. Synthesis of 4-(4-dimethylamino)phenyl-1-methylpyridinium iodide and 4-(4-hydroxyphenyl)-1-methylpyridinium iodide

General:

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich and were used without further purification. When necessary, solvents were dried by passing them through a column of alumina under argon. Flash chromatography was performed on SILICYCLE silica gel (230-400 mesh). Nuclear Magnetic Resonance spectra were recorded at 300K on Bruker 300 or 400 MHz Fourier transform NMR spectrometers. Proton chemical shifts are expressed in parts per million (ppm, δ scale) and referenced to residual protium in the NMR solvent (CDCl₃, δ 7.26; CD₃OD, δ 3.30). Data for ¹H NMR are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, bs = broad singlet), and coupling constant in Hertz (Hz). Carbon chemical shifts are expressed in parts per million (ppm, δ scale) and are referenced to the carbon resonance of the NMR solvent (CDCl₃, δ 77.0; CD₃OD, δ 49.0). Mass spectra were recorded on a JOEL LCmate (ionization mode: APCI⁺).

Preparation of APP+ Iodide:

The structure and synthesis of APP+ iodide has previously been reported,¹³⁴, ⁴¹, ⁴² and all data including ¹H NMR data for intermediate 1 and APP+ Iodide are consistent with previous reports (see below).
**Intermediate 1**

To a mixture of 4-bromopyridine HCl (0.21 g, 1.03 mmol), 4-(dimethylamino)phenylboronic acid pinacol ester (169.9 mg, 1.09 mmol), and cesium carbonate (0.99 g, 3.04 mmol) in tetrahydrofuran (THF, 0.5 mL) and dimethylformamide (DMF, 1.5 mL) was added palladium (II) acetate (0.014 g, 5 mol%) and tricyclohexylphosphine tetrafluoroborate (0.046 g, 10 mol%). The reaction mixture was refluxed overnight, after which time the reaction mixture was filtered through a celite plug and concentrated. The crude product was purified by flash chromatography (silica, CH₂Cl₂:methanol = 99:1 → 95:5) to give compound 1 as a tan solid (31%). ¹H NMR (400 MHz, Chloroform-d): δ 8.56 (d, J = 4.9 Hz, 2H), 7.61 – 7.55 (m, 2H), 7.46 (dd, J = 4.7, 1.5 Hz, 2H), 6.82 – 6.76 (m, 2H), 3.02 (s, 6H).

**APP+ iodide**

To a solution of compound 1 (0.016 g, 0.081 mmol) in acetonitrile (1 mL) was added iodomethane (0.04 mL, 0.123 mmol) and the resulting mixture was refluxed for 4 h. After this time, the solvent was evaporated and the crude product was dried under high vacuum prior to purification by flash chromatography (silica, CH₂Cl₂:methanol = 95:5 → 90:10). The fractions were collected, evaporated, dissolved in deionized water and lyophilized to give APP iodide as a bright orange powder (70%). ¹H NMR (400 MHz,
Methanol-$d_4$ $\delta$ 8.55 (d, $J$ = 7.1 Hz, 2H), 8.17 (d, $J$ = 7.2 Hz, 2H), 7.94 (d, $J$ = 9.2 Hz, 2H), 6.90 (d, $J$ = 9.2 Hz, 2H), 4.24 (s, 3H), 3.12 (s, 6H). LRMS (APCI+, M – CH$_3$): Calc’d for C$_{13}$H$_{14}$N$_2$ 198.26 m/z, measured 199.21 (M$^+$)

B. Photophysical Characterization of RJK150 and APP Iodide

General
Ultraviolet absorption spectra were measured on a BioTek H1MF plate reader/spectrophotometer operated through Gen5 Data Collection and Analysis software. Fluorescence measurements (emission/excitation) were carried out on a Jobin Yvon Fluorolog 3 fluorescence spectrofluorometer.

Absorption
UV absorption spectra were taken by adding APP+ (2µL of 10 mM stock solution in DMSO) to 998µL of PBS buffer, DMSO, ethanol, or CHCl$_3$ (final probe concentration = 20 µM) in a quartz cuvette.

Emission/Excitation
Fluorescence Emission/Excitation spectra were taken by adding APP+ (2µL of 1 mM stock solution in DMSO) to 998µL of PBS buffer or CHCl$_3$ (final probe concentration = 2 µM) in a quartz cuvette.
C. $^1$H NMR Spectra
D. Cell Culture Studies

**Epifluorescence Microscopy in hDAT-EM4 and EM4 cells.** An EM4 cell line stably expressing hDAT (hDAT-EM4) and an empty vector-transfected EM4 cell line to serve as a control were kindly provided by Drs. Jonathan Javitch and Mark Sonders of the Department of Psychiatry at Columbia University Medical Center. Cells were grown in DMEM + Glutamax (Invitrogen) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). For fluorescence microscopy experiments, cells were plated on poly-D-lysine (Sigma-Aldrich, 0.1 mg/mL) coated six-well plates (Falcon) at a density of 100,000 cells/well and were incubated until confluence (4 days at 37°C in a humidified atmosphere containing 5% CO₂). The medium was then removed by aspiration and wells were carefully washed with PBS (2 mL/well). Cells were then treated with 900µL of experimental medium (DMEM minus phenol red containing 25 mM HEPES (Invitrogen) with 1% FBS (Atlanta Biologicals)) for 3h. To investigate intracellular localization of APP+, solutions of APP+ (20 µM), Mitotracker Deep Red (200 nM) (Invitrogen), or a mixture of both in 100 µL experimental medium (all prepared from stock solutions in DMSO) were added to wells for final concentrations of 2 µM APP+ and 20 nM mitotracker in mL of experimental medium. After incubating at 37°C for 10 minutes, images were taken using a Leica DMI 4000B inverted epifluorescence microscope equipped with a Leica DFC 360 FX digital camera controlled through Leica LAS AF 6000E software. Bright field and fluorescence images were acquired sequentially (BF acquisition
time = 37 ms). Fluorescence images were acquired using filter cubes (APP+, ex = 440 ± 25 nm, em = 550 ± 25 nm, 500 ms acquisition time; Mitotracker Deep Red, ex = 580 ± 20 nm, em = 660 ± 25 nm, 500 msec acquisition time). Using these filters, no crosstalk was observed between fluorophores. All images of each fluorophore were adjusted to the same brightness and contrast level, respectively, using ImageJ (National Institutes of Health).

**Epifluorescence Microscopy in VMAT2-HEK and TetR-HEK cells.**

A HEK cell line stably expressing rVMAT2 (VMAT2-HEK) and an empty vector-transfected HEK cell line to serve as a control (TetR-HEK) were kindly provided by Professor Robert Edwards of the Department of Neurology at the University of California San Francisco (UCSF). Cells were grown in DMEM + Glutamax (Invitrogen) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen). For fluorescence microscopy experiments, cells were plated on poly-D-lysine (Sigma-Aldrich, 0.1 mg/mL) coated six-well plates (Falcon) at a density of 100,000 cells/well and were incubated until confluence (4 days at 37°C in a humidified atmosphere containing 5% CO₂). The medium was then removed by aspiration and wells were carefully washed with PBS (2 mL/well). Cells were then treated with 900µL of experimental medium (DMEM minus phenol red containing 25 mM HEPES (Invitrogen) with 1% FBS (Atlanta Biologicals)) for 1-3h. Cells were then treated with 2 µM APP+ for 50 minutes followed by addition of Mitotracker Deep Red for a final concentration of 50nM Mitotracker Deep Red. Cells were incubated for an
additional ten minutes before images were acquired in presence of both dyes. Brightfield and fluorescence images were acquired as described above, with sequential exposures using filter sets optimized for each fluorophore (as described above for APP+ and Mitotracker Deep Red). Controls indicated that crosstalk between fluorophores in the different filter sets was negligible.

**Procedures for Examination of APP+ in Acute Mouse Brain Slice**

These protocols and all imaging controls in the brain can be found in our report detailing the evaluation of APP+ behavior as a potential FFN{(1)} and will be included in the thesis of Matthew R. Dunn (graduate student, Sames Lab).
V: References


Chapter V

Fluorescent Substrates for Plasma Membrane Transporters of the Uptake2 System
I: Introduction

The Uptake2 System

Regulation of the synaptic and extrasynaptic concentrations of monoamine neurotransmitters is essential to proper function of the central nervous system, and perturbations of this homeostasis have been implicated in a number of neuropsychiatric disorders. While the transporters principally responsible for clearing the extracellular milieu of monoamine neurotransmitters are the Na\(^+\) and Cl\(^-\) dependent, high affinity members of the SCL6A family of monoamine transporters (MATs: DAT/NET/SERT, collectively referred to here as the Uptake1 system), there also exists a secondary system of low affinity, high capacity transporters known as the Uptake2 system. These transporters include the Plasma Membrane Monoamine Transporter (PMAT) and the Organic Cation Transporter 3 (OCT3), both of which are expressed widely in the brain.(1, 2) These transporters have garnered interest recently due to their ability to translocate monoamines. A detailed study comparing the substrate scope of hOCT3 and hPMAT was recently reported by the Wang laboratory at the University of Washington, and the results are reproduced in Table 1 (hOCT3) and Table 2 (hPMAT) below.

**Table 1: hOCT3 Substrate Efficiencies**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{max}) (pmol/mg protein/min)</th>
<th>(V_{max}/K_m) ((\mu)l/mg protein/min)</th>
<th>(V_{max}/K_m) relative to MPP(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP(^+)</td>
<td>166±11</td>
<td>15,664 ± 821</td>
<td>94.7 ± 1.1</td>
<td>100%</td>
</tr>
<tr>
<td>TEA</td>
<td>921±161</td>
<td>2,760 ± 92</td>
<td>3.03 ± 0.43</td>
<td>3.2%</td>
</tr>
<tr>
<td>Dopamine</td>
<td>1033±127</td>
<td>22,676 ± 484</td>
<td>22.1 ± 3.2</td>
<td>23%</td>
</tr>
<tr>
<td>Serotonin</td>
<td>988±264</td>
<td>11,562 ± 3,109</td>
<td>11.7 ± 0.2</td>
<td>12%</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>923±172</td>
<td>30,134 ± 2,674</td>
<td>32.9 ± 1.3</td>
<td>35%</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>458±37</td>
<td>12,760 ± 605</td>
<td>28.0 ± 3.6</td>
<td>30%</td>
</tr>
<tr>
<td>Histamine</td>
<td>641±24</td>
<td>34,604 ± 47</td>
<td>54.0 ± 2.1</td>
<td>57%</td>
</tr>
</tbody>
</table>

The Michaelis-Menten constants-(\(K_m\)), maximum uptake rates (\(V_{max}\)), and specificity constants (\(V_{max}/K_m\)) of different substrates for hOCT3, as well as
the percentage of substrate efficiency compared to MPP+. (Reproduced from Duan and Wang 2010) [3]

Table 2: hPMAT Substrate Efficiencies

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/mg protein/min)</th>
<th>$V_{max}/K_m$ (µl/mg protein/min)</th>
<th>$V_{max}/K_m$ relative to MPP+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP+</td>
<td>111 ± 3</td>
<td>6,557 ± 942</td>
<td>59.2 ± 10.0</td>
<td>100%</td>
</tr>
<tr>
<td>TEA</td>
<td>8,759 ± 3,175</td>
<td>15,246 ± 3,023</td>
<td>1.79 ± 0.30</td>
<td>3.0%</td>
</tr>
<tr>
<td>Dopamine</td>
<td>406 ± 4</td>
<td>22,402 ± 3,166</td>
<td>55.1 ± 1.3</td>
<td>93%</td>
</tr>
<tr>
<td>Serotonin</td>
<td>283 ± 40</td>
<td>14,194 ± 2,381</td>
<td>50.1 ± 1.4</td>
<td>85%</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1,078 ± 107</td>
<td>8,822 ± 1,323</td>
<td>8.16 ± 0.41</td>
<td>14%</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>951 ± 59</td>
<td>7,252 ± 195</td>
<td>7.65 ± 0.68</td>
<td>13%</td>
</tr>
<tr>
<td>Histamine</td>
<td>4,379 ± 679</td>
<td>42,374 ± 4,098</td>
<td>9.72 ± 0.57</td>
<td>16%</td>
</tr>
</tbody>
</table>

The Michaelis-Menten constants ($K_m$), maximum uptake rates ($V_{max}$), and specificity constants ($V_{max}/K_m$) of different substrates for hPMAT, as well as the percentage of substrate efficiency compared to MPP+. (Reproduced from Duan and Wang 2010). [3]

**Uptake2 System and its Relevance in CNS Disease**

There is emerging evidence implicating low affinity, high capacity transporters of the Uptake2 system as potential contributors to monoamine clearance from the extracellular space under certain circumstances. [3-6]. For example, it has been found that expression of OCT3 is upregulated when SERT is knocked out, [7] and that this upregulation has a compensatory effect on 5HT clearance from SERT deficient mice. [4] It has thus been suggested that OCT3 can act as a compensatory uptake mechanism for 5HT when SERT activity is reduced. [8] Additionally, mice lacking OCT3 have less anxiety than wild type littermates. [9] Interestingly, PMAT has been shown to transport DA and 5HT more efficiently than OCT3, while OCT3 transports norepinephrine more efficiently. [3] In fact, studies using a brain synaptosome uptake assay and in vitro hybrid depletion analysis have estimated that PMAT may contribute up to 20-30% of total brain 5HT uptake under
normal conditions.\textsuperscript{3, 10} Importantly, both PMAT and OCT3 are not inhibited by currently prescribed antidepressants such as the selective serotonin reuptake inhibitors (SSRIs).\textsuperscript{10} Thus, it is possible that OCT and PMAT may be pharmacologically relevant in a number of neuropsychiatric disorders; potent and selective inhibitors of these transporters may constitute an augmentation therapy to SSRIs in cases of drug inefficacy.\textsuperscript{6}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{neurotransmitter_transporters.png}
\caption{A possible example of the relationship between high-affinity reuptake transporters (MATs, Uptake1 system) and low-affinity transporters (Uptake2 system), shown in the hippocampus. 5HT (black dots) is released from serotonergic neurons and modulates the activity of fast synapses. It has been shown that transporters of the Uptake2 system (OCT3 in red, PMAT in blue) can contribute to the clearance of monoamines escaped from the synapse, and may compensate for diminished Uptake1 activity (SERT, depicted in green).}
\end{figure}

**Uptake2 System and the FFN Program**

Study of FFN probe activity at Uptake2 transporters is important for three reasons. First, it is possible that OCT3 or PMAT may contribute to FFN loading into
cells in a manner not entirely dependent on VMAT2 or DAT/NET/SERT. This information is important when interpreting FFN results from the brain. Second, a FFN or FFN-like Uptake2 substrate could prove useful in the study of OCT3 and/or PMAT activity and distribution in brain tissue. Given the potential involvement of these transporters in disease or drug treatment inefficacy as discussed above, a selective OCT3 or PMAT FFN (or fluorescent FFN-like substrate) could be advantageous to the study of these transporters as alternative means to clear monoamines from the extracellular milieu or to load VMAT2-expressing cells with neurotransmitters. Third, a well-characterized fluorescent substrate of the Uptake2 transporters could serve as a tool for the pharmacological study of OCT3 or PMAT as a fluorescence-based alternative to radiolabeled substrates. As part of our broad FFN program, we therefore became interested in identifying fluorescent substrates for OCT3 and PMAT.

A fluorescent analog of MPP+, 4-(4-(dimethylamino)phenyl)-1-methylpyridinium (ASP+), has previously been identified as substrate of Uptake2, and has been widely used to study OCT3 expression and pharmacology.\(^{11-13}\) ASP+ was also used as the first fluorescent substrate for study of high affinity plasma membrane monoamine neurotransmitter transporters DAT, NET, and SERT (see Chapters 3 and 4).\(^ {14} \) More recently, a smaller pyridinium analog, APP+, was developed to overcome a number of drawbacks inherent to ASP+; it is a superior SERT substrate by comparison to ASP+, and it displays less background uptake in cell culture.\(^ {15,16} \) Although APP+ has been suggested to be a substrate for Uptake2 system,\(^ {15} \) characterization of its substrate activity at these transporters has yet to
be reported. As part of a broad program aimed at imaging neurotransmission with fluorescent substrates of solute carriers in the brain, we became interested in the scope of the utility of this compound.\(^{17}\) Since APP+ is a small fluorescent analog of the Uptake2 substrate MPP+, we were especially curious to test the substrate activity of APP+ in cultured cells stably expressing hOCT3 or hPMAT.

Because fluorescent pyridiniums are not acceptable FFNs due to background labeling of organelles and poor fluorescence in aqueous media,\(^{17}\) we were also interested to test FFNs as possible substrates of Uptake2 transporters. In this chapter, I report the results of systematic screening and advanced studies of FFNs, FFN-related probes, and APP+ and related pyridiniums in HEK cells transfected with hOCT3 (hOCT3-HEK) or hPMAT (hPMAT-HEK). I also comment on the implication of these results with respect to interpretation of FFN results in the brain, the possibility of adapting FFNs for the study of these transporters, and the use of fluorescent substrates as tools for fundamental pharmacological studies of the Uptake2 system.
II: Results and Discussion

Screening of FFNs and Related Probes as Possible Substrates for Uptake2

A number of FFNs and FFN-like fluorescent probes were screened for substrate activity at hOCT3 and hPMAT by using isogenic Flp-HEK cells developed by the Wang lab\(^3\). A number of FFNs and FFN-like fluorescent compounds were screened for hOCT3 and hPMAT-dependent accumulation in transfected HEK cells (Flp-HEK transfected with hOCT3, hPMAT, or a pcDNA empty vector). The screened compounds were primarily produced within the Sames Lab as potential FFNs or related fluorescent probes, and were selected for this study based on a variety of factors: low lipophilicity, acceptable photostability (as determined by previous studies), some were known Uptake1 or VMAT2 substrates (previous studies),\(^{18-20}\) some were known specifically not to be Uptake1 or VMAT2 substrates, and others were included based on similarities to known Uptake2 substrates.

The screen uncovered a variety of different behaviors of the various compounds, and representative results are shown (Figure 2, Figure 3). The results of this screen have been compiled qualitatively and are indicated in Table 3 (7-hydroxycoumarins), Table 4 (7-aminocoumarins), and Table 5 (pyridiniums and other motifs). Each compound was screened at the same concentration and over the same time period in hOCT3-HEK, hPMAT-HEK, and pcDNA-HEK (serving as a control for passive diffusion/background uptake), and substrate activity was initially determined by comparing transporter-dependent accumulation of the compound with background staining on an epifluorescence microscope with excitation and
emission wavelengths filtered appropriately for each fluorophore (see Figure 3 for representative images).

Figure 2. Representative images of some FFNs and FFN-related probes in cells transfected with pcDNA (empty vector control, left column), hOCT3 (center column), or hPMAT (right column). A) Representative images of transporter-independent uptake, in this case NG54. B) AGH093 is a substrate for hOCT3 and for hPMAT. C) FFN202 is a substrate for OCT3 and not appreciably a substrate for PMAT, likely due to the increased steric bulk of the 6-position relative to AGH093. All treatments were 20µM for 1h. AGH093 and FFN202 images are representative images of 3 independent experiments, at least 3 images per well.
Figure 3: A) Epiflourecence image of FFN202 in hOCT3-HEK cells, B) brightfield image of the same frame, and C) overlay of brightfield and fluorescence images (20µM, 1h treatment). The compound is excluded from the junction of the cells, visible in clusters. Images are representative of 5 independent experiments of FFN202 uptake in hOCT3 HEK.

For compounds identified as possible substrates, substrate activity was confirmed by pharmacological inhibition at each transporter with respective inhibitors, namely corticosterone for hOCT3 and decynium-22 (D22) for hPMAT. Of these select candidates, qualitative comparisons between each were made directly within the same experiment on the same day to compare relative brightness, from which the qualitative results were based. Figure 4 and Figure 5 are representative inhibition studies conducted on each potential substrate).
Table 3: 7-Hydroxycoumarins screened for Uptake2 Substrate Activity\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>hOCT3</th>
<th>hPMAT</th>
<th>Name</th>
<th>Structure</th>
<th>hOCT3</th>
<th>hPMAT</th>
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<td>*</td>
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\textsuperscript{a}Qualitative comparisons of probe accumulation as a function of Uptake2 transporter expression. X = no observable transporter-dependent uptake, * = minimal detectable increase in fluorescence compared to background uptake in control cells, ** = clear increase in fluorescence, *** = bright probe accumulation cleanly inhibited by pharmacological agents: a definite substrate. \textsuperscript{b}All 7-
hydroxycoumains with any substrate activity at hOCT3 or hPMAT displayed a
general cytosolic staining pattern as indicated by representative images in Figure 2.
AGH093 is cleanly inhibited by D22 at PMAT but is not as consistently bright as
in hOCT3-HEK cells.

Table 4: 7-Aminocoumarins Screened for Uptake2 Substrate Activitya

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>hOCT3</th>
<th>hPMAT</th>
<th>Name</th>
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<td>G180</td>
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</table>

aNo transporter-dependent uptake was observed with any of the 7-aminocoumarins tested. X = no observable transporter-dependent uptake.
Table 5: Pyridiniums and Other Fluorescent Probes Screened for Uptake2 Substrate Activity

<table>
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<th>Name</th>
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<th>hPMAT</th>
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<tr>
<td>NG54</td>
<td><img src="image" alt="NG54 Structure" /></td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

*Qualitative comparisons of probe accumulation as a function of Uptake2 transporter expression. X = no observable transporter-dependent uptake, * = minimal detectable increase in fluorescence compared to background uptake in control cells, ** = clear increase in fluorescence, *** = bright probe accumulation cleanly inhibited by pharmacological agents: a definite substrate. APP+ appears to be accumulated to a very small degree in hPMAT-HEK cells compared to control cells, although the amount is inconsequential compared to the bright staining afforded in the presence of hOCT3 (see Figure 6 for representative images).

The goals of this screen were threefold. First, I was interested in exploring the general substrate scope of hOCT3 and hPMAT with respect to sterically-demanding fluorescent compounds. Second, as many of the screened compounds are in use in advanced systems in the hands of collaborators (e.g., acute brain slice preparations), I was curious to test for any potential contributions of Uptake2 to FFN behavior in these systems, or possibly to discover a Uptake2 system selective probe (compared to Uptake1 or VMAT). Third, I was curious to see if any potentially good substrates could be developed into a screening agent for OCT3 or PMAT activity and inhibition.
There are a number of important negative results to highlight. None of the 7-aminocoumarins, many of which are FFNs employed in advanced systems, appeared to act as substrates for Uptake2 transporters. These negative results suggest that labeling results in advanced systems are not likely skewed by Uptake2 transport activity. Additionally, NG54 is a substrate for DAT, NET, and SERT (Chapter 3) and VMAT2 (Chapter 2), but shows no OCT3 or PMAT dependent uptake. Correspondingly, NG54 displays a high degree of colocalization with TH-GFP in the mouse dorsal striatum (Chapter 3), indicating clean labeling of dopaminergic neurons.

**Figure 4:** FFN202 is inhibited by corticosterone at hOCT3. Top Panel – 60 minute treatment of Flp-HEK cells with 20µM FFN202 shows that FFN202 is accumulated by hOCT3-HEK cells (center column) compared to hPMAT-HEK (right column) or pcDNA-HEK (left column). Bottom Panel – Treatment with 100µM corticosterone inhibits FFN202 uptake into hOCT3-HEK cells and has no effect on uptake of FFN202 into hPMAT-HEK or pcDNA-HEK cells.
Substrate activity at Uptake2 system transporters was observed with several of the 7-hydroxycoumarins, and confirmed with inhibition (Figures 4 and 5 are representative). From these results, we can draw some preliminary conclusions about the substrate tolerance of OCT3 and PMAT with respect to these fluorescent probes (Figure 6). It appears that hOCT3 is more tolerant of diverse ethylamine regiochemistry (both the 3- and 4-position afforded substrate activity, Table 3), the length of alkyl group linking the amine to the arene (a methylamine analog of AGH093, compound AGH113, is a weak substrate for OCT3 but not PMAT, Table 3), and steric bulk in the 6-position (FFN202 is a strong OCT3 substrate but not a PMAT substrate, while the smaller 6-fluoro substituted AGH093 is a substrate for both, Table 3).
Figure 6: Summary of tolerated 7-hydroxycoumarin substrates at hOCT3 and hPMAT. For each, the alkylamine moiety is only tolerated in the 3 or 4 positions, and additional steric bulk on the arene is only allowed at the 6-position.

Many of the 7-hydroxycoumarins are also used as advanced FFNs, as many including FFN102, FFN202, and AGH093 are well-characterized substrates for Uptake1 transporters DAT and NET (unpublished results). The fact that these compounds also display activity toward Uptake2 transporters highlights the fact that we must be aware of this secondary activity when analyzing and interpreting data from primary culture, brain preparations, or in vivo systems. For example, it has been noticed that FFN202 and AGH093 label some cells in acute mouse brain tissue with morphology consistent with labeling of cell bodies such as astrocytes or oligodendrocytes (Figure 7). These results also suggest that with proper optimization and characterization in brain slice preparations, some of these probes may prove useful in studying Uptake2 expression and activity in conjunction with aminergic signaling.
**Figure 7:** Representative image of FFN202 staining cell bodies in the dorsal striatum of an acute mouse brain slice preparation. The bright oval structures are cell bodies of presumably astrocytes, and the relatively faint fine punctate staining are dopamine presynaptic terminals. The bright, elongated structures are blood vessels. Scale bar = 20 µm. This representative image was obtained by Matt Dunn (graduate student colleague) during studies of FFN202 uptake and release from dopaminergic presynaptic elements in this region of the brain, and highlights the importance of understanding secondary uptake mechanisms in brain tissue.

This screen also uncovered an interesting result concerning fluorescent pyridiniums, which may satisfy the third goal of this screening program. APP+ (discussed in detail in Chapter 4) was found to be an excellent substrate for hOCT3 at low concentrations and short treatment times (2µM, 10 min). Preliminary results suggested that APP+ might be useful as a fluorescent substrate to study transporter activity and inhibition; it was characterized more rigorously in additional experiments (see below).

**APP+ is an Excellent Substrate for hOCT3 but a Poor Substrate for hPMAT**

In the initial screen described above, it was found that incubation of isogenic Flp-HEK cells stably transfected with an empty vector (pcDNA-HEK), human PMAT (hPMAT-HEK), and human OCT-3 (hOCT3-HEK), with APP+ (2 µM, 10 min) uncovered an interesting phenomenon; APP+ is accumulated rapidly by action of hOCT3 but not hPMAT, with minimal relative background uptake in control pcDNA-
HEK cells (Figure 8). The resulting intracellular fluorescent label is consistent with reported behavior of APP+,(15,17) and the intracellular localization into mitochondria affords a more stable signal than that of the FFNs, which tend to leak or wash out through the plasma membrane over time (data not shown). Uptake of APP+ in OCT3-HEK cells can be inhibited by the OCT3 inhibitor corticosterone (100 μM, Figure 6D). These images suggest that APP+ is a substrate for OCT3 and is well suited for imaging applications, whereas it is at best a poor substrate for PMAT (Figure 8B). Substrate activity of APP+ at OCT3 is not surprising, considering the reported robust transport activity of OCT3 towards the structurally related ASP+ and MPP+. However, the relatively small uptake by PMAT towards APP+ is somewhat unexpected, as MPP+ is an excellent substrate for PMAT. These data would suggest that PMAT is a more sterically discriminating transporter than OCT3, at least within this pyridinium motif. On the basis of these results, we chose to pursue fluorescence-based studies of the behavior of APP+ at OCT3.

Figure 8: APP+ is an excellent fluorescent substrate for hOCT3 and a poor substrate for hPMAT. (A) pcDNA-transfected, (B) hPMAT-transfected, and (C) hOCT3-transfected HEK293 cells were treated with 2μM APP+ in experimental medium for 10 minutes before imaging. In this time, uptake into empty-vector transfected cells and hPMAT-transfected cells is negligible while uptake by hOCT3 is robust. (D) hOCT3-dependent uptake of APP+ over the same time period was confirmed by
inhibition with corticosterone (100 µm) which significantly reduces APP+ accumulation. Scale bar = 10 µm.

**Kinetics of APP+ Uptake by hOCT3**

Examination of initial rates of fluorescence accumulation of different concentrations of APP+ was employed to determine the apparent Km of APP+ at hOCT3, in collaboration with Dr. Adam Henke. Flp-in HEK cells stably expressing hOCT3 were grown on a 96-well microplate and treated with concentrations of APP+ ranging from 5 µM to 150 µM, and fluorescence accumulation was monitored for 4.5 minutes (see Experimental Section IV). The apparent Michaelis constant (Km) of APP+ at OCT3 was determined to be 26.0 ± 4.6 µM (mean of 3 independent experiments ± SEM; Figure 9A). This result is consistent with reported values for ASP+ at OCT2 (~24-42 µM), and surprisingly compares to the reported apparent Km of APP+ at DAT (30 µM). The hill coefficient was calculated to be ~1.2, suggesting no cooperativity in transport.
Figure 9: Quantitative analysis of APP+ as a substrate for hOCT3. A) Apparent $K_m$ of APP+ at hOCT3 was determined by initial rates experiments to be $26.0 \pm 4.6 \mu M$ (mean ± SEM). Shown is the average of three independent initial rates experiments. Error bars represent SEM. B) Dose response curves of some select OCT3 inhibitors (mean of two independent experiments, two identical plates each, error bars = SEM). APP+ uptake is normalized to controls: uninhibited uptake = 100%, 2mM MPP+ cotreatment = 0%. EC₅₀ values for corticosterone, phenoxybenzamine, and desipramine were determined to be $19.6 \pm 3.9 \mu M$, $9.0 \pm 1.4 \mu M$, and $40.8 \pm 4.2 \mu M$, respectively (means ± SEM, n = 2). $K_i$ values were calculated using the Cheng-Prusoff equation, and were determined to be $16.5 \mu M$ for corticosterone, $14.4 \mu M$ for phenoxybenzamine, and $34.2 \mu M$ for desipramine. C) Structures of the fluorescent hOCT3 substrate APP+ and OCT3 inhibitors corticosterone, phenoxybenzamine, and desipramine.

**Inhibition of APP+ cell uptake by reported OCT3 inhibitors**

In order to determine the applicability of APP+ as a screening agent or research tool to study inhibitor interaction with hOCT3, we set out to confirm the $K_i$ values of some known inhibitors of hOCT3 using the APP+ cell assay. Although OCT3 is a low affinity transporter for monoamines, the $K_m$ of APP+ for OCT3 was
determined to be moderately low (~25 µM), and we found that 5 µM is a sufficient concentration of APP+ to furnish reproducible results from inhibition experiments. Compounds tested for potency of APP+ inhibition include corticosterone, phenoxybenzamine, and desipramine (Figure 9B). These compounds were selected as their inhibition potencies have been compared directly in the same study using hOCT3, and they are structurally distinct from one another. The Cheng-Prusoff equation was used to convert measured IC₅₀ values to Kᵢ values of the inhibitors. I was able to measure IC₅₀ values for corticosterone, phenoxybenzamine, and desipramine, which were determined to be 19.6 ± 3.9 µM, 9.0 ± 1.4 µM, and 40.8 ± 4.2 µM, respectively (means ± SEM, n = 2). Kᵢ values were calculated using the Cheng-Prusoff equation, and were determined to be 16.5 ± 3.3 µM for corticosterone, 7.6 ± 1.2 µM for phenoxybenzamine, and 34.2 ± 3.5 µM for desipramine.

Our measured inhibition potencies of compounds known to interact with OCT3 compare variably with those previously reported using other substrates including [³H]-MPP+.²¹ For example, while the Kᵢ of phenoxybenzamine measured here (7.6 ± 1.2 µM) is essentially the same as measured by scintillation counting of [³H]-MPP+ (6.13 ± 2.04 µM),²¹ the Kᵢ of corticosterone measured with APP+ was found to be considerably higher (16.5 ± 3.3 µM) than that found by [³H]-MPP+ (0.29 ± 0.04 µM).²¹ The considerably higher value for corticosterone is consistent with inhibition potencies measured using the related pyridinium, ASP+,¹¹ and may suggest a unique substrate-specific inhibition profile, which is a well-known phenomenon in low affinity transporters with large or multiple substrate and/or
inhibitor binding sites. The shifted $K_i$ for corticosterone as measured by APP+ compared to that measured with radiosubstrate assays using $[^3H] \cdot MPP^+$ is somewhat problematic, and demonstrates that APP+ may miss or misrepresent activities or inhibition potencies of experimental inhibitors in a high-throughput (HT) setting.

**Applicability of APP+ for High Throughput Screening**

The $Z'$-Factor is an important parameter used to describe the applicability of a particular assay for HT applications. Using the standard method for determining this value,$^{(22)}$ we were unable to reliably achieve a $Z'$-factor for an APP+-based OCT3 inhibition assay greater than $Z' = 0.5$ (5 μM APP+, 100 μM corticosterone positive control, 15 min incubation). Our results indicated a positive $Z'$-factor but were highly variable ($Z' \sim 0.3$), suggesting that this compound could be used in a +/- assay as currently optimized, but further optimization would be required for rigorous and conservative high throughput screening. One additional drawback is that substrate-specific inhibition of APP+ may contribute to missed or misrepresented hits in a HT campaign, as inhibition of APP+ may not relate particularly closely to inhibition of endogenous substrates.

**Potential Utility of APP+ at the Uptake2 System**

Based on these results above, it is possible that APP+ can be used as an alternative to radiolabeled substrates in the study of OCT3, although there are some concerns as mentioned above. Since APP+ is already marketed by Molecular
Devices in the Neurotransmitter Transporter Uptake Assay Kit developed for use in HT applications for DAT, NET, and SERT\(^{23}\) there is significant precedent for the use of this motif as a fluorescent substrate of plasma membrane transporters as a screening agent for inhibition assays\(^{16,24}\) ASP+ has been used previously in the pharmacological study of OCT3\(^{11}\) although APP+ is reported to overcome limitations of ASP+, including background uptake\(^{15,16}\) suggesting that APP+ may be more suitable for the study of OCT3 as well. Indeed, when the same concentrations of ASP+ and APP+ are compared in HEK293 cells, ASP+ background uptake is markedly greater, presumably due to a higher rate of passive diffusion as ASP+ is a more lipophilic pyridinium\(^{15}\) As such, the use of APP+ may lead to a more sensitive HT assay than one employing ASP+. Potent and selective inhibitors identified from such an assay could serve as an augmentation therapy to SSRI treatment in cases of treatment resistant depression, or after SSRI inefficacy manifests from long-term treatment. Although a number of drugs have already been shown to interact with OCT3 and PMAT, there is a debate over the relevance of these molecules in eliciting the phenotypic response;\(^{11,25}\) a more selective and potent inhibitor would be needed for further study.

As we have shown in brain tissue, APP+ labels catecholaminergic axons and cell bodies (Chapter 4)\(^{17}\) At concentrations of APP+ required to label cell bodies, there is additional staining of non-catecholaminergic structures, a likely consequence of APP+ uptake by OCT3. As APP+ can be used as an imaging agent in brain tissue, it may prove a useful research tool when investigating areas innervated with OCT3-expressing neurons or glia with corresponding sparse monoaminergic
innervations. APP+ may also facilitate fluorescence microscopy studies of OCT3 activity and upregulation in specific areas of the brain of Uptake1 system (MAT) knockout animals.
III: Conclusions and Outlook

A number of FFNs and FFN related fluorescent probes, including all advanced FFNs used in brain tissue studies, have been screened for substrate activity at the Uptake2 transporters hOCT3 and hPMAT using epifluorescence microscopy. These transporters have been suggested to contribute significantly to monoamine clearance from the extracellular space, and may prove to be viable pharmacological targets. As such, fluorescent probes may be useful for study of Uptake2 system activity in tissue or for identification and characterization of novel Uptake2 inhibitors. It is important to understand the behavior of FFNs at Uptake2 transporters for our interpretation and analysis of FFN results in complex settings of primary tissue culture, brain slice preparations, or in vivo applications. Interesting trends have been noticed with FFN structure and substrate activity at hOCT3 and hPMAT, most notably that the regiochemistry of the ethylamine is important for transport, as well as the steric of substituents on positions 6 and 8, with substitution of position 8 with halogens being completely excluded. It can be noted, however, that AGH093 and FFN202 are the brightest FFNs in hOCT3-HEK, and AGH093 is brightest of all in hPMAT-HEK, each relative to background uptake in empty vector-transfected control cells. The above results will affect our interpretation of observed behavior of these compounds in more advanced systems.

We are beginning a collaboration with Professor Joanne Wang to study PMAT activity in the hippocampus with FFNs. Many of the compounds found here to have substrate activity at hOCT3 and/or hPMAT are known to be VMAT2 substrates as well, including all the hydroxycoumarins with Uptake2 activity. A lead for this study
will be the PMAT/VMAT substrate AGH093 that, in the presence of OCT3 inhibitors in the hippocampus, could be used as a tracer of SERT-independent 5HT uptake and release, as PMAT is suggested to be expressed on presynaptic serotonergic neurons and AGH093 is not a substrate for SERT.\textsuperscript{(6)}

Additionally, we have observed the interesting result that APP+ is a good substrate for hOCT3, although is a relatively poor substrate for hPMAT in HEK cells stably expressing these transporters. We have determined the apparent $K_m$ of APP+ at hOCT3, and I compare inhibition constants of known hOCT3 inhibitors obtained with APP+ with the reported values obtained using $^3$H-MPP+. With further optimization, APP+ may be applicable to high-throughput screening for OCT3 inhibitors, and it may prove useful as a fluorescent alternative to radiolabeled substrates in fundamental experiments on the pharmacology of OCT3.

APP+ is likely a better choice for optimization of HTS experiments than many of the FFNs, as APP+ is sequestered into mitochondria and chromatin where it fluoresces brightly and remains quite stable as an intracellular label. FFNs, on the other hand, exhibit general cytosolic labeling which is less stable, as the compound can leak from the cells more readily via passive diffusion across the plasma membrane or through reverse transport through OCT3 or PMAT. FFN206, for example, is an excellent HTS candidate for VMAT2 activity and provides a stable signal for at least an hour as it is sequestered into VMAT2-expressing organelles and cannot easily diffuse from the cell. While FFNs are not likely appropriate for HT applications for Uptake2 transporters, quantitative examination and characterization of FFNs as hOCT3 and hPMAT substrates is still an important future
endeavor, as they may represent useful imaging agents for Uptake2 expression and activity in the brain, as discussed above.
IV: Experimental

Cell culture

Isogenic Flp-in HEK cells stably transfected with either hOCT3, hPMAT, or an empty vector (pcDNA)(3) were maintained in Dulbecco’s Minimal Essential Medium (DMEM) with Glutamax (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Atlantis Biologicals), 150 µg/mL hygromycin-B (to maintain the transgene), 100 µg/mL Penicillin, and 100 U/mL Streptomycin (Invitrogen). Cells were routinely cultured in 10 cm polystyrene culture plates (Falcon), were subcultured before reaching confluence (every 3-4 days), and were maintained in a humidified atmosphere at 37 °C containing 5% CO₂.

Uptake of FFNs by hOCT3 and hPMAT

Flp-in HEK cells expressing either hOCT3, hPMAT, or an empty vector were subcultured into 6-well polystyrene plates (Falcon) coated with poly-D-lysine at a density of ~200,000 cells/well, or into 12-well plates with the same coating at a density of ~100,000 cells/well and were allowed to grow for 3 days in complete medium. Prior to the experiment, wells were aspirated and washed with 2 mL DPBS, and were subsequently refilled with 900 µL clear experimental medium (DMEM without phenol red, 25 mM HEPES, 1% FBS). Cells were incubated for 60 min at 37°C. For inhibition experiments, cells were preincubated for 60 min with corticosterone (100 µM) or D22 (50 µM) in experimental medium. Uptake was initiated by adding 100 µL of a 200 µM solution of FFN in clear medium (or clear medium + corticosterone 100 µM or D22 50 µM for inhibition studies) for a final
concentration of 20 µM probe in each well. Cells were incubated for 60 min, after which time experimental medium containing the probe was aspirated, wells were washed with 1 mL DPBS, and were refilled with 1 mL warm experimental medium. Images were taken using a Leica DMI4000B inverted epifluorescence microscope using a filter cube for corresponding to the excitation/emission wavelengths of the respective probe. Images of each probe were adjusted to the same levels of brightness and contrast using ImageJ (NIH).

**Imaging APP+ uptake in HEK cells**

APP+ iodide was prepared as described previously\(^{(17)}\) (Chapter 4 of this work). For microscopy studies of APP+ uptake, cells were subcultured into 6-well polystyrene plates (Falcon) coated with poly-D-lysine (Sigma-Aldrich), at a density of ~200,000 cells/well and were allowed to grow for 3 days to confluence. Prior to the experiment, wells were aspirated and washed with DPBS (2 mL), and were subsequently refilled with 900 µL experimental medium consisting of DMEM without phenol red, supplemented with HEPES (25 mM), glutamine (2 mM), 1% FBS, 100 µg/mL penicillin, and 100 U/mL streptomycin. Cells were incubated for 60 min. Uptake was initiated by adding 100 µL of a 20 µM solution of APP+ iodide in clear medium for a final concentration of 2 µM in each well. Fluorescence images were taken after 10 minutes of uptake in the presence of the probe (filter cube parameters: ex = 440 ± 25 nm, em = 550 ± 25 nm, 250 msec acquisition time). All microscopy was conducted on a Leica DMI 4000B inverted epifluorescence microscope connected to a Leica DFC FX 360 camera controlled through Leica LAS.
AF 6000E software. All images were adjusted to the same levels of brightness and contrast using ImageJ (NIH).

**Determination of Apparent K_m of APP+ at hOCT3**

hOCT3-HEK cells were plated onto white, clear bottom 96-well plates (Falcon) pre-coated with poly-D-lysine (Sigma Aldrich) at a density of ~50,000 cells/well and were incubated for 24 hours in complete growth medium. Before the experiment, complete medium was aspirated, wells were washed with 200 μL PBS, and treated with experimental medium (100 μL) or experimental medium containing the OCT3 inhibitor corticosterone (100 μM) for 30 minutes. 2x concentrated solutions of APP+ in experimental medium (100 μL) or experimental medium containing corticosterone were added to the plate for final concentrations indicated. Fluorescence accumulation was measured (λ_ex = 450 nm, λ_em = 516 nm) every 1.5 minutes for 4.5 minutes using a BioTek H1MF plate reader controlled through Gen5 software. Background uptake for each concentration was defined as uptake of APP+ in inhibited wells, and was subtracted from each corresponding uninhibited value. Rates of APP+ uptake were determined from the slope of the resulting linear uptake curves, and were plotted as a function of APP+ concentration. Data were analyzed using GraphPad Prism 5. The K_m value was calculated using the Michaelis-Menten nonlinear regression analysis. The Hill coefficient was determined using a sigmoidal dose response fit with variable slope constrained through 0.
Dose-Response Studies of Inhibitors using APP+ as a Fluorescent OCT3 Substrate

hOCT3-HEK cells were cultured into 96-well plates as described above. On the day of the experiment, complete medium was removed and replaced with 100 μL serum-free experimental medium containing vehicle control or experimental compounds at 2x the final concentrations. Cells were pre-incubated for 15 minutes before addition of 100 μL of serum-free experimental medium containing 10 μM APP+, for a final in-well concentration of 5 μM APP+ and a 1x concentration of experimental compounds as reported. Uptake was conducted for 20 minutes before medium was removed, wells were quickly washed with 200 μL PBS, and finally filled with PBS (120 μL, pH = 7.4). Fluorescence accumulation was measured using a BioTek H1MF plate reader at the same excitation and emission maxima as above. EC50 values from each experiment were determined using GraphPad Prism 5, and corresponding Ki values were calculated using the Cheng-Prusoff equation.

Determination of Z’-Factor of APP+ at OCT3

Cells were cultured into 96-well plates as above. On the day of the experiment, two 96-well plates were washed with PBS as above and filled with 90 μL serum-free experimental medium per well, containing either a DMSO vehicle control (0.11%) or corticosterone in DMSO (111 μM). Half of the plate (48 wells) was treated with the vehicle control while half was treated with corticosterone. The plates were preincubated for 15 minutes at 37°C before addition of APP+ (50 μM, 10 μL/well) for a final concentration of 5 μM APP+ in each well containing either
vehicle control or corticosterone (100 μM). Plates were incubated for 20 min at 37°C, after which time wells were washed with 200 μL PBS and finally filled with 120 μL PBS. Fluorescence accumulation was immediately measured on a plate reader as described above. Z'-factor was calculated from the means and standard deviations of each control data set as described previously. (22)
V: References


Part II

New Small-Molecule Inducers of Glial Cell Line-Derived Neurotrophic Factor (GDNF) from C6 Glioma Cells
Chapter VI

New Small-Molecule Inducers of GDNF from C6 Glioma Cells(1)
"In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything must die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.”

-Santiago Ramón y Cajal, 1928(2)

I: Introduction

Neuroplasticity and Chemical Neuroscience

When Ramón y Cajal famously elucidated the discrete nature of neurons in the brain, it was widely assumed that neural tissue was static, fundamentally incapable of changing after reaching maturity. It is now well understood that neuroplasticity, the ability of the brain to change its structure and signaling network organization in response to environmental conditions, is critically important for learning and behavior, as well as the development and treatment of disease.(3) Given the dynamic nature of the brain’s structure and organization, as well as the extreme complexity of its connections, it has not yet been possible to fully elucidate and characterize the phenotypes that underlie specific functions in healthy brain tissue. It is presently not possible to fully characterize the causal relationships between brain structure changes and disease.

Recently, the Sames Group has become interested in the development of small molecules capable of modulating brain function in order to compensate for genotypic or phenotypic deficiencies characteristic of disease. Our interest in imaging monoaminergic neurons, particularly dopaminergic neurons,(4,5) motivated us to consider novel pharmacological mechanisms to restore the health of dopaminergic neurons that have been damaged by neurodegenerative disease or
substance abuse, or to fundamentally alter the structure of dopaminergic neurons that are malformed in a number of other pathologies. It is well known that growth factors, including neurotrophic factors, are responsible for the development of neurons and the maintenance of their physiology and health, and have been demonstrated to have reparative and restorative properties in the case of neurodegenerative disease.(6) Unfortunately, these signaling proteins are not drug candidates, as they are unstable in the blood and do not readily cross the blood brain barrier (BBB).(7, 8) If a drug could be developed with the capability of inducing a mature brain to produce neurotrophic factors or to modulate or potentiate how the brain responds to the low levels of endogenously released and active growth factors, it could serve as a useful therapy for neurodegenerative disease. This chapter details some preliminary work toward this goal, describing experiments that served as a springboard for a program of RTK-modulating isoquinuclidines.

**Glial Derived Neurotrophic Factor (GDNF) and its Cognate Receptor, Ret**

During the early stages of development, growth factors including neurotrophins and neurotrophic factors are widely expressed in the brain, and are essential to the development and differentiation of neurons in the central nervous system (CNS).(9) Upon reaching maturity, this expression is significantly attenuated. Glial-Derived Neurotrophic Factor (GDNF) is one specific neurotrophic factor that has been shown to be critical for neural development,(10) particularly in the context of dopaminergic neurons.(11) GDNF has been shown to signal through the receptor tyrosine kinase Ret (rearranged during transfection, Figure 1).(12)
Figure 1: A representation of GDNF-induced Ret signaling. Ret (rearranged during transfection) is the receptor tyrosine kinase for which GDNF is a ligand. In a complex equilibrium not fully characterized, GDNF binds with a membrane-anchored co-receptor GFRα1, which recruits two subunits of Ret. The resulting close spatial orientation of the cytosolic catalytic domain of the Ret subunits causes an autocatalytic phosphorylation of the kinase domain and subsequent activation of the indicated signaling cascades. Adapted from Dr. Teresa L. Jacques.\textsuperscript{13}

Ret is a tyrosine kinase expressed by numerous cell types, including kidney epithelial cells and dopamine neurons.\textsuperscript{12, 14} Ret is activated by a number of GDNF family ligands (GFLs: GDNF, neurturin, artemin, and persephin) upon binding with cognate GFRα-family coreceptors (GFRα1, GFRα2, GFRα3, and GFRα4, respectively).\textsuperscript{12} GDNF has been implicated in addiction (specifically opioids, cocaine, and alcohol)\textsuperscript{15, 16} and neuropathic pain.\textsuperscript{12} GDNF has also been shown to protect the brain after ischemia (stroke)\textsuperscript{17, 18} and has been demonstrated to be effective in protecting nigrostriatal neurons in models of Parkinson's disease.\textsuperscript{19} It has also been found that GDNF improves cognition in aging mice when transgenically expressed in astrocytes.\textsuperscript{20} RET has been identified as a proto-
oncogene, as gain of function mutations leading to constitutive kinase activation have been shown to cause certain forms of cancer.\(^{21}\) Despite the classification of Ret as an oncogene, Ret/GDNF represents an interesting pharmacological target for recovery of dopamine neurons damaged (or plastically modulated) by drug and alcohol abuse,\(^{22, 23}\) CNS trauma,\(^{8, 9, 17}\) or neurodegeneration.\(^{19}\) Ret is also a potentially promising target for inducing plasticity in pathologies causally related to dopaminergic dysfunction.

**Ibogaine**

We became interested in GDNF through a collaborator, Professor Dorit Ron, (UCSF) who has found that the natural product Ibogaine (Figure 2) as well as the related noribogaine, mediates GDNF release in the midbrain of rats.\(^{24, 25}\) Professor Ron has demonstrated that the beneficial effects of ibogaine are related to its ability to induce production of GDNF, at which point autocrine or paracrine signaling with the released GDNF establishes a positive regulatory loop, leading to the long-term phenotypic changes associated with neuronal repair.\(^{24, 26}\)

Ibogaine is a naturally occurring alkaloid found in the west-African plant *Tabernanthe iboga* and has been used for centuries in traditional ceremonies. Ibogaine has complex pharmacology, interacting with the N-methyl-D-aspartate receptor (NMDAR), nicotinic acetylcholine receptors, adrenergic receptors, the \(\kappa\)-opioid receptor (KOR), serotonin receptors (specifically 5HT\(_2\) and 5HT\(_3\)) as well as the monoamine transporters and the enigmatic sigma receptors \(\sigma1R\) and \(\sigma2R.\(^{27, 28}\) It has been shown that ibogaine has anti-addictive effects in rigorous animal
studies\textsuperscript{,29} and anecdotal reports in humans. The complex pharmacological profile of ibogaine is responsible for hallucinatory effects as well as ataxia, ventricular tachycardia, and nausea\textsuperscript{,28,30} Ibogaine is currently a Schedule I drug in the US. As such, there would be benefit in finding a compound to act similarly, with respect to ibogaine’s beneficial effects on dopamine neurons, without such promiscuous pharmacology. Our group hypothesized that from a small library of ibogaine-related structures, specifically arylethyl-isoquinuclidines, it would be possible to identify compounds with similar GDNF-inducing effects.

**Generation and Initial Biological Characterization of a Library of N-arylethyl Isoquinuclidines**

A library of isoquinuclidine compounds related to ibogaine was synthesized by Dr. Xiaoguang Li. The goal was to examine the ability of each compound to induce expression and release of neurotrophic growth factors or otherwise induce similarly neurotrophic responses as ibogaine with fewer deleterious side effects, such as hallucinatory symptoms, cardiac symptoms, etc. (Figures 2,3).

**Figure 2:** Ibogaine, and the general scheme of analogs tested herein. Structural variation in the library was included at positions R\textsuperscript{1} and R\textsuperscript{2}, the length of linker was varied, as was the hybridization state of the C5-C6 bond (see preliminarily-screened structures in Figure3).
Measurement of growth factor release into culture medium by enzyme-linked immunosorbent assay (ELISA) is widely used to test growth factor secretion by various conditions. To establish an assay, Dr. Shu Li screened a number of cell lines reported to release GDNF using the corresponding positive control inducers for each cell line, and detected GDNF release by a commercial ELISA kit (Promega E\textsubscript{max} GDNF Immunoassay System). It was found that SH-SY5Y, Neuro2A, SK-N-AS, and primary human cortical astrocytes each produced GDNF levels below the detection limit of the kit after 48-hour treatment with the respective positive controls. It was also found that C6 cells provided a detectable background release of GDNF, and several experimental isoquinuclidines released GDNF under preliminary screening conditions (Figure 3).

C6 cells, originally transformed from a rat glioma tumor,\textsuperscript{(31)} were ultimately chosen for wider study based on this preliminary optimization as well as the numerous reports documenting GDNF release from C6. In fact, several compounds have been identified as GDNF releasers in C6 cells, including riluzole, amitriptyline, and antipsychotics; receptor tyrosine kinases have been implicated either as direct or transactivated targets for signaling mechanisms related to GDNF release induced by many of these compounds.\textsuperscript{(32-36)} C6 cells proved to be an excellent system for screening compounds for capacity to release GDNF, as active forms of a number of RTKs are expressed. Numerous potential targets funnel through MEK to activate the promoter for GDNF, cAMP response-element binding protein (CREB), ultimately resulting in upregulation of GDNF transcription, translation, and subsequent release.
One drawback to using C6 cells was the previously documented phenotypic instability, resulting in extreme variability from experiment to experiment and
across lots (provided by ATCC).\cite{13,37,38} GDNF release consistently decreased as a function of culture age, as previously reported,\cite{37} and as a consequence experiments were reproduced within a tight range of passages. Variations in background GDNF release also proved problematic for calculating fold/control levels. Consequently, many of the results below are presented as representative experiments showing an effect (inhibition, activation, etc.) and statistical parameters are calculated for biological replicates within the same experiment. In these cases, experiments are presented as representative of an effect when statistical significance of intraassay biological replicates is reproducible across experiments.
II: Results and Discussion

Conventional ELISA is a More Sensitive Method for Detecting GDNF Release

The release of GDNF from C6 cells was initially screened by Dr. Shu Li using an in-situ ELISA protocol, due to reported high detection sensitivity of this method (Figure 3). GDNF release for each compound was calculated as a ratio of the amount of GDNF released by experimental compounds to the amount released by vehicle control (DMSO 0.1% v/v), and expressed as "fold/control." In-situ ELISA involves culturing a monolayer of cells on an ELISA detection plate that has been pre-coated with a monoclonal capture antibody. Once GDNF is released into the culture medium from cells, it is theoretically captured by the monoclonal antibody. To terminate the experiment, treatment medium is removed and cells are disaggregated/dissociated from the plate by action of detergent and gentle rocking. As seen in Figure 3 above, the best GDNF releasers as detected by in-situ ELISA were found to release ~2-3 fold/control.

In order to develop a more robust assay for GDNF release, I decided to optimize a conventional ELISA. It was initially thought that the GDNF released into conditioned media, when not captured by a monoclonal antibody as in in-situ ELISA, autocrine signaling would amplify signal initiated by inducing compounds. It was observed by treating C6 cells with XL-008, a strong inducer of GDNF as determined using in-situ ELISA, that release of GDNF can be as high as ~ 40 fold/control (Figure 4, see note about phenotypic instability above). Conversely, it is possible that the increased sensitivity afforded by conventional ELISA relates to the method of detection. For the less sensitive in-situ ELISA, the fact that cells are growing on the
monoclonal antibody-treated surface or the detergent based wash could contribute to decreased sensitivity of GDNF detection.

While in-situ ELISA is thought to be a more sensitive detection method in for some applications, conventional ELISA offers a number of advantages for our experiments. First, it allows multiple measurements of conditioned media from each replicate well, as the treatment wells are not limited to small, 96-well format. For example, I chose to pursue a 12-well format, with each treatment in at least duplicate per experiment, each read in triplicate with the GDNF ELISA kit. Further, the increased number of cells in each of these larger wells facilitates the study of intracellular processes via western blot or other ELISA-based experiments conducted on cellular lysates collected immediately after the conditioned media were removed at the termination of the experiment.

**Figure 4:** Conventional ELISA affords greater detection sensitivity than in-situ ELISA. Shown are results for XL-008 (10 µM, 48h)-dependent GDNF release, indicating a statistically significant release of GDNF by XL-008 and not by the inactive, structurally-related negative control 18-MC.\(^{(25)}\) Cells were grown in a 12-well format, treatments were conducted in triplicate wells, and conditioned media from each treatment were measured in triplicate for GDNF release. Shown are the
means ± SEM of 5 independent experiments. GDNF release by XL-008 was found to be statistically significant by ANOVA followed by Tukey’s post test (*** p < 0.001).

**Dose-Responsive GDNF Release and Toxicity by Isoquinuclidines**

In order to confirm pharmacological induction of GDNF release by the experimental compounds, dose-dependence was studied. C6 cells were treated with a varying concentration of compound over 48 h, and GDNF release into the conditioned media was measured. Indeed, GDNF release by two isoquinuclidines studies in depth, XL-008 and XL-026 (for structures see Figure 3) reproducibly displayed a dose-dependent relationship (Figure 5). These particular compounds were chosen based on release properties in preliminary experiments using in-situ ELISA, as well as the reproducibly high amounts of GDNF detected from our preliminary conventional ELISA results with XL-008. The potency (EC$_{50}$) for XL-008 was determined to be 2.5 ± 0.2 µM (mean ± SEM, n = 4 independent experiments), and XL-026 was found to be 22.4 ± 6.6 µM (n = 3 independent experiments) (Figure 5 A,C).

Toxicity can be approximated by measuring release of lactate dehydrogenase (LDH) into the culture medium, as LDH is known to leak through perforated membranes upon cell death. When toxicity was determined for the same experiments above by LDH release into the medium relative to intracellular LDH in untreated control cells, it became evident that a dose-dependent relationship existed, as well (Figure 5 B,D).
Figure 5: Isoquinuclidines XL-008 and XL-026 each release GDNF and lactate dehydrogenase (LDH, an approximation of cytotoxicity) in a dose-responsive manner. A) The EC$_{50}$ of GDNF release for XL-008 was determined to be 2.5 ± 0.2 µM (mean ± SEM, n = 4 independent experiments). Shown is a dose-response curve from a representative experiment. B) A highly reproducible bell-shaped dose response toxicity curve (as measured by LDH released into conditioned media) was found for XL-008, with a local minimum at 50 µM. Shown is the toxicity curve associated with the dose response of GDNF from A, and is representative. C) XL-026 exhibited a less potent dose-dependent relationship for GDNF release (EC$_{50}$ = 22.4 ± 6.6 µM, mean ± SEM, n = 3 independent experiments). D) The corresponding toxicity curves for XL-026 were never bell-shaped as for XL-008. All curves are representative examples of individual experiments, and GDNF and LDH curves correspond to the same experiment.

An interesting and highly reproducible toxicity result was noticed for XL-008. The toxicity as estimated by LDH release was consistently and statistically significantly lower at 40 and 50 µM XL-008 than the apparent maximum at 10 µM.
(ANOVA of biological replicates within experiment followed by Tukey’s post-hoc analysis, p < 0.01, 3 representative examples). This interesting result, as well as morphological analysis of the cells under these conditions (Figure 6), led us to the hypothesis that XL-008 may be altering the phenotype of C6 cells, which are notoriously phenotypically unstable. C6 cells are known to differentiate under stress or when treated with cAMP or certain small molecules,[39, 40] characteristically producing more glial fibrillary acidic protein (GFAP), and behaving more like an astrocyte than a tumor. It is known that under these conditions, C6 cells display spindly morphology (as in Figure 6C).

![Figure 6: XL-008 induces morphological changes in C6 cells in a dose-dependent manner. A) DMSO vehicle control (0.1% v/v) treatment leaves cells with typical fibroblastic morphology. B) XL-008 (10 µM) begins to have a noticeable effect of cell size and shape, as cells become considerably more rounded. C) XL-008 (40 µM) consistently induces spindle-like morphology (highlighted by arrow) from considerably more lymphoblastic cells. All images are representative of typical experiments, 400X magnification.](image)

While it is possible that toxicity of XL-008 is truly lower at 50 µM concentrations due to some phenotypic shift, the appearance of the morphology of the cells suggested that toxicity was indeed occurring (cells appeared round and lymphoblastic with spindle-like processes). Because the LDH determination is made
in the presence of all components of the 48-hour conditioned medium including remaining experimental compound and metabolites, it was thought that the compound might be inhibiting enzymatic activity of the LDH present in conditioned media. Titration of constant amounts of LDH from cellular lysate and colorimetric reagent with increasing concentrations of XL-008 resulted in no decrease in apparent LDH activity at even the highest concentrations of XL-008 in two independent experiments (Figure 7). Consistent measurements of approximately 20% LDH activity (as expected) suggests that XL-008 is not inhibiting LDH activity, although it is not possible to rule out LDH inhibition by metabolites.

**Figure 7:** No significant inhibition of LDH activity by XL-008 from 1-50 µM in a 5:1-diluted lysate (20% LDH activity expected). Representative of two independent experiments.

**GDNF Release by XL-008 and XL-026 is Not Simply Caused by a Leak of Cytosolic or Stored GDNF into the Conditioned Medium**

Because the compounds investigated to this point had displayed apparent release-associated toxicity in dose response studies, it was necessary to determine if
the released GDNF was true induction of protein synthesis as a consequence of induced protein synthesis, or if the compounds were simply releasing previously translated GDNF as a function of toxicity-related membrane permeabilization. We treated C6 cells with cycloheximide, a small molecule inhibitor of translation initiation and translational elongation at the ribosome. As cycloheximide is known to potently inhibit protein synthesis, it is a useful pharmacological tool for probing the dependence of protein synthesis on GDNF release. In fact, it was found that 1µg/mL cycloheximide reproducibly inhibited GDNF synthesis as caused by XL-008 (Figure 8, representative of 2 experiments) and XL-026 (2 experiments by Dr. Teresa Jacques). Cycloheximide did not have an appreciable effect on background GDNF release.

**Figure 8:** Cycloheximide (1 µg/mL) inhibits GDNF release (representative example shown from 2 independent trials, and 2 trials showing similar inhibition of XL-026 in collaboration with Dr. Teresa Jacques). ***p < 0.001 indicates statistical
significance compared to XL-008, determined by ANOVA followed by Tukey’s post-test.

In order to conclusively show that GDNF is being synthesized in C6 cells in response to the experimental isoquinuclidines, the total GDNF in experimental wells was measured after 48-hour treatments. Previously, only released GDNF had been measured in conditioned media, leaving the possibility that cells were simply leaking GDNF as a function of toxicity, similar to LDH. To accomplish this analysis, after the 48 hour treatment period, conditioned media were collected and GDNF was measured as before; the cell monolayers were lysed using conditions optimized for detection of GDNF from lysate using the same ELISA kit. If the compounds were simply inducing the cells to leak GDNF, it would be expected that the total GDNF in the system would be unchanged as measured by this method. It was noticed, however, that there is a consistent increase in total GDNF in both the extracellular and intracellular samples (sums are presented in Figure 9, representative of 2 independent experiments for both XL-008 and XL-026).

These experiments used higher concentrations of isoquinuclidines in order to observe the effects of massive toxicity on GDNF and LDH as detected using the GDNF and LDH kits, respectively, from Promega. Analysis of higher concentrations of XL-008 acting on C6 cells reveals that the strange “bell” shaped LDH activity curve remains with a local minimum around 50 µM, and LDH activity emerges to a global maximum thereafter as a function of culture death.
Figure 9: GDNF is synthesized, and not simply leaked into the conditioned media, by action of isoquinuclidines XL-008 and XL-026. For these experiments, intracellular and extracellular GDNF were collected, measured using an optimized protocol for detection of intracellular GDNF, and summed. A) Dose response of total GDNF induced in C6 cells by XL-008. The lower GDNF values at higher concentrations of XL-008 are a direct result of massive toxicity (intended in this experiment). B) Dose response of relative extracellular LDH (toxicity) shows the same local minimum at 50 µM, above which toxicity increases. Note: this detection was saturated, so the % LDH activity is an inflated representation of toxicity at lower values. C) Similar dose-dependent release for XL-026. D) Corresponding toxicity for XL-026. All figures are from representative experiments, 2 independent experiments. Error bars represent SEM of biological replicates (n = 2) within the representative experiment. Red arrows are included in XL-008 experiments to highlight the divergence observed between GDNF (total) and LDH release at 50 µM XL-008.

From these data, it can be concluded that there is a close relationship between release and toxicity up to a point where massive toxicity precludes GDNF synthesis. While it is not possible under these conditions to separate GDNF release from toxicity with certainty, it is clear that GDNF is being produced by the cells as
GDNF release is inhibited by the protein synthesis inhibitor cycloheximide and total GDNF increases as a function of increasing isoquinuclidine concentration.

**Release of GDNF by XL-008 is MAPK/ERK Dependent**

GDNF is known to be expressed under control of the promoter CREB, which is activated by the signaling kinases ERK 1/2. ERK 1/2 are in turn activated by the kinase MEK, which specifically phosphorylates ERK. MEK/ERK signaling represents the convergence of a number of direct and transactivation-related signaling mechanisms.\(^{(42)}\) In order to preliminarily investigate the mechanism of GDNF release and the effects of intracellular signaling, C6 cells were treated with XL-008 (10 µM, 48 h) in the presence of the MEK inhibitors PD98059 and U0126 (30 µM and 10 µM, respectively, with an additional 30 minute pre-treatment). The result was a < 90% inhibition of GDNF release by XL-008 (Figure 10, mean of 3 independent experiments).

**Figure 10:** GDNF release by XL-008 is inhibited by MEK1/2 inhibitors PD98059 and U0126. These structurally unrelated inhibitors of MEK 1/2 significantly reduce GDNF release. Data represent mean ± SEM of 3 independent experiments. (** p <
0.01 indicates statistical significance for each inhibitor-treated condition compared to XL-008, determined by ANOVA followed by Tukey’s post-hoc analysis, n = 3 independent experiments). XL-008 release was significant compared to vehicle control during the same experiments (# p < 0.01, ANOVA followed by Tukey’s post-hoc analysis).

**Effect of RTK Inhibitors on GDNF Release**

Since ERK1/2 signaling was found to be responsible for XL-008-dependent GDNF release, the upstream receptor tyrosine kinases (RTKs) were pharmacologically evaluated for their potential role in GDNF release by XL-008. It is known that C6 cells release GDNF in response to fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) through direct signaling with the cognate RTKs.\textsuperscript{13, 37} In an experiment to compare the effects of RTK inhibitors on FGFb-dependent and XL-008-dependent GDNF release, cells were pre-treated for 30 minutes with the indicated RTK inhibitors (Figure 11), followed by a 48 hour cotreatment with inhibitor and releaser (FGFb or XL-008, or DMSO vehicle control). GDNF release by the growth factor FGFb is completely inhibited by PD173074, a reported FGFR-selective inhibitor (Figure 11). As concurrently found by Dr. Teresa Jacques in the same system, inhibition of PDGF-dependent GDNF release by KRN633 and SU-5416,\textsuperscript{13} reported PDGFR/VEGFR inhibitors,\textsuperscript{43, 44} validates that these RTK inhibitors suppress GDNF-release caused by direct activation of the corresponding growth factor receptors (FGFR and PDGFR) in our C6 system. Partial inhibition of FGF by the PDGFR/VEGFR inhibitors (KRN633, SU-5416) was ascribed to likely inhibitor cross reactivity. These RTK inhibitors are all known to compete with ATP at the intracellular kinase domain of the RTKs, which has a notoriously conserved
sequence across families of RTKs. Related to this is the dearth of truly selective RTK inhibitors.\textsuperscript{(45)}

As VEGFR and PDGFR inhibitors KRN633 and SU-5416 were found to inhibit GDNF release by XL-008 greater than 90%, but the FGFR inhibitor PD173074 was found to inhibit GDNF release by approximately 60% (\textbf{Figure 11}, representative of 3 independent experiments together with Dr. Teresa Jacques), it was hypothesized that either VEGFR or PDGFR could be involved in GDNF release by our isoquinuclidines. As discussed above, these inhibitors are not entirely selective, and it was thought that incomplete inhibition of XL-008 by FGFR inhibitors was simply due to inhibitor cross-reactivity with the responsible RTK, analogous to the partial inhibition of FGF by KRN633 and SU5416 (VEGFR/PDGFR inhibitors).
**Figure 11:** RTK inhibitors reduce release of GDNF by XL-008. A) Inhibition of XL-008- and FGFb-dependent GDNF release by RTK inhibitors reveals differences in inhibition profiles. SU-5416 and KRN633 are VEGFR/PDGFR selective inhibitors, and inhibit XL-008 nearly completely, while only inhibiting FGFb-dependent release by approximately 50%. PD-173074 is a FGFR inhibitor, and inhibited FGFb-dependent release completely, while only partially inhibiting XL-008. Genistein, a pan tyrosine kinase inhibitor, abolished release by both the growth factor and isoquinuclidine. Error bars represent SEM of intra-assay means of duplicate treatment wells in a representative experiment, repeated three times in collaboration with Dr. Teresa Jacques. B) Quantification of inhibition percentages.
from the indicated experiment and C) structures of utilized RTK inhibitors are shown.

Based on these inhibition experiments, it was thought that an RTK was involved in upstream signaling in the MAPK/ERK pathway. In an attempt to isolate the RTK responsible for signaling (and possibly the target of isoquinuclidine activity), Dr. Teresa Jacques investigated the potencies of exemplar FGFR, PDGFR, VEGFR, and EGFR inhibitors. From comparison of measured inhibition potencies (IC$_{50}$s) to literature values, we proposed that PDGFR was likely involved in upstream signaling of GDNF release by XL-008. This hypothesis was supported by the observation that PDGF is capable of inducing GDNF in C6 cells.$^{(13,37)}$

**Figure 12:** Overview of possible mechanisms by which XL-008 and related isoquinuclidines could be signaling to induce production of GDNF (blue structure, top left) in C6 cells. A) It is possible that these compounds are acting through a G-protein coupled receptor (GPCR), which either directly activates MEK or transactivates a RTK. B) The compounds could interact directly with the extracellular or catalytic domains of an RTK, leading to catalytic autophosphorylation. Additionally, the compound could interact with an intracellular adaptor protein. C) Activation of a stress response is also a possibility, either lysosomal (green ovals) or endoplasmic reticulum (purple structures). Other possibilities include activation of a matrix metalloprotease (not shown) leading to the release of a growth factor, which signals via RTKs. Any of these are possible
mechanisms, as GDNF release was found to be inhibited by MEK1/2 and RTK inhibitors (above).

In order to determine the pharmacological target for these compounds, numerous studies were conducted by Dr. Teresa Jacques. Detailed pharmacological inhibition data implicate RTK involvement. At this time, we can not rule out direct activation of a GPCR or transactivation of an RTK by action of a GPCR (Figure 12A), direct activation of an RTK (Figure 12B), or any other means of directly or indirectly modulating RTK activity, such as through interactions with the σ-receptors. It is known that stress-related processes can induce GDNF in C6 cells via RTK/MAPK signaling. Endoplasmic reticulum (ER) stress and lysosomal stress from alkalization by vATPase inhibitors are among the processes shown to induce GDNF release from C6 cells (Figure 12C). Gene silencing experiments are currently underway in the Sames lab, with the goal of understanding which RTK (or RTKs) are involved in isoquinuclidine induction of GDNF release. While the exact molecular target remains elusive, it has been found that XL-026 and XL-008 can modulate FGFb-dependent signaling and GDNF release (see below).

*N-Arylethyl Isoquinuclidines Potentiate GDNF release by FGFb*

In order to examine the effect of FGFb on isoquinuclidine-induced GDNF release, C6 cells were treated for 48 hours with either FGFb, isoquinuclidine of varying concentration, or a cocktail of FGFb with the isoquinuclidine of varying concentration. It was found that under most conditions, the release of GDNF caused
by a cocktail of FGF and isoquinuclidine was a greater than additive release compared to isoquinuclidine or FGFb alone (Figure 13). As XL-008 is approaching $E_{\text{max-apparent}}$ at 10 $\mu$M, it may be true that cells are physically incapable of producing more GDNF in these conditions. Thus, the effect of FGFb treatment in conjunction with 10 $\mu$M XL-008 is minimal compared to the effect on 10 $\mu$M XL-026, which at 10 $\mu$M is below the $E_{50}$. More than additive effects are noticed with treatment of FGFb with lower concentrations of XL-008 as well as with all concentrations of XL-026 below 10 $\mu$M. This implies that the compound is either potentiating FGFb signaling, or vice-versa. A full quantitative study to confirm these results is underway in Sames group, using optimized 24h treatment conditions to largely remove contributions of toxicity.

Figure 13: XL-008 and XL-026 act in concert with FGFb to release increased amounts of GDNF. Potentiation of GDNF release from C6 p#41 in 12-well plates. Shown are the means of duplicate biological replicates each measured in triplicate. Error bars represent SEM of biological replicates. Statistical analysis by ANOVA followed by Tukey's post-test indicates that all "potentiations" by isoquinuclidine + FGF are significant compared to their parent isoquinuclidine release. Results are representative of 3 independent experiments shared with Dr. Teresa Jacques.
It has been demonstrated that riluzole induces GDNF release from C6 cells in a FGFR-dependent manner,\(^{(36)}\) and it is also known that signaling pathways including GPCR-related signaling converge with and modulate the activity of FGFb in C6 cells.\(^{(48)}\) FGFR2 is currently considered an attractive pharmacological target, as FGF signaling dysregulation has been implicated in major depression,\(^{(49)}\) and mood disorders,\(^{(50)}\) and FGF2 cannulation directly into brain tissue of rats has been shown to have antidepressant effects.\(^{(51)}\) Thus, XL-008 and related isoquinuclidines warrant further study, as potentiation or amplification of FGFR signaling in the brain, whether direct or indirect, could play important roles in neuroprotection, treatment of depression or mood disorders, or recovery from traumatic brain injury through potentiating RTK signaling from endogenous levels of FGF.

**N-Arylethyl isoquinuclidines induce GDNF in C6 cells: Additions to the Library of Compounds\(^{(1)}\)**

In search of a more potent GDNF releaser, and in order to explore the structure activity relationships present in the process underlying GDNF release, a number of new N-Arylethyl isoquinuclidines were synthesized by Andrew Kruegel and Dr. Souvik Rakshit and screened by myself for GDNF induction in C6 cells. This screen was accomplished using the 12-well format conventional ELISA from experiments above, with all compounds treated at 10 µM in biological replicates \((n = 2 \text{ wells per experiment})\). Many of these compounds were screened at 1 µM as well, with the hope of identifying a compound as active or nearly as active at 1 µM as at 10 µM, thus displaying sub-micromolar activity. Unfortunately, none of the tested
compounds released more GDNF than XL-008 at 1 µM (data not shown). GDNF release into conditioned medium was determined for each compound as above. Each compound claimed to release statistically significant GDNF at 10 µM did so in each of three independent experiments, as determined from means of intraassay replicates compared to DMSO vehicle control (0.1% v/v). Results are summarized below in Tables 1, 2, and 3 (adapted from reference 1). Negative results are summarized in Figure 15, showing compounds releasing statistically insignificant amounts of GDNF compared to the DMSO control. Toxicity for each compound was found to be below 20%, as determined by LDH release.
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<th>GDNF Increase ± SEM (fold/vehicle control)</th>
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*a All results represent fold-increase of GDNF release by C6 glioma cells treated with the indicated compound for 48 hours, normalized to the vehicle control (conventional ELISA, see experimental section). *b Results are from a representative experiment and were determined to be statistically significant (ANOVA) compared to the vehicle control. All compounds induced a statistically significant increase of GDNF in each of 3 independent experiments.
Table 2: 7-endo-Series\textsuperscript{a,b}

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<tr>
<td>SORA-II-073</td>
<td><img src="image" alt="Structure" /></td>
<td>13.82 ± 2.01</td>
</tr>
<tr>
<td>SORA-I-104</td>
<td><img src="image" alt="Structure" /></td>
<td>10.87 ± 0.84</td>
</tr>
<tr>
<td>SORA-I-103\textsuperscript{c}</td>
<td><img src="image" alt="Structure" /></td>
<td>16.03 ± 1.66</td>
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<tr>
<td>SORA-I-105</td>
<td><img src="image" alt="Structure" /></td>
<td>14.71 ± 0.11</td>
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<tr>
<td>ACK-II-186</td>
<td><img src="image" alt="Structure" /></td>
<td>14.39 ± 0.51</td>
</tr>
<tr>
<td>SORA-I-102</td>
<td><img src="image" alt="Structure" /></td>
<td>11.86 ± 0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All results represent fold-increase of GDNF release by C6 glioma cells treated with the indicated compound for 48 hours, normalized to the vehicle control (conventional ELISA, see experimental section). \textsuperscript{b}Results are presented from a representative experiment and were determined to be statistically significant (ANOVA) compared to the vehicle control. All compounds induced a statistically significant increase of GDNF in each of 3 independent experiments unless otherwise indicated. \textsuperscript{c}SORA-I-103 was tested twice and afforded statistically significant results in both trials.
Using the same analysis as above, there were a few screened compounds that did not display any significant GDNF release into the conditioned medium compared to DMSO control (Figure 15). Interestingly, XL-026 was a strong releaser of GDNF, although the exo-ethyl isoquinuclidine benzofurans were not at the screened concentration (10 µM). While it is conceivable that these diastereomers are substrates for a metabolic enzyme that deactivates them, the significantly different activities of the diastereomers may suggest that the GDNF-releasing effect is not non-specific. Preliminary results comparing ACK-090 to XL-026 suggest that the exo-ethyl isoquinuclidines may also act as releasers of GDNF with a lower potency.
(data not shown). Related, various hydroxyl, acetate, or ether substituents on the 7-position were found to be inactive at 10 µM.

**Figure 15:** Other isoquinuclidines affording statistically insignificant release of GDNF at 10 µM. Interesting results include the lack of release by the 7-exo-ethyl diastereomers of the benzofuran XL-026 (SORA-I-081 and ACK-I-090) as well as the related 7-exo-benzyl compound SORA-II-059.
III: Conclusions and Outlook

From these studies, it was found that several isoquinuclidine-based compounds are capable of releasing GDNF from C6 glioma cells, a widely-used model for astroglia in the brain. Select compounds identified from a screen were chosen for further evaluation. Dose-dependent release of GDNF was confirmed, GDNF synthesis was found to be induced by action of the compounds, and the beneficial MEK/ERK signaling cascade was determined to be involved in the observed GDNF synthesis and release. Inhibition of RTKs with a small library of RTK inhibitors implicated the involvement of these beneficial signaling receptors. It was also found that XL-008 positively modulates the effect of FGFb with respect to GDNF synthesis and release.

With Dr. Teresa Jacques, a number of potential direct- and transactivation mechanisms were investigated, and it appears clear from pharmacological data that RTKs are involved somewhere in the signaling sequence. While the GDNF-induction of FGFb is amplified and potentiated by action of XL-008, we cannot conclude that FGFR2 is the direct molecular target of these compounds. FGFR2 may indeed be the target of a transactivation mechanism from other tyrosine kinases, GPCRs, metalloproteases, or become modulated by adapter proteins.

With Madalee Gassaway, these experiments have been developed into a 96-well format for more efficient screening of increased numbers of conditions. By shortening the isoquinuclidine treatment time to 24 h, Madalee has optimized conditions to largely eliminate toxicity while retaining statistically significant release of GDNF within each experiment. Using this platform, it has been confirmed
that XL-008 indeed modulates FGFb-dependent signaling and GDNF release. As such, XL-008 may represent a potentially useful modulator of FGFR activity, whether direct or indirect, if this mechanism of action is retained in vivo. Additionally, in searching for a more potent analog, a large number of isoquinuclidines have been identified as GDNF inducers in C6 cells, although unfortunately none appear more potent than XL-008.

The early work described here, including optimization of the GDNF detection, characterization of GDNF synthesis and release, isoquinuclidine toxicity studies, and preliminary pharmacological inhibition experiments served as a springboard for ongoing advanced studies of N-arylethyl isoquinuclidine-induced signaling mechanisms, neurotrophic factor induction, and applications thereof.
IV: Experimental

General Cell Culture

C6 cells from two separate lots were purchased from American Type Culture Collection (ATCC, Manassas, VA) at passage 37, and displayed slightly different behavior as discussed above. Cells from lot #58987834 were used between passages 44-49. Cells from lot # 59673008 were used between passages 41-44. Cultures were grown on 10 cm polystyrene culture plates (Falcon), were maintained at 37°C in a humidified atmosphere containing 5% CO₂, and were subcultured at a 1:10 ratio approximately every three days upon reaching confluence. Complete growth medium consisted of DMEM (high glucose with GlutaMAX, Life Technologies) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

GDNF Release Assay: GDNF Conventional ELISA in C6 glioma cells

Conventional ELISAs were used to determine compound-induced increases of GDNF concentration in conditioned media of C6 glioma cells, and have afforded increased sensitivity compared to in-situ ELISA in our hands. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. GDNF release was measured for each compound in 3 independent experiments. Briefly, C6 cells were seeded into sterile 12-well culture plates (Falcon) at a density of 300,000 cells per well and were recovered for 24h in 1mL complete growth medium (94% high glucose DMEM, 5% FBS, 1% penicillin/streptomycin). Wells were then washed with
1mL sterile DPBS and were incubated for an additional 24h with 0.5mL low-serum medium (98.5% high glucose DMEM, 0.5% FBS, 1% penicillin/streptomycin). On the following day, experimental compounds or DMSO vehicle control were added each to duplicate wells in an additional 0.5mL of low-serum medium. The final concentration of each compound was 10 µM in 1mL low-serum medium containing 0.1% DMSO. Plates were incubated with compounds and vehicle control for 48h. Conditioned media were then collected from each well, and GDNF concentrations were measured in triplicate on 96-well ELISA plates using the GDNF E\textsubscript{max} Immunoassay System from Promega (Madison, WI) according to the manufacturer’s instructions. A BioTek H1MF plate reader (Burlington, VT) was used to measure absorbance from the resulting colorimetric reaction. Total GDNF release for each compound was calculated from the standard curve using Microsoft Excel. Data are normalized to the vehicle control and are thus presented as fold increase of GDNF concentration in experimental wells over that of the vehicle control. Data presented here are interassay means from the indicated number of independent experiments ± interassay standard error of the mean (SEM) calculated from means of independent experiments. Statistical significance was determined by ANOVA followed by Tukey’s post-hoc analysis using GraphPad Prism.

**Inhibition of GDNF synthesis and release with small-molecules**

C6 cells were subcultured into 12-well plates at a density of \(~300,000\) cells/well, and were allowed to recover in complete medium for 24 hours. Wells were washed once with PBS and treated as above with low serum, high glucose
experimental medium for 23.5 hours. 30 minutes prior to initiation of treatment with experimental compounds, inhibitors were added for the indicated final concentrations. Treatment was initiated by adding solutions of experimental compounds or experimental compounds + inhibitors to wells. Final conditions were 10μM experimental compounds or 10μM experimental compounds + indicated concentration of inhibitors. DMSO (0.2%) served as a vehicle control. Final volumes were 1 mL per well, and each respective condition was run in duplicate. Plates were incubated for 48h at 37°C, 5% CO₂. After 48h, conditioned experimental medium was removed from wells and frozen o/n at -80°C. GDNF in each sample was measured in triplicate on a 96-well ELISA plate as described above (Promega). For MEK inhibition experiments, data presented here are interassay means from the indicated number of independent experiments ± interassay standard error of the mean (SEM) calculated from means of independent experiments. Statistical significance for was determined by ANOVA followed by Tukey's post-hoc analysis using GraphPad Prism. Tested for significance were both GDNF release caused by the experimental isoquinuclidine compared to vehicle control, and the effect of inhibitors compared to uninhibited release. For cycloheximide and RTK inhibition experiments, data are presented for a representative experiment, and means and standard errors are determined from intraassay biological replicates.

**Determination of Total GDNF**

Experiments were conducted as above in 12-well plates. GDNF and LDH in each sample were measured in triplicate on 96-well plates. Extracellular GDNF or
LDH were measured in undiluted conditioned media as before using Promega’s GDNF ELISA or the Cytotox96 LDH kit, respectively. Intracellular GDNF was collected by lysing cells in 200 µL of a custom, lysis buffer (see below), and diluting the resulting lysates 10X before detection, to minimize the concentration of detergent present during antibody/antigen association (ELISA capture).

In order to optimize lysis conditions for GDNF detection, 4 different conditions were tested for their compatibility with Promega’s GDNF assay. Cells from an independent 48-hour treatment with complete medium (unused monolayers of basal treatment) were lysed with each condition and were then assayed both neat and diluted 1:2. Final in-well concentrations of detergent were compared between lysis buffers containing 1%, 0.5%, 0.1%, and 0.05% Triton-X 100 in tris buffered saline (TBS), each with and without a protease inhibitor cocktail (P8340, Sigma-Aldrich) and phosphatase inhibitor cocktail 2 (P5726, Sigma-Aldrich). Additionally, the same conditions were tested for the ability to accurately detect a known concentration of GDNF (500 pg/mL). It was determined that lysis conditions containing protease and phosphatase inhibitors, with dilution conditions resulting in 0.05% Triton-X 100 in the GDNF ELISA buffer were optimal for detection of GDNF and LDH from the lysate.

**Cytotoxicity Counter Assay**

A counter assay was also run using the same conditioned medium. Triplicate measurements of each sample were made using Promega’s Cytotox 96 LDH assay kit according to the manufacturer’s instructions. Untreated cells were lysed using the
lysis buffer included in the kit, and a standard curve was generated from the intracellular lysate to approximate the percentage of total LDH released as a function of toxicity in experimental conditioned media.

Screening of Related Isoquinuclidines for ability to release GDNF

C6 cells were treated as above for conventional ELISA experiments. All compounds were screened at 10 µM concentration. Data presented herein are intra-assay means from a representative experiment ± intra-assay standard error of the mean (SEM) calculated from biological replicates. Each compound from Tables 1-3 afforded a statistically significant increase of GDNF release over the DMSO control in each of three independent experiments unless otherwise noted. Statistical significance within each experiment was determined by ANOVA followed by Tukey’s post-hoc analysis using GraphPad Prism. Cytotoxicity for each compound was below 20% as measured by LDH levels in conditioned media (Cytotox 96, Promega).
V: References


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