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N-Alkyl 4-Methylamphetamine enantiomers and the implication for potential modulation of abuse liability and enhancement of psychoactive drug targeting.

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Thesis submitted to the faculty of Virginia Commonwealth University in order to fulfill the degree requirements of Master of Science in Physiology and Biophysics.

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Community Health B.S.
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Virginia Commonwealth University
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Abstract

N-ALKYL 4-METHYLAMPHETAMINE ENANTIOMERS AND THE IMPLICATION FOR POTENTIAL MODULATION OF ABUSE LIABILITY AND ENHANCEMENT OF PSYCHOACTIVE DRUG TARGETING.

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Drugs of abuse have a long history in humanity. Currently however, a subject of great interest is the phenylalkylamine family of drugs. Not only is the abuse liability of interest but also the potential therapeutic expansion of the capabilities of this family of drugs by utilizing the unique stereospecific effects of the newly discovered hybrid compounds. Based upon prior data of N-Alkyl 4-MA the enantiomers of N-Methyl, N-Ethyl, and N-Propyl were analyzed in hDAT, hNET, and hSERT. It was found that there was a negative correlation between chain length and potency and dopaminergic component. In agreement with the currently established paradigm it was also found that in almost all cases the S(+) enantiomer was the more potent.

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I. Scientific Background

1. Drugs of Abuse

Humanity has a long and widespread history of psychoactive drug use. Archaeological and historical evidence suggests that 13,000 years ago the betel nut, who's active compound is arecoline, was chewed in Timor. Records also indicate use of the betel nut almost 11,000 years ago in Thailand, as well as the use of the coca plant, active ingredient cocaine, in the western regions of South America about 5,000-7,000 years ago (Sullivan & Hagen, 2002) . The list of evidence for psychoactive drug abuse goes on with similar stories found across the world. Psychoactive drug abuse truly became an issue however when artificial synthesis and isolation of the active compounds began, the most notable being the synthesis of amphetamine by Romanian chemist Lazar Edeleanu in 1887 (History of Amphetamines, 2017).

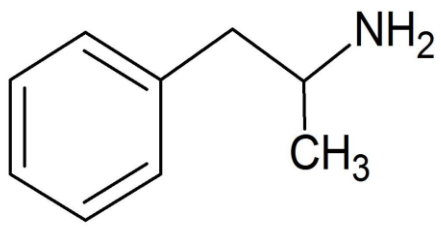


Figure 1.1 Structure of Amphetamine (Alere, 2017)

It was not until the early 1920's however, that amphetamines were perceived as pharmaceutically useful. The explosion of amphetamine abuse began during the Second World War, militaries around the world even supplied their soldiers with amphetamine rations throughout the war. Amphetamines were then also prescribed by physicians for everything from weight-loss and fatigue to depression and ADHD. It was not until the 1960's that widespread

consumption of amphetamines began to show on a large scale the negative effects of amphetamine abuse. It was also in the 1960's that research began to indicate that amphetamines were in fact quite addictive, instead of simply habituating like caffeine. The initial epidemic of

abuse was ascertained to be iatrogenic in nature, however a newer problem arose beginning in the 1980's of a culture of illicit drug which resulted in a resurgence of the amphetamine abuse epidemic that is present today (Rasmussen, 2008).

In tandem with the rise of abuse of amphetamines, was the abuse of other stimulants such as cocaine, and ecstasy (MDMA). With the crackdown by the FDA and the federal government, many began to synthesize synthetic amphetamines or designer drugs in order to avoid the current regulation by the government. In 2012, when bath salts became an issue discussed nationally, they were still available for legal purchase over the counter or online (Gershman & Fass, 2012). However as news stories began to pour in the federal government took action and scheduled and banned many of the known compounds circulated as "bath salts".

Investigations into the underlying mechanisms behind drug abuse and abuse liability have identified the dopaminergic pathways as the prominently affected areas in substance abuse. The dopaminergic pathways primarily consist of the mesolimbic system in the brain. The two regions of greatest interest in the mesolimbic/dopaminergic pathway are the Nucleus Accumbens (NA) and the Ventral Tegmental Area (VTA) (Alcaro et al., 2007).

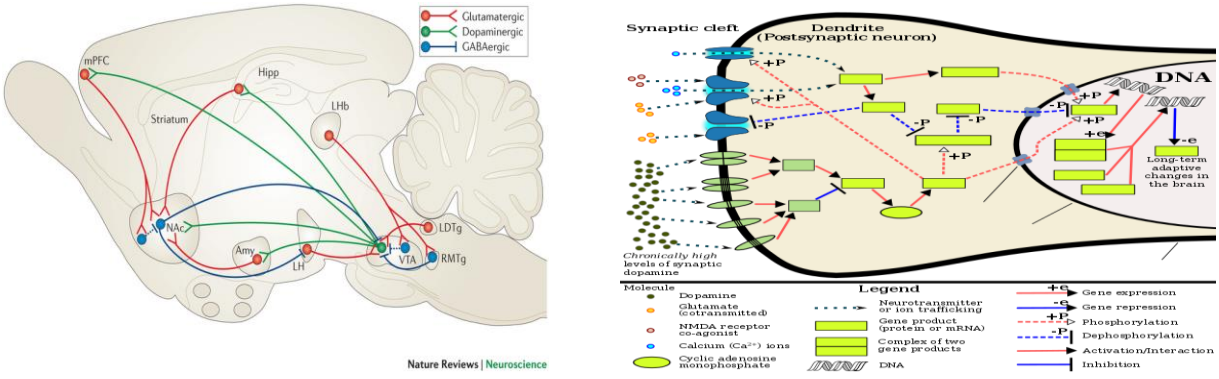


Figure 1.2 Illustration of Meso-lymbic pathway and Dopaminergic tracts and interactions with other tracts (Russo and Nestler, 2013) **Figure 1.3** Illustration of addiction pathway (https://commons.wikimedia.org/wiki/File:%CE%94FosB.svg)

The neurons in the VTA and NA undergo a depletion of dopamine and dopamine receptors upon chronic use of many drugs of abuse leading to increased substance seeking behavior to continually meet the increasing demand for the substance (Blum et al., 2015). It is yet to be discovered how dopamine specifically contributes to the addiction state, initially it was thought that dopamine's hypothesized role in hedonic behavior was what lead to addiction. However, experiments have demonstrated that dopamine is not required for a hedonic response in animal studies (Berridge & Robinson 1998). Nonetheless it has been established that all drugs of abuse elicit a notable increase in synaptic dopamine in the nucleus accumbens. The primary source of this increase in synaptic dopamine has been identified as the ventral tegmental area where the somas of the dopamine releasing neurons are located (Johnson & North 1992, Jones et al. 1998, Tapper et al. 2004, Waldhoer et al. 2004, Justinova et al. 2005).

2. Abuse Liability

Abuse liability in drugs targeting the nervous system is typical measured by a ratio of dopaminergic to serotonergic activity. Those that, either by neurotransmitter release stimulation or re-uptake inhibition, increase synaptic concentrations of dopamine are typically considered to be at high risk for abuse. Recent findings however found that certain drugs that have moderate to significant serotonergic activity can decrease abuse liability of certain compounds.

Effects of Test Drugs on Transporter-Mediated Release from Rat Brain Synaptosomes

Test drug	[^{3}H]MPP$^+$ release via NET EC_{50} (nM)	[^{3}H]MPP$^+$ release via DAT EC_{50} (nM)	[^{3}H]5-HT release via SERT EC_{50} (nM)	NET/DAT ratio	DAT/SERT ratio
Mephedrone	62.7±17.1	49.1±8.32	118.3±25.9	0.78	2.41
Methylone	152.3±33.2	133.0±11.2	242.1±48.3	0.87	1.82
MDMA	54.1±8.9	51.2±6.3	49.6±5.4	0.95	0.97
Methamphetamine	13.8±3.1	8.5±1.4	1291.7±241.6	0.62	152.0

Table 2.1 Transporter Mediated Release from Rat Brain Synaptosomes (Bonano et al., 2014)

One of the prominent examples is 3,4-Methylenedioxymethamphetamine otherwise known as MDMA or ecstasy. MDMA has significant serotonergic and dopaminergic activity but shows relatively low abuse liability in animal studies. Meanwhile methamphetamine which is a highly addictive substance contains a significantly larger dopaminergic component. In addition a trend observed when modifying the *para* position of the phenyl group in methcathinone that the larger & more bulky the substituent group the more the molecule had decreased affinity for DAT and increased in SERT. This was demonstrated both by the ICSS and synaptosome experiments (Bonano et al., 2014).

Abuse liability in human subjects is a significantly intricate concept and methods to establish clear criteria for liability have yet to yield a simple answer. However currently as accepted by researchers and the United States Drug Enforcement Agency (DEA), the typical profile of a drug that has high abuse liability includes positive subjective effects, i.e. "euphoria" or a"high" sensation (Horton et al., 2013), but in experimental settings the DAT vs SERT selectivity of drugs is a good index to predict abuse liability (Bonano et al., 2014).

3. Monoamine Transporters

In addition to the regulatory mechanisms of the dopaminergic systems, the potential of effects of adrenergic and serotonergic systems have been implicated in the modulation or attenuation of drugs of abuse and abuse liability. These neuronal tracts, contain specific types of neurons called monoamine neurons that produce, store , and release monoamine neurotransmitters. The monoamine neurotransmitters in this case are norepinephrine, dopamine, and serotonin (Rothman & Baumann, 2003). These neurons contain the corresponding monoamine transporters (MATs) as well, in humans these would be hSERT, hDAT, and hNET.

The monoamine transporters all belong to the solute carrier 6 (SLC6) gene family formed by structurally related proteins that use the Na+ driving force to concentrate substrates against their chemical gradient (Kristensen et al., 2011). These MATs are localized to the perisynaptic clefts of the neurons and are crucial for the monoamine re-uptake and termination of monoamine transmission (Torres, Gainetdinov, & Caron, 2003). The MAT's contain 12 transmembrane domains with some conserved structures in between different transporters. There are 617 residues in hDAT, 620 residues in hNET, and 630 residues in hSERT. (Bruss, Hurt, Chen).

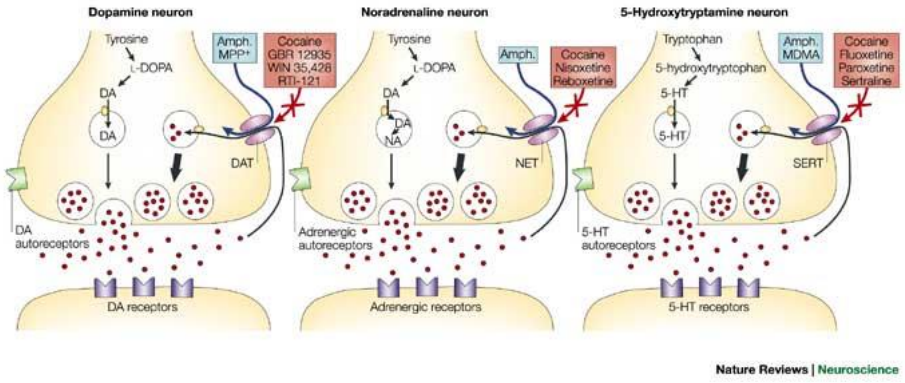


Figure 3.1 Illustration of localization of MATs (Torres et al., 2003)

The initially proposed mechanism of coupling was that the substrate would bind to the externally facing conformation of the transporter and that sodium and chloride mediated conformational changes in the transporter caused it to push into its inward facing conformation and transport the substrate along with a specific number of ions (Jadetzky, 1966). Experiments conducted on platelet serotonin (5HT) transport seemed to indicated that chloride ions specifically were required to transport serotonin in either direction. Nelson and Rudnick further established that it was not an electrogenic demand for chloride but an allosteric one. They did so by substituting other more electronegative compounds, that did not improve transport, as well as

changing the membrane potential. Even after changing membrane potential chloride was need for serotonin transport. In the experiments chloride ions lowered the Km and raised the Vmax for 5HT transport strongly supporting the assertion that chloride had a direct effect on the transporter. In addition, the transport of serotonin required the presence of intracellular potassium for efficient transport of 5HT (Nelson & Rudnick 1982; Nelson & Rudnick 1979). It was further discovered that serotonin transport required sodium to be bound and transported. The transport seemed to depend on the stoichiometric coupling of sodium and 5HT due to the simple kinetics and saturability of the transporter with relation to the sodium gradient. It was also concluded, based on the experimental data, that serotonin and sodium were transported in a 1:1 ratio (Talvenheimo, Fishkes, Nelson & Rudnick, 1983) . Based upon the prior experiments if 1 Na+ goes in and 1 K+ goes out in the transport of serotonin it should be an electroneutral mechanism with no current (Gu et al, 1994).

Unexpectedly, however, an experiment conducted by Mager et al in 1994 in *Xenopus* oocytes expressing a cloned rat 5-HT transporter showed three separate currents. The first current that was identified was a transport associated current upon addition of 5HT to the solution, and was maintained while 5HT was in solution and expired after removal of 5HT. The second current they identified was a transient current that occurred in the absence of 5HT and triggered by strong hyperpolarization and was blocked by the addition of 5HT. The third and final current was a small leak current observed in the absence of 5HT and it is blocked by uptake inhibitors. The most notable of the three currents, with regards to the function of the transporters, was the first current (the substrate-induced current). According to prior research 5HT transport was purported to be electroneutral and yet there was a clear transport associated current in the

oocytes. The experiment also postulated that the conducting states of SERT reflected the behavior of ionic channels rather than carriers (Mager et al, 1994).

Further experiments demonstrated that the premise of the alternating access theory to be inaccurate or incorrect. In a cut-open oocyte voltage clamp experiment Adams and DeFelice discovered the transporter was simultaneously accessible to sodium and chloride ions binding internal and externally, while also asserting that the large transport associated current was indicative of the transporter behaving like an ion channel rather than a solute carrier. Varying internal and external ionic concentrations were used while simultaneously measuring current across the membrane to validate these assertions. It was also posited that K+ did not in fact compete with 5HT binding on the inward facing portion of the transporter based on competition binding assays (Adams & Defelice, 2003).

Further experiments in hDAT also supported the hypothesis that the MATs mechanism was akin to that of an ion channel. Using two electrode voltage clamping experiments in *Xenopus* oocytes it was established that the transport current seen in hDAT were significantly greater than expected based on the assumed fixed stoichiometric ratio of 2 Na+:1Cl-:1 Dopamine (DA+) (Sonders et al., 1997). The effect was also demonstrated in hNET in which the stoichiometric prediction did not match the measured current generated by the transport. However the assertions in the final experiments seek to reconcile the original and rising theories behind monoamine transport, suggesting that the MATs have multiple modes of transport (Galli, Blakely, DeFelice, 1996).

The drugs that act on the transporters typically fall into two categories. The first category being those termed ‘inhibitors’ that bind the transporter to block neurotransmitter uptake. The second type termed ‘substrates’ bind to the transporter, are subsequently transported into cells,

and typically evoke release of neurotransmitters by reverse transport (Kahlig et al, 2005; Khoshbouei et al, 2003; Rothman and Baumann, 2006; Scholze et al, 2000). Many drugs that are substrates on the specific transporters share structural and biochemical similarities to the endogenous neurotransmitters associated with the transporter. It should be noted that while each transporter corresponds to a specific neurotransmitter, both hNET and hDAT transport dopamine and norepinephrine.

Not only can substrates and blockers be differentiated by the binding mechanism and downstream effects, but also by electrophysiological signature. Substrates, by way of the transport mechanism & transporter, induce an inward sodium current inducing a localized depolarization of the membrane. Meanwhile blockers hinder the constitutive "leak" current producing an apparent outward hyperpolarizing current. (Cameron et al, 2013; Solis, 2017; Solis et al, 2012). It has been suggested that the magnitude of substrate-induced current of a substrate is correlated with long-term depletion of neurotransmitter in the brain (Baumann et al, 2014a; Fleckenstein et al, 2007).

4. Voltage-gated Calcium Channels

Monoamine transporters induced current by taking advantage of the sodium differential across the membrane and use the gradient of ions to transport neurotransmitters/substrates (Nelson & Rudnick, 1979). The depolarization by the monoamine transporters leads to affected activity of membrane ion channels. Membrane ion channels are membrane proteins that form pores in the lipid bilayer and help to sustain the resting membrane potential. In addition, voltage-gated channels that modulate ion conduction as a function of the electrical potential across the membrane are important to generate action potentials in excitable cells (Hille, 2001).

What makes calcium channels such an important researched topic with relation to MATs is the role of calcium as a secondary messenger. Calcium signaling controls cellular functions from vesicle release to muscular contraction, and is one of the most important secondary messengers in the body. Calcium entry that is coupled with transporter activation is also an excellent tool for measuring compound activity upon the monoamine transporters. Upon activation of voltage-gated calcium channels there is an increase in intracellular level of calcium that triggers presynaptic vesicular fusion and neurotransmitter release (Catterall, 2000).

The intracellular concentration of Ca2+ is maintained at a concentration of about 100nM (Triggle, 2006), while extracellular concentrations are 10,000 times greater at about 1mM creating a massive gradient. Calcium becomes toxic above certain concentrations and due to its role as a secondary messenger intracellular concentrations must be tightly regulated (Simmons, 1988). A portion of the pumps that participate in calcium homeostasis reside in the plasma membrane, and a portion reside on the endoplasmic reticulum, where calcium may also be stored and released from. The plasma membrane Ca2+ ATPase (PMCA) functions together with the Na+/Ca2+ exchanger (NCX) at the plasma membrane in order to maintain the Ca2+ homeostasis. The NCX is classified as low-affinity, high-capacity, this classification indicates that it can help clear large amounts of calcium at high concentrations normally to restore resting levels of Ca2+ after activation (Brini and Carafoli, 2001). PMCA on the other hand is a high-affinity and low-capacity pump that keeps the resting level of calcium in quiescence.

The L-Type calcium channels are distinguished by their relatively long activation and high-voltage threshold. T-type channels on the other hand are distinguished by their low voltage threshold. Both types are found in neurons (Tsien et al., 1988). Using whole-cell voltage clamping of dorsal root ganglion neurons N-type channels were identified. The N-type channels

require both a strong negative potential for removal of inactivation as well as a strong depolarization for activation (Nowycky, 1985).

Ca^{2+} channel	Ca^{2+} current type	Primary localizations	Previous name of α_1 subunits	Specific blocker	Functions
$Ca_V1.1$	L	Skeletal muscle	α_{1S}	DHPs	Excitation-contraction coupling Calcium homeostasis Gene regulation
$Ca_V1.2$	L	Cardiac muscle Endocrine cells Neurons	α_{1C}	DHPs	Excitation-contraction coupling Hormone secretion Gene regulation
$Ca_V1.3$	L	Endocrine cells Neurons	α_{1D}	DHPs	Hormone secretion Gene regulation
$Ca_V1.4$	L	Retina	α_{1F}		Tonic neurotransmitter release
$Ca_V2.1$	P/Q	Nerve terminals Dendrites	α_{1A}	ω-Agatoxin	Neurotransmittler release Dendritic Ca^{2+} transients
$Ca_V2.2$	N	Nerve terminals Dendrites	α_{1B}	ω-CTx-GVIA	Neurotransmitter release Dendritic Ca^{2+} transients
$Ca_V2.3$	R	Cell bodies Dendrites	α_{1E}	None	Ca^{2+}-dependent action potentials
		Nerve			Neurotransmitter release
		Terminals			
$Ca_V3.1$	T	Cardiac muscle Skeletal muscle Neurons	α_{1G}	None	Repetitive ring
$Ca_V3.2$	T	Cardiac muscle Neurons	α_{1H}	None	Repetitive ring
$Ca_V3.3$	T	Neurons	α_{1I}	None	Repetitive ring

Table 4.1 Calcium channel sub-types (Catterall, 2000)

P-type calcium channels were first identified in Purkinje neurons in 1989 (Llinas, Sugimori & Cherksey, 1989). The defining characteristic of the P-type channel was that they could be blocked by at low concentrations peptide toxin ω-Aga-IVA while the T-type, L-type, & N-type currents were not affected (Mintz, Adams & Bean, 1992). The Q-type channels are distinguished by their lower sensitivity to blockage by peptide toxin ω-Aga-IVA . Finally the R-Type, first recorded in cerebellar granule neurons, are distinguished by their resistance to the subtype-specific organic and peptide calcium channel blockers (Tsien & Randall, 1995).

Upon purification and analysis of calcium channels it was discovered that, unlike cardiac and skeletal muscles Ca2+ channels, neuronal Ca2+ channels contain α1, α2δ, and □ subunits but no □ subunit. The α1 is the primary subunit forming the actual ionic pore and the voltage sensing domain. Whereas the other subunits act as modulators of the activity of the calcium channel (Ahlijanian, 1990).

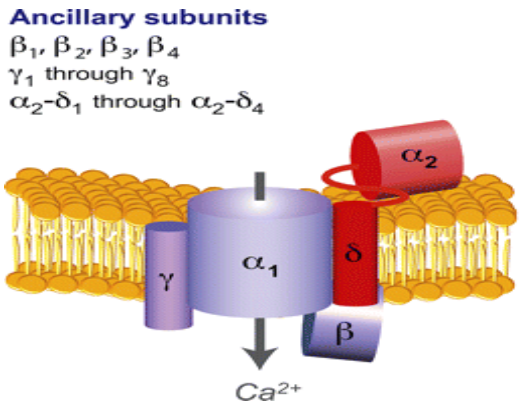


Figure 4.1 Illustration of Calcium Channel Structure (Khosravani, 2006)

In experimental studies it was determined that expression of the α1 subunit alone was sufficient to produce functional Calcium channels. Despite being functional, the expressivity of the channels with only the α1 subunit was low. In addition the channel kinetics and voltage dependence of the calcium current were abnormal (Perez-Reyes et al., 1989). It was later discovered by using muscle cells and including different subunits that the inclusion of the α2δ subunit in addition to the □ subunit greatly normalized the behavior of the calcium channels and presented data in line with "normal" gating function (Lacerta et al., 1991). And in 1996 it was established, by use of transgenic animals, that □ subunit was necessary for L-type channels to function (Gregg et al., 1996).

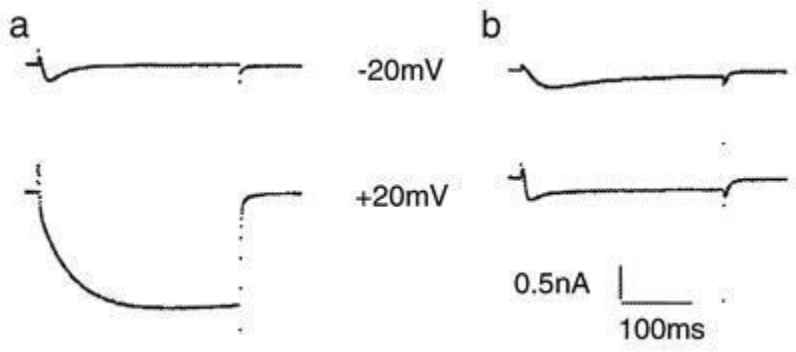


Figure 4.2 Voltage clamp experiments on myocytes of control animals (a) and of □-null animals (b) (Gregg et al., 1996).

After demonstrating that the □1-□4 subunits were present in the cerebral cortex by immunoblotting, the effects of each subtype were analyzed. It was determined using oocytes with the α1C (CaV1.2) subunit that the □1,3, & 4 subunits were permissive to voltage dependent calcium channel facilitation, whereas the □2 subunit was not. (Pichler et al., 1997; Cens, Mangoni, Richard, Nargeot & Charnet, 1996) Using oocytes again it was ascertained that the □3 subunit allowed for larger currents and had a faster inactivation time than both the □2a and □2b subunits (Hullin et al., 1992).

5. Monoamine Transporters & Calcium Channel Coupling

Using whole cell voltage clamping a significant difference was observed not only between the L-type (CaV 1.2 & 1.3) and N-type channels studied (CaV 2.2), but also among the L-types. The CaV1.3 channels opened at very negative potential requiring very little change in voltage to activate them, and reaching half-conductance at negative voltages. The CaV 1.2 channels opened at moderately negative potentials requiring a moderate to significant change in voltage to activate, and reaching half-conductance at a slightly negative voltage. While the CaV 2.2 opened at potentials closer to 0 mV requiring a very significant change in voltage to activate, and

reaching half-conductance at values above 0 mV.

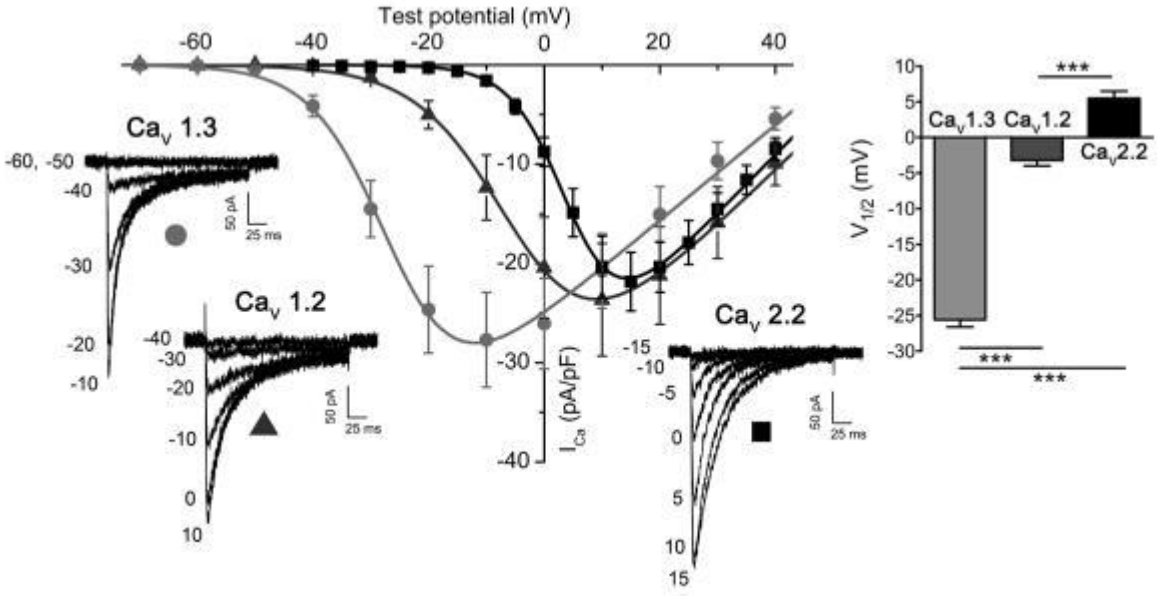


Figure 5.1 Voltage dependence of CaV1.2, CaV1.3 and CaV2.2- mediated Ca2+ currents: HEK293T cells were co-transfected with β3, α2δ, and EGFP expression plasmids plus alternatively CaV1.3, CaV1.2 or CaV2.2 plasmids. The Ca2+ current (ICa) recordings were carried out at room temperature under constant perfusion. Test pulses in 5 mV steps for CaV2.2 or 10 mV steps for CaV1.2 and CaV1.3 were applied from a holding potential of −80 mV. Representative responses are shown for CaV1.3 (light grey circle), CaV1.2 (dark grey triangle) and CaV2.2 (black square) and the magnitude of the test potentials are indicated in mV(Cameron et al. 2015).

Through experimentation with HEK293 cell lines expressing the human SERT and DAT it was discovered the transport of both endogenous and exogenous substrates not only induced a current but also indirectly activated voltage-gated calcium channels.

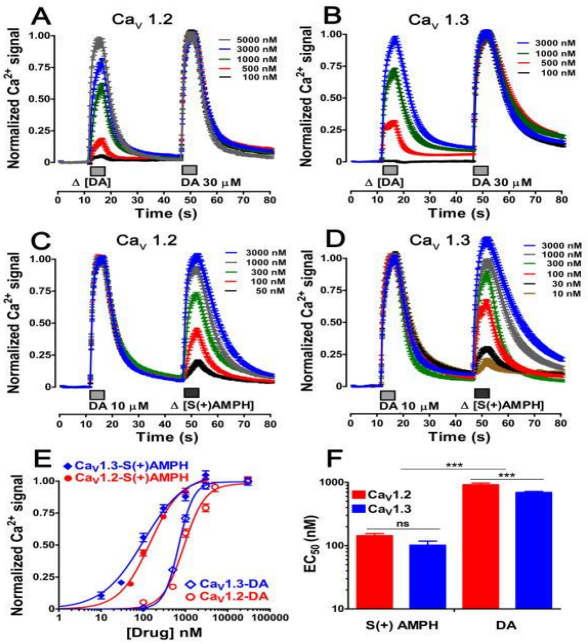


Figure 5.2 S(+)AMPH is more potent than DA producing Ca2+ signals in L-type Ca2+ channel-expressing Flp-hDAT cells. Traces and dose response in CaV 1.3 & CaV 1.2 channels (Cameron et al., 2015).

The experiments established that the transporter-associated current was capable of opening CaV 1.3 channels, which are commonly found in serotonergic neurons responsible for pacemaking. It was also discovered that transport could activate CaV1.2 calcium channels, abundant in cardiac cells but are also present in the central nervous system. The transport was not able to activate the neuronal-type CaV2.2 channels that were transiently transfected into HEK293 cells (Ruchala et al., 2014; Cameron et al., 2015).

6. N-Alkyl 4-Methylamphetamine

A drug first synthesized and studied in the 1950's, 4-methlyamphetamine (4-MA) temporarily faded into obscurity until about 2009. 4-MA made its resurgence in the European drug market, being marketed/sold as "speed" (EMCDDA, 2012). Preclinical research established that 4-MA is a non-selective transporter substrate, releasing both dopamine & serotonin from neurons *in vitro* and *in vivo* (Bauman et al., 2011; Wee et al., 2005). While there is only a modicum of investigation into the pharmacological effects of 4-MA there is even less

investigation into the 4-MA analogs. It was for this reason that Solis et al. decided to investigate the effects of lengthening the N-alkyl chain of 4-MA and its effects on the interactions at DAT, NET, and SERT. The amine group was the primary site of modification based upon prior amphetamine and cathinone studies that explored amine, phenyl, and alkyl group substitutions (Rothman et al, 2012; Saha et al, 2015; Simmler et al, 2014).

Solist et al. conducted, ICSS, synaptosome, HEK cell calcium assay, and *Xenopus* oocyte two-electrode voltage clamp experiments to analyze in depth the effects of lengthening the amine substituents.

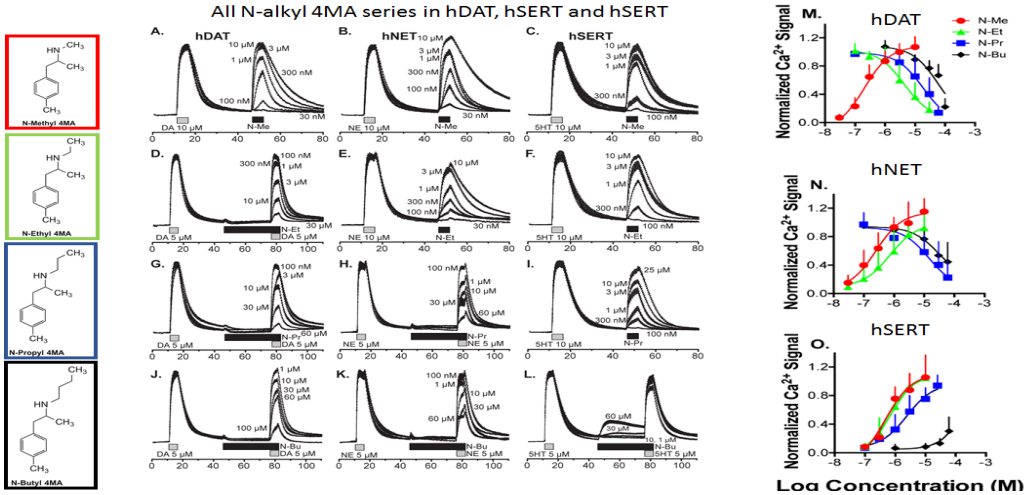


Figure 6.1 Trace overlays and dose response curves of HEK cell Calcium Screening assays of N-Alkyl 4MA analogs (Solis et al, 2017)

It was discovered that the N-alkyl chain length is negatively correlated with the drug potency on the transporters across all experimental condition tested. It was also discovered that as the chain length increased the drug shifted from a substrate to a blocker in some of the transporters. In hDAT N-Methyl 4-MA was a substrate while N-Ethyl, Propyl, and Butyl 4-MA were all blockers. In hNET the transition happened between N-Ethyl and N-Propyl, while in hSERT the transition did not happen at all (Solis et al., 2017). In addition studies done in synaptosomes and using the Ca2+ assays to study the pharmacology of these drugs, agreed that lengthening the N-alkyl chain of 4MA, gradually decreases the DA releasing properties but

keeping the 5HT releasing effect of these drugs. This observations were further corroborated in *in vivo* assays using ICSS studies in rats, showing a decrease in abuse liability as the N-alkyl chain is lengthened on the 4MA scaffold.

II. Experimental Objectives

The objective of this study is to determine the potency and efficacy of stereoisomers of *N*-alkyl- 4-Methyl Amphetamines to produce or to block electrical currents through monoamine transporters. The understanding of the pharmacological profile that these compounds have on hDAT, hSERT and hNET are important to predict: 1) its abuse liability and 2) its potential therapeutical use.

III. Materials & Methodology

1. Experimental Procedure

To analyze the drug effects in hSERT, hDAT, and hNET previously established Human Embryonic Kidney (HEK) cell lines with the transporters expressed were utilized. The cells were stored in liquid nitrogen for long term storage. The HEK cells would be removed from the liquid nitrogen, plated in Dulbeco's Modified Eagle Medium (DMEM) in addition to 1% penicillin & streptomycin and 10% Hyclone fetal bovine serum. The cells were stored in incubators at physiological temperature and pH.

Three days prior to planned Calcium screening assay, the cells would be transferred to 96-well plates that had the wells coated in Matrigel (Corning) so as to insure that cells would not be dislocated when exposed to the perfusion of solution. The typical confluency at initial plating was about 20-40%.

After the cells had attached to the matrigel they needed to be transfected with the necessary calcium channels and subunits. For the purpose of consistency all cell lines were transfected with CaV 1.2 alpha, beta 3 subunit, alpha 2 delta subunit, and EGFP. The addition of EGFP to the transfection mix was to assist in locating transfected cells underneath the microscope for analysis.

The transfection agent used was Fugene 6. The transfection mix was injected directly above the cells in the DMEM media and allowed to continue transfection for a minimum of 4 hours and a maximum of 7 hours. After the transfection was complete the media was drained and replaced with DMEM containing 1ug/mL of doxycycline to induce expression of the transporters. Three days after transfection is completed and the media is changed the cells are removed from the incubator and the plate is cut so that only select numbers of wells remain exposed to the room temperature during the experiment. After cutting the plate the lanes to be used for experimentation immediately are drained of their current media and loaded with 50uL of imaging solution with 2 ug/mL of Fura-2AM. After a 25 minute loading time solution is drained again and the cells are washed for 20 minutes with imaging solution.

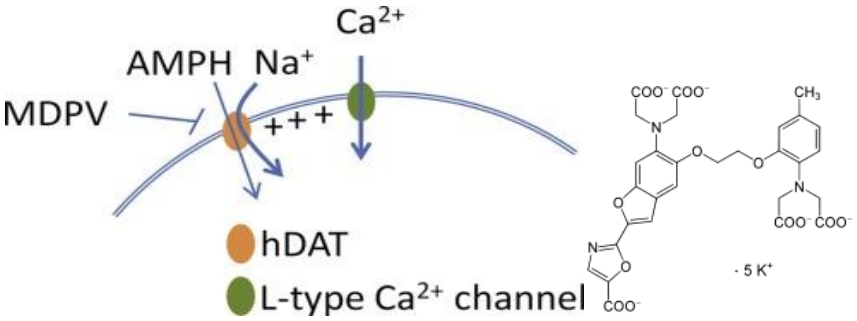


Figure 8.1 Illustration of Calcium as a biosensor (Cameron, et al, 2015) Structure of Ratiometric imaging dye Fura-2 (Fura-2).

The plates are then placed on an Olympus oil microscope with an attached imaging, recording, and perfusion system managed by the LiveAcquisition software. The camera takes 3 pictures per second at two different wavelengths. The two protocols used are the substrate and

blocker protocols. For both protocols the perfusion system is placed slightly above the cells, with suction to prevent overflow, and the cells are washed with imaging solution (IS) for 10-20 seconds. The beginning of each protocol is similar, they both start with a 10 second IS wash were images are acquired.

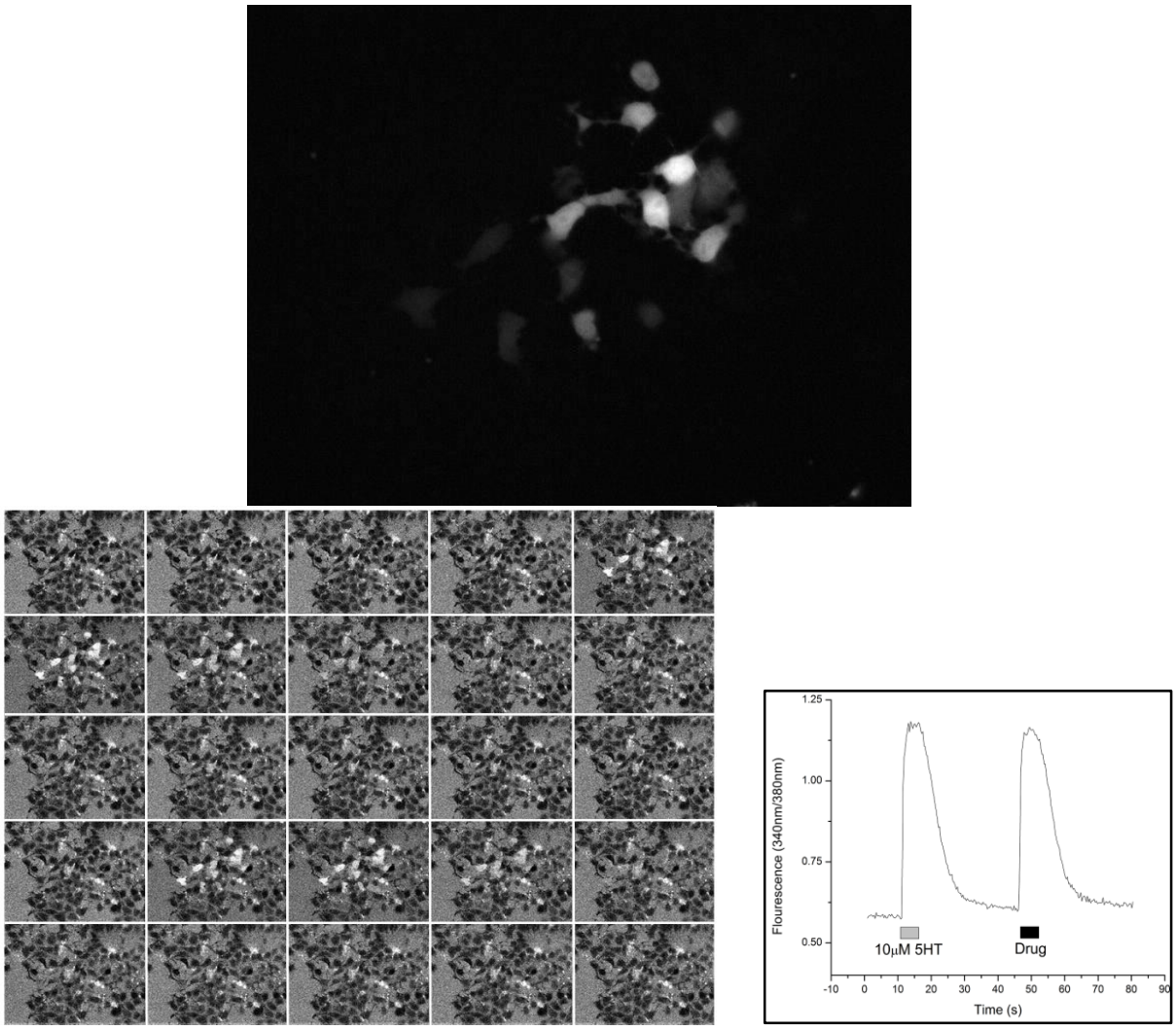


Figure 8.2 Image of GFP fluorescence under the microscope. Montage of images & Fluorescence Ratio Trace captured from a representative experiment. R(-)-N-Methyl 4-MA in hSERT.

Then both protocols have a 5s exposure to the control (5HT, DA, or NE), with images being captured. After the 5 second exposure to the control there is a 30 second wash with images being captured. In the substrate protocol after the 30 second wash there is a 5 second exposure to the compound being tested followed by another 30 second wash and the completion of the

experiment. In the blocker protocol the initial 30 second wash is followed by a 30 second exposure to the test drug, followed by a 5 second exposure to the control and the test drug at the same time, followed by a 30 second wash and completion of the experiment.

2. Data Analysis

After the experiments are done the images are analyzed by an offline analysis software. Regions of interest (ROIs) are selected manually after visual and objective analysis determined the cells to be within the accepted range. The primary measure for selecting cells/placing ROIs is to make sure the cells are not dislocated, do not burst, or are not affected by a calcium wave from other cells that is not due to the experiment. Typically about 5-7 cells are selected per well, each concentration from a dose response has approximately 3-5 wells for experimentation, and experiments are typically done on 2-3 separate days to evaluate consistency within the data gathered. Typical cells that had a clear response to the control and were not dislocated or affected by a calcium wave were assumed to be acceptable for analysis.

Data was then compiled and statistically analyzed to determine the EC/IC 50 values of each compound. To perform the analysis the selected ROIs for each well were compiled into separate files depending on the concentration being tested. Next the average of all the ROIs was used to select a maximum value range for the control and the test peaks. The maximum test values was divided by the maximum control values. For substrates the ratio corresponded to the excitation by the test drug, while for blockers it corresponded to the amount of signal from the control that was blocked. Trace overlays, created in Origin, had all values normalized to the maximum response of the control, so that all traces would be displayed as values between 0.0 and 1.0.

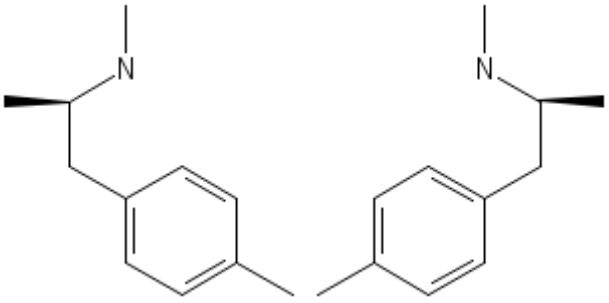
Dose response curves were generated using GraphPad's Prism software by inputting the data and using the equation below:

$$Y(x) = (Y\text{max}) / 1 + 10\text{exp}[(\log EC50 - \log x) * n]$$

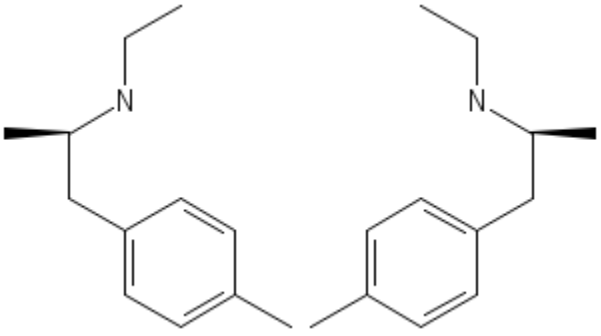
In the equation, x is the concentration of experimental compound; $Y(x)$ is the measured response; EC50 is the concentration of compound that gives rise to the half-maximal response; and n is the Hill slope parameter.

3.Compounds under study

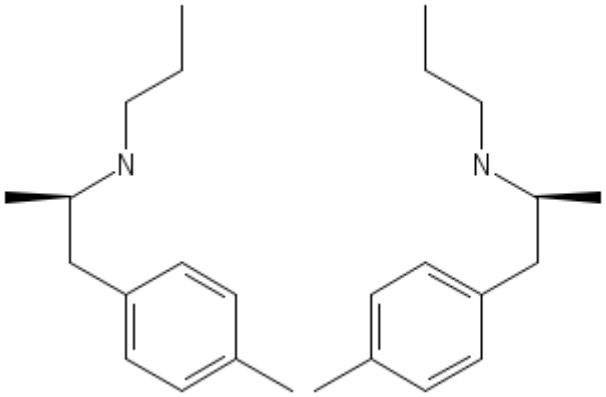
The compounds analyzed in the research presented in this paper are the enantiomers of N-methyl-1-(4-methylphenyl)propan-2-amine, N-ethyl-1-(4-methylphenyl)propan-2-amine, and N-propyl-1-(4-methylphenyl)propan-2-amine. The names are abbreviated below as S(+)/R(-)-N-Methyl 4-MA, S(+)/R(-)-N-Ethyl 4-MA, and S(+)/R(-)-N-Propyl 4-MA.



S(+)-N-Methyl 4-MA R(-)-N-Methyl 4-MA



S(+)-N-Ethyl 4-MA R(-)-N-Ethyl 4-MA



S(+)-N-Propyl 4-MA R(-)-N-Propyl 4-MA

Figure 10.1 Structure of tested compounds

IV. Results & Figures

As we have shown before the co-expression of voltage-gated Ca2+ channels and MATs in HEK cells is a useful tool to study the interaction between a test drug and MATs (Cameron et al., 2015; Ruchala et al., 2014). As described above, substrates of MAT activate an inward current through the transporter that depolarizes the plasma membrane and this depolarization is strong enough to open L-type Ca channels (Cameron et al., 2015; Ruchala et al., 2014). Since cells used in this study are loaded with the Ca2+ sensor (fura 2), the opening of the Ca2+ channels produce an instantaneous intracellular Ca2+ signal that is visualized as an increase in the ratio of fluorescence emission 340 nm/380 nm using fluorescence microscopy (fig 8.2).

Using this experimental setting we are able to detect substrates of monoamine transporters, since they produce reliable Ca2+ signals, whereas blockers do not generate Ca2+ signals but can antagonize the action of a known substrate.

The racemate N-Methyl 4MA showed an EC50 of 0.21 ± 0.02 uM, 0.25 ± 0.04 uM and 0.48 ± 0.10 uM to produce Ca2+ signals when tested in cells expressing CaV1.2 and hDAT,

hNET or hSERT respectively (Solis, 2017). The S(+) N-Methyl 4MA enantiomer tested in the same experimental setting showed an EC50 of 0.25 ± 0.035 uM, 0.19 ± .01 uM and 0.461 ± 0.04 uM for hDAT, hNET and hSERT, respectively. These results clearly show that the S(+) enantiomer of N-Methyl 4MA is responsible for most of the potency of the racemate compound. When the R(-)Methyl 4MA was tested in comparable experimental conditions, the EC50 observed were 3.20 ± 1.19 uM, 0.19 ± 0.01 uM and 3.70 ± 0.40 uM for cells expressing hDAT, hNET and hSERT, respectively. As seen for others amphetamine analogs, the R(-) enantiomer of N-Methyl 4MA show about one log unit less potency than the S(+) enantiomer for hDAT and hSERT cells. In contrast, S(+) and R(-) enantiomer of N-Methyl 4MA are equipotent in hNET expressing cells. In all cases studied the S(+) and R(-) enantiomers of N-Methyl 4MA were fully efficacious in generating Ca2+ signals suggesting that both enantiomers work as substrates in all three MATs.

The racemate N-Ethyl 4MA showed 4.35 ± 0.61 uM (IC50), 0.73 ± 0.18 uM (EC50) and 0.68 ± 0.09 uM (EC50) to block or produce Ca2+ signals when tested in cells expressing CaV1.2 and hDAT, hNET or hSERT respectively (Solis, 2017). The S(+) N-Ethyl 4MA enantiomer tested in the same experimental setting showed 2.73 ± 0.16 uM (IC50), 0.57 ± .08 uM (EC50) and 0.57 ± 0.01 uM (EC50) for hDAT, hNET (with CaV1.3) and hSERT, respectively. The data obtained using the CaV1.2 sub-type does not exhibit enough of a dynamic range to be an accurate predictor of the EC50 in hNET with N-ethyl enantiomers. For the sake of the results description the data gathered by Harris will be used for comparison (Harris, 2016). When the R(-)Ethyl 4MA was tested in comparable experimental conditions, the IC/EC50 observed were 44.49 ± 17.04 uM (IC50), 1.17 ± 0.21 uM (EC50) and 1.62 ± 0.14 uM (EC50) for cells expressing hDAT, hNET and hSERT, respectively. As seen for others amphetamine analogs, the

R(-) enantiomer of N-Ethyl 4MA show around one log unit less potency than the S(+) enantiomer for hDAT, hNET (when using data gathered with CaV1.3 channels), and hSERT cells (Harris, 2016). In hDAT the S(+) and R(-) enantiomers of N-Ethyl 4MA were fully efficacious in blocking Ca2+ signals suggesting that both enantiomers work as blockers in hDAT. In hSERT the enantiomers generated an equivalent calcium signal were fully efficacious substrates. However in hNET the enantiomers did not generate Ca2+ signals equivalent to the control and only reached 80% efficacy for the S(+) and 65% efficacy for the R(-) as substrates. These results suggest that the S(+) enantiomer of N-Ethyl 4MA is responsible for most of the potency of the racemate compound.

The racemate N-Propyl 4MA showed 18.26 ± 3.60 uM (IC50), 14.35 ± 3.56 uM (IC50) and 0.68 ± 0.09 uM (EC50) to block or produce Ca2+ signals when tested in cells expressing CaV1.2 and hDAT, hNET or hSERT respectively (Solis, 2017). The S(+) N-Propyl 4MA enantiomer tested in the same experimental setting showed 6.60 ± 2.30 uM (IC50), 6.66 ± 1.50 uM (IC50) and 0.872 ± 0.12 uM (EC50) for hDAT, hNET and hSERT, respectively. When the R(-) N-Propyl 4MA was tested in comparable experimental conditions, the IC/EC50 observed were 37.92 ± 2.79 uM (IC50), 26.00 ± 3.51 uM (IC50) and 1.62 ± 0.14 uM (EC50) for cells expressing hDAT, hNET and hSERT, respectively. In hDAT the S(+) and R(-) enantiomers of N-Propyl 4MA were fully efficacious in blocking Ca2+ signals suggesting that both enantiomers work as blockers in hDAT. In hSERT the S(+) generated a fully efficacious Ca2+ signal acting as a substrate. The R(-) blocked calcium signal in hSERT at low concentrations but did generate some Ca2+ signal at very high concentrations giving the impression that it is a weak/partial substrate. In hNET the enantiomers both blocked Ca2+ signal, the S(+) was almost fully efficacious, and since R(-) only blocked ~50% of the signal at 30uM, further concentrations were

not tested. These results suggest that the S(+) enantiomer of N-Propyl 4MA is responsible for most of the potency of the racemate compound.

Drug	**hDAT (uM +/- SEM)**	**hNET (uM +/- SEM)**	**hSERT (uM +/- SEM)**
N-Methyl 4MA	**0.21 ± 0.02**	**0.25 ± 0.04**	**0.48 ± 0.10**
(S+)-N-Methyl 4MA	0.25 ± 0.035*	0.19 ± .01	0.461 ± 0.04*
(R-)-N-Methyl 4MA	3.20 ± 1.19	0.19 ± .01	3.70 ± 0.40
N-Ethyl 4MA	**4.35 ± 0.61**	**0.73 ± 0.18**	**0.68 ± 0.09**
(S+)-N-Ethyl 4MA	2.73 ± 0.16*	0.23 ± ~0 [CaV1.2] 0.57 ± .08 [CaV 1.3]*	0.57 ± 0.01 *
(R-)-N-Ethyl 4MA	44.49 ± 17.04	N/A** [CaV 1.2] 1.17 ± 0.21 [CaV1.3]	1.62 ± 0.14
N-Propyl 4MA	**18.26 ± 3.60**	**14.35 ± 3.56**	**2.44 ± 0.39**
(S+)-N-Propyl 4MA	6.60 ± 2.30*	6.66 ± 1.50*	0.872 ± 0.12

(R-)-N-Propyl 4MA	37.92 ± 2.79	26.00 ± 3.51	4.39 ± 1.38

Table 11.1. Calculated EC/IC50. Darkened cells indicate compounds that were blockers, while cells with no fill indicate substrates.* indicates that the S(+) enantiomer IC/EC50 values are significantly different at $p<0.01$ compared to the R(-) enantiomer based upon an unpaired t-test. ** Signal too small to estimate a reliable potency. CaV1.3 data from (Harris, A.C.). All data gathered using CaV1.2 unless stated otherwise.

Drug	**hDAT (nM +/- SEM)**	**hNET (nM +/- SEM)**	**hSERT (nM +/- SEM)**
(S+)-N-Methyl 4MA	109.2%±4.5	101.7%± 1.9	94% ± 2.9
(R-)-N-Methyl 4MA	98%±13.7	90.1%± 1.8	107% ± 7.6
(S+)-N-Ethyl 4MA	95.3%± 2.5	57.2%± 3.1 [CaV1.2] 87.5% ±2.1 [CaV 1.3]	91.3% ± 3.6
(R-)-N-Ethyl 4MA	100% ± 18.9	~35% [CaV 1.2] 67.5%± 2.9 [CaV1.3]	96% ± 3.3
(S+)-N-Propyl 4MA	100%± 3.0	~90%	83% ± 2.8
(R-)-N-Propyl 4MA	76% ± 3.5	>50%	59.8%± 7.2

Table 11.2 Calculated Efficacy, Darkened cells indicate compounds that were blockers, cells with no fill indicate substrates.

The IC/EC50 curves below and the traces overlays are visual representations of the data discussed and in the table above.

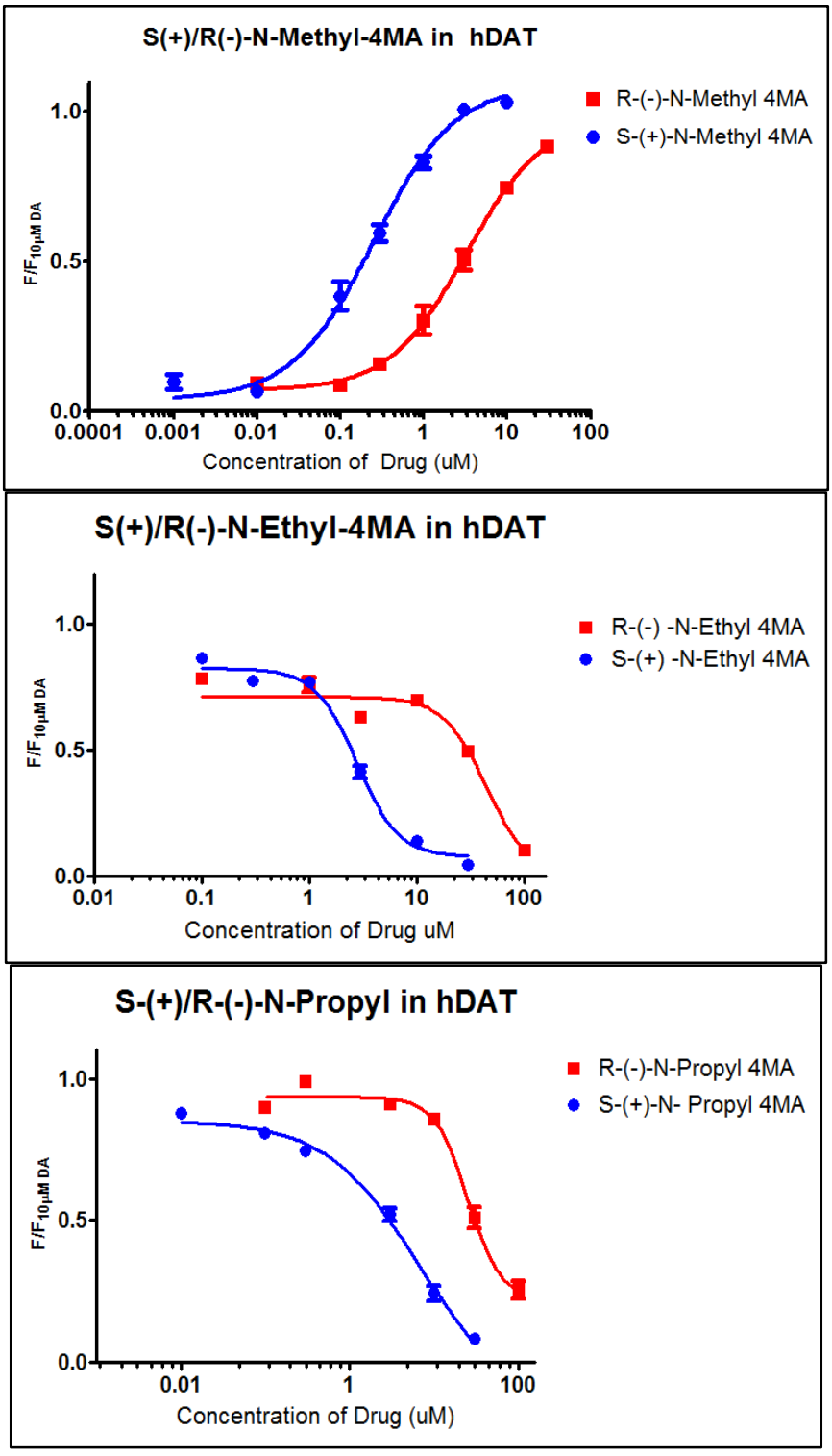


Figure 11.1 hDAT EC/IC50 Dose Response Curves

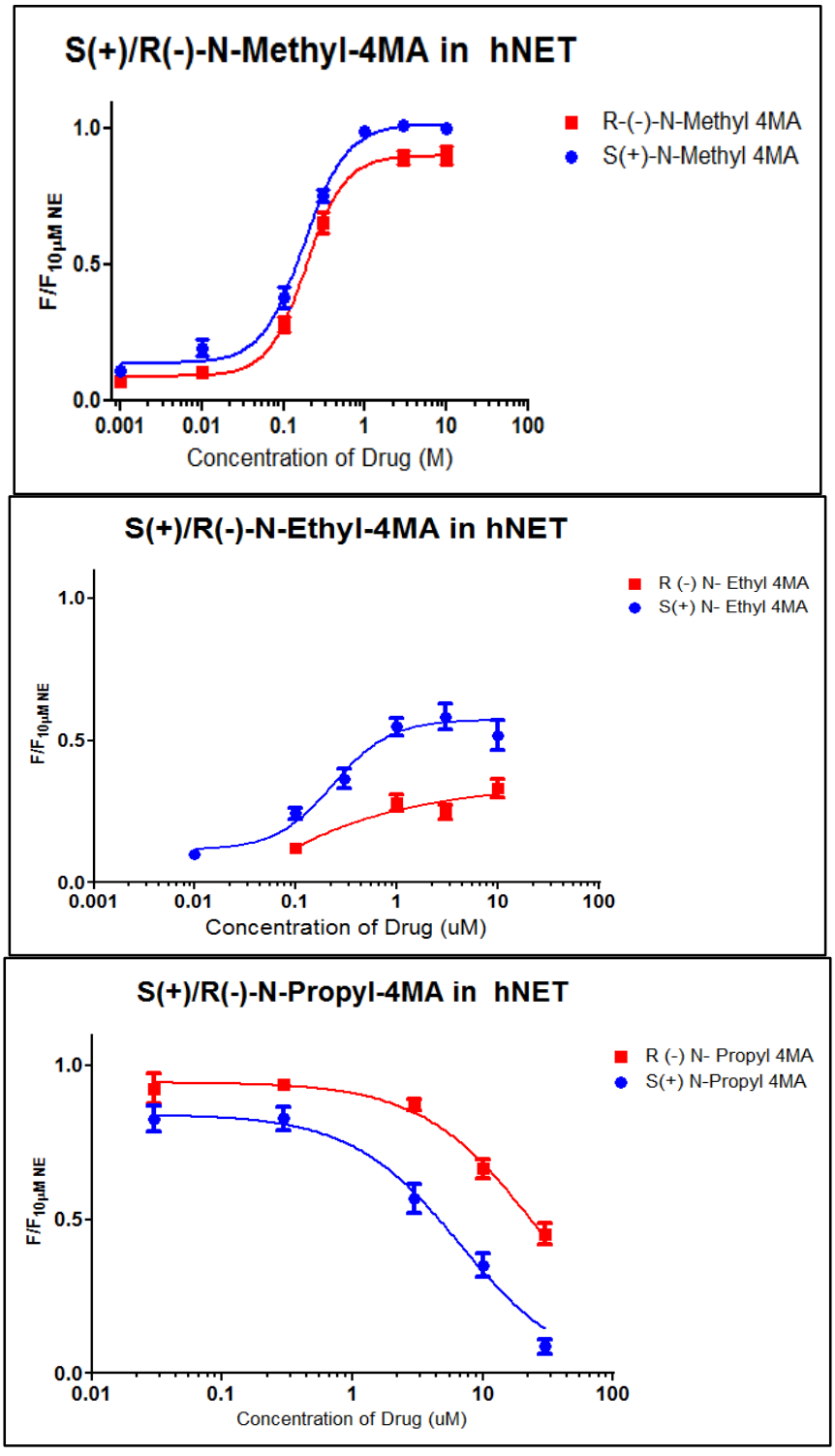


Figure 11.2 hNET EC/IC50 Dose Response Curves

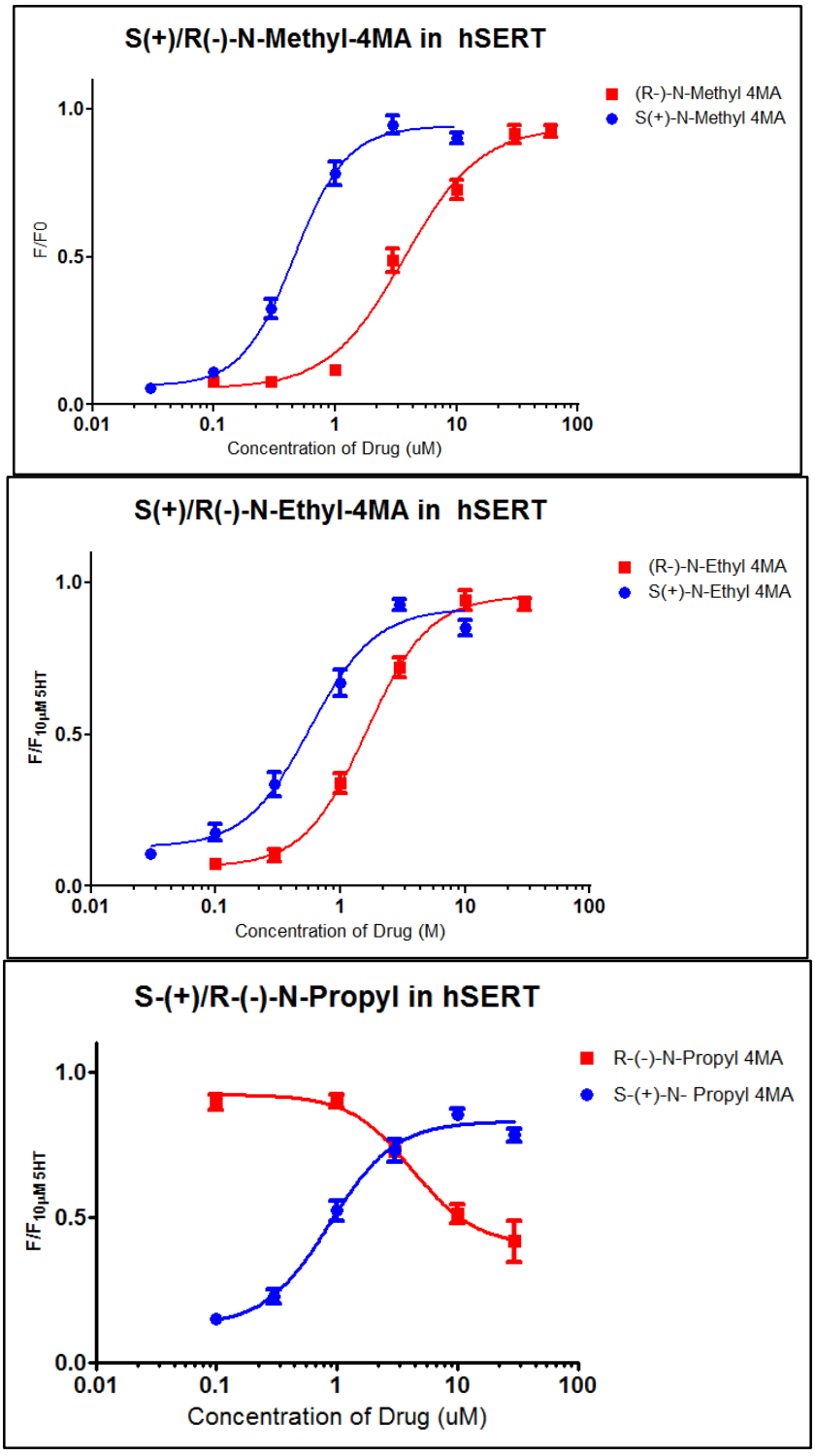


Figure 11.3 hSERT EC/IC50 Dose Response Curves

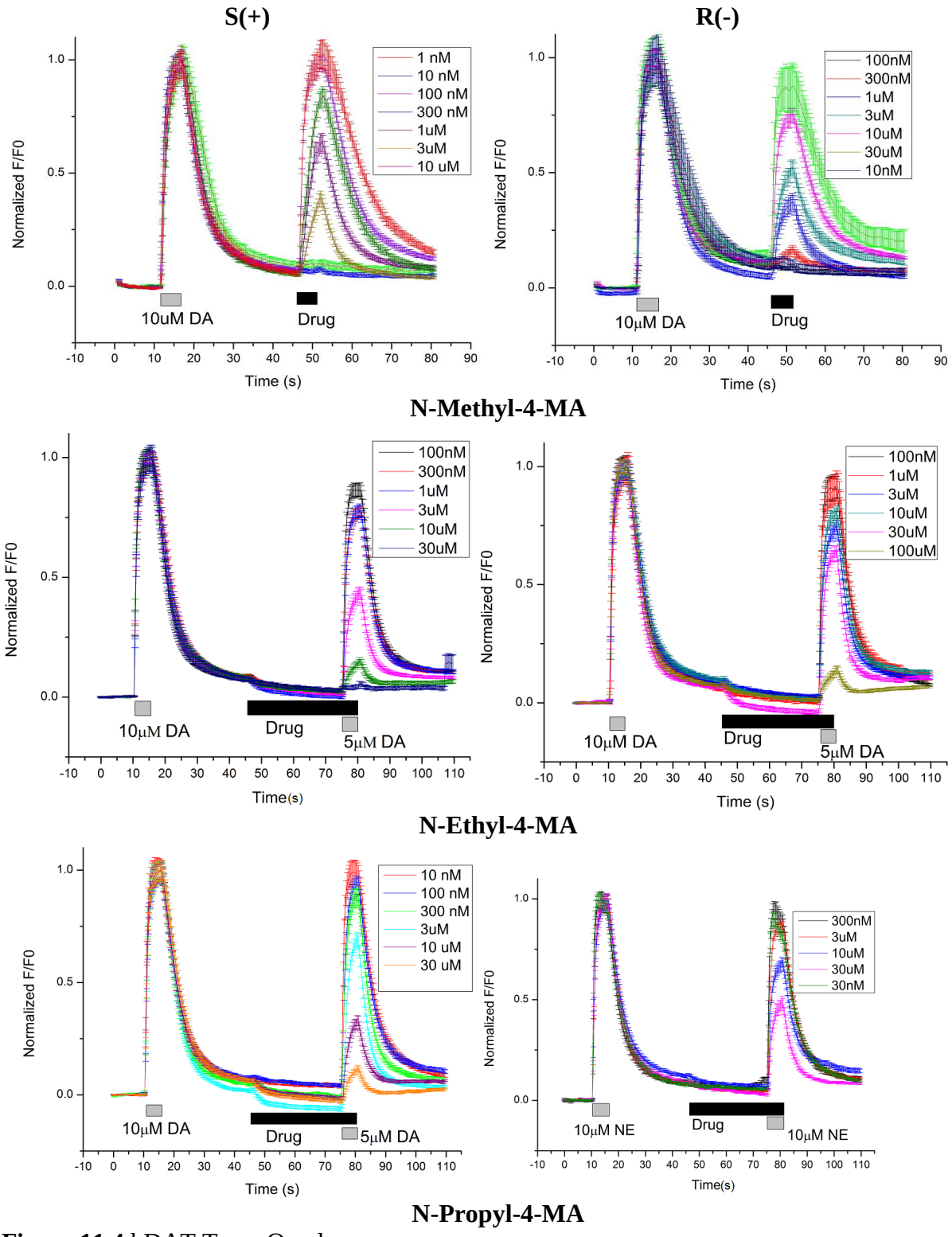


Figure 11.4 hDAT Trace Overlays

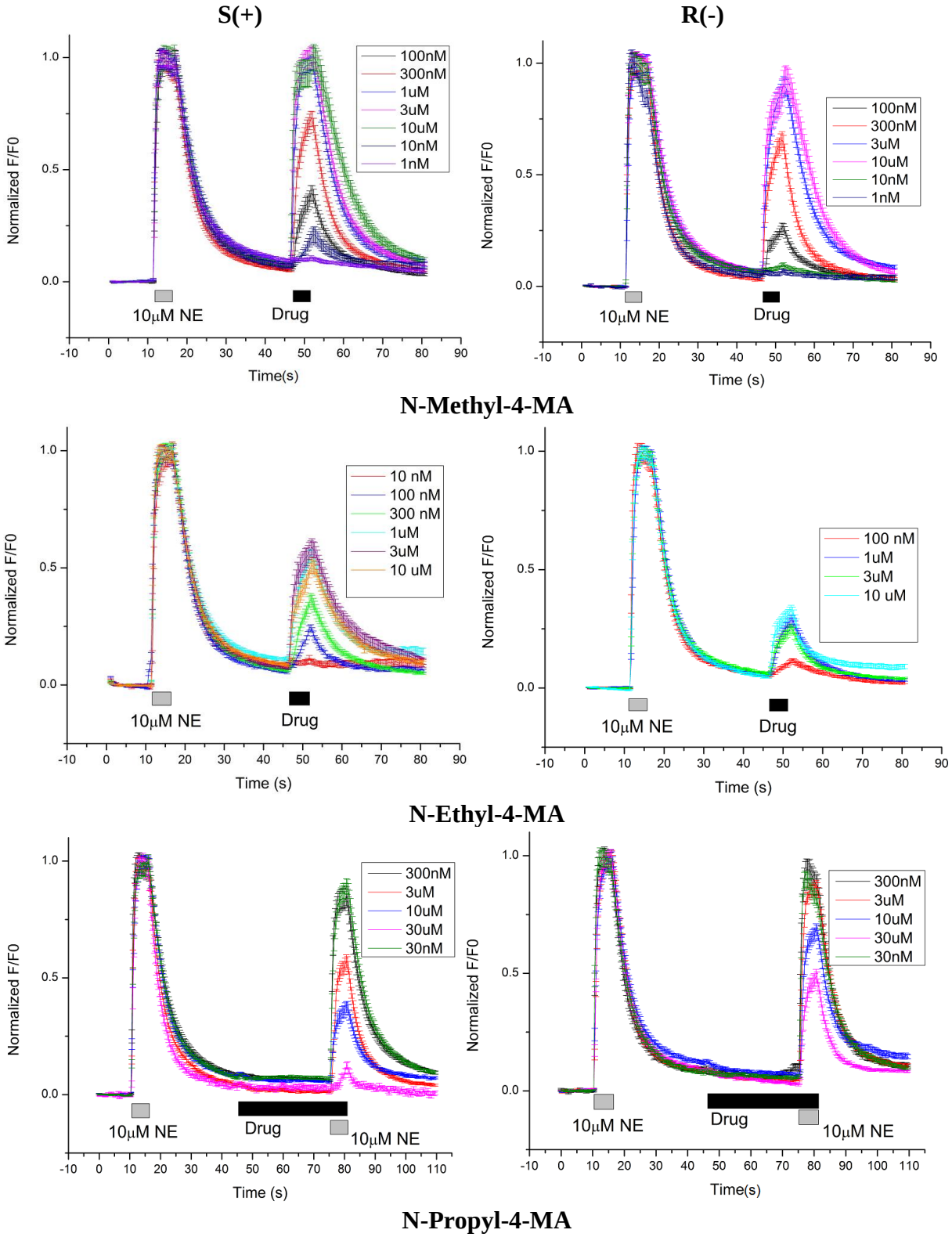


Figure 11.5 hNET Trace Overlays

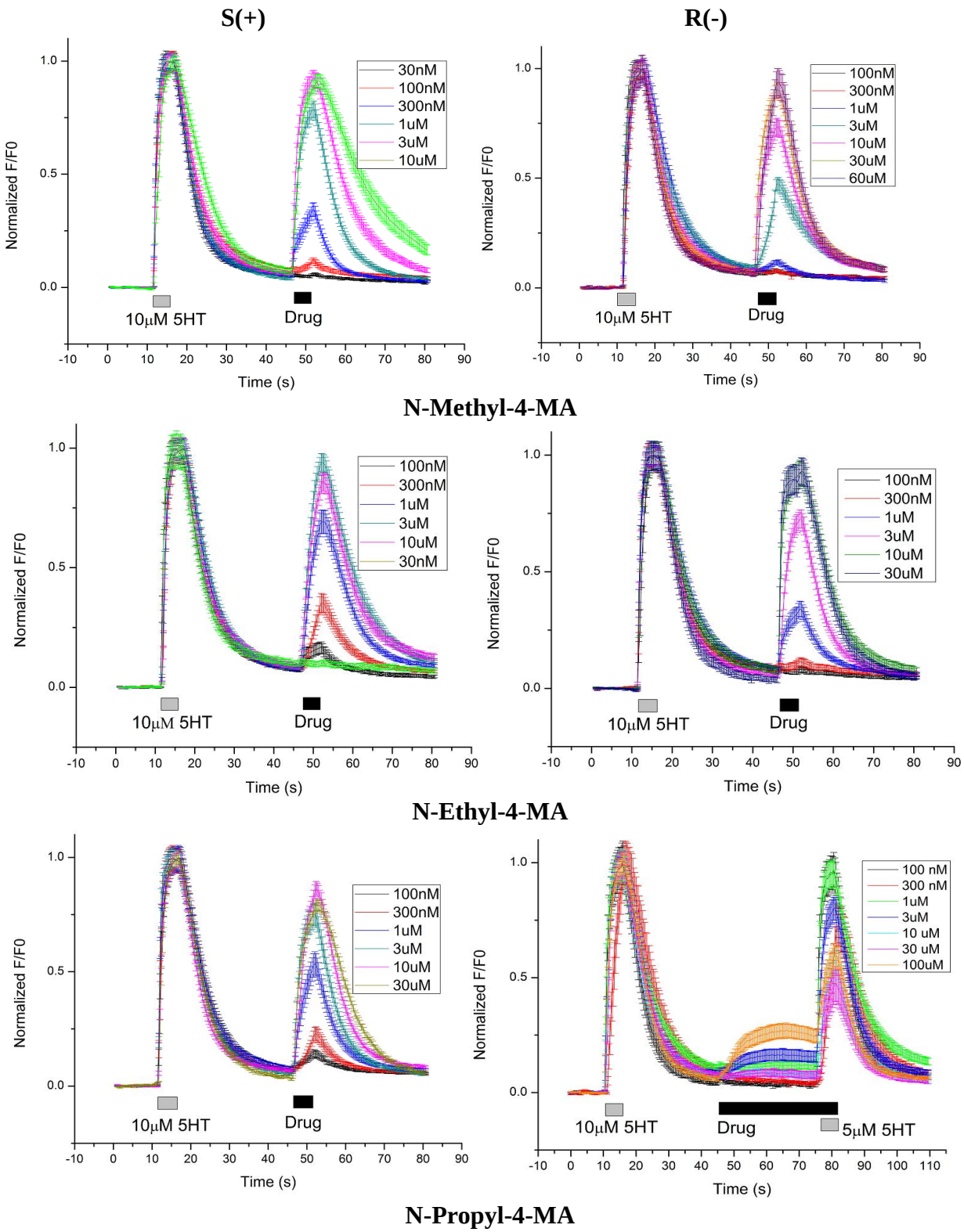


Figure 11.6 hSERT Trace Overlays

V. Discussion

Drug stereospecificity has long been a subject of interest in the pharmacological community. Stereospecificity of a compound is conferred by having at least one chiral center or a plane of symmetry. A chiral center is a carbon atom that has four different substituent groups. A plane of symmetry is essentially a point in space that if the molecule was divided by a line at that point each side of the molecule would be mirror image of the other side. Research over the years has consistently indicated a trend, at least in amphetamines, of the (+) enantiomer being more potent than the (-) enantiomer (Arnold, 2000) . Adderall, a commonly prescribed medication for attention deficit disorder, is a combination of D(+) and L(-) amphetamine salts in a 3:1 ratio favoring the D(+) enantiomer. The specific formulation of Adderall takes advantage of the specific effect of each enantiomer. The D/dextro-amphetamine typically acts as more of a CNS stimulant, while the L/levo-amphetamine acts more peripherally as well as eliciting a better response from some children (Smith & Davis, 1977; Westfall & Westfall, 2010; Lewin, Miller, Gilmour, 2011; Anthony, 2013; Arnold, 2000). This blend of the stereoisomers, is just one example of how pharmaceutical formulation can optimize drug action when stereospecific effects is taken into account.

As described in the result section the S(+) and R(-) enantiomers of the N-Alkyl 4-MA drugs seems to be consistent with the trends observed in D/(+)-Amphetamine and L/(-)-amphetamine by Holmes and Rutledge in 1976. Their data showed that (+)-Amphetamine was notably more potent in SERT and DAT and mildly more potent in NET. Methamphetamine also demonstrates stereospecific activity akin to that of amphetamine. The (+) stereoisomer being similarly potent to the (-) stereoisomer in hNET, and more potent in hSERT and hDAT. The results presented here for N-Methyl-4MA enantiomers (that structurally are very similar to

Methamphetamine) almost directly mirrors this pattern and seems to strengthen the evidence behind the current stereoselective paradigm in amphetamines and amphetamine like compounds (Holmes & Rutledge, 1976) (Rothman & Baumann, 2003). Interestingly, when the N-Alkyl chain is lengthened to obtain N-Ethyl 4MA and N-Propyl 4MA the equipotency of enantiomers on hNET gradually disappears, suggesting that small substituents in the N-alkyl position is important to preserve the lack of stereoselectivity of amphetamines on hNET.

When we studied N-Ethyl 4-MA enantiomers in hNET cells transfected with CaV1.2 the results showed that both enantiomers work as substrates, and are not fully efficacious to generate Ca2+ signals. The data obtained for R(-) N-Ethyl 4MA was not robust enough to get a reliable dose response curve (fig 12.1). The decreased efficacy of N-Ethyl 4MA compounds compared to NE, can be explained by a restricted ability of these compounds to generate substrate-associated currents on hNET; this could produce a reduced depolarization that would activate less Ca2+ channels, resulting in smaller Ca2+ signals. The efficacy of N-Ethyl 4MA enantiomers to generate Ca2+ signals were significantly increased when CaV1.3 (Harris et al, 2016) was used instead of CaV1.2 in comparable experimental settings (fig 12.1). CaV1.3 shows a 20 mV left shift in the voltage sensitive compared to CaV1.2 (Ruchala et al., 2014; Cameron et al., 2015), in other words, CaV1.3 can sense smaller levels of depolarization than CaV1.2. Thus, the enhanced Ca2+ signals measured when CaV1.3 was used, suggest that N-Ethyl 4MA enantiomers produce smaller currents associated to hNET activation compared to N-Methyl 4MA and NE. It would be of great interest to study the effect of R(-) N-Ethyl 4-MA on release studies in synaptosomes, the prediction would be that these compound would produce partial release.

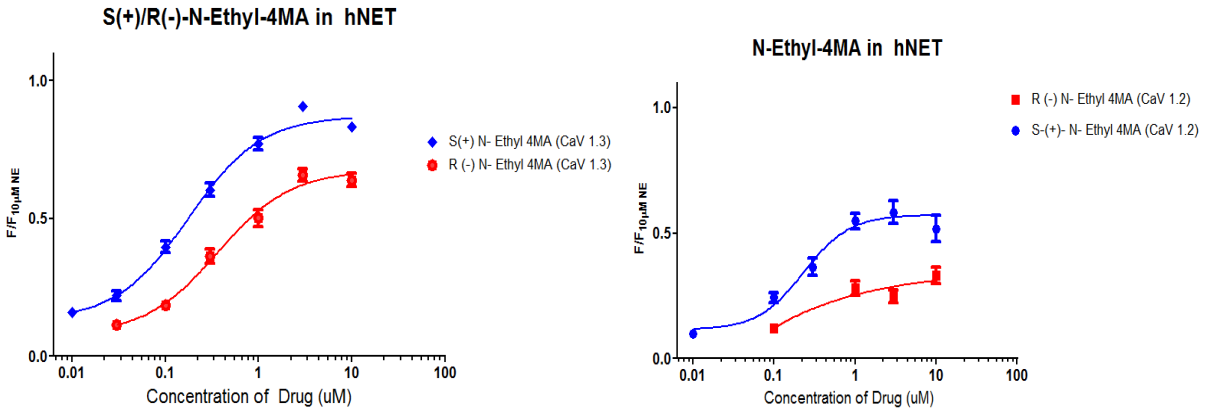


Figure 12.1 N-Ethyl in hNET transfected with CaV 1.3 channels (Harris, A.C.). N-Ethyl in hNET transfected with CaV 1.2 channels.

Until recently the current paradigm with regards to calcium channels and minimum subunits required for 'normal' function states that there must be an α1 subunit along with and α2δ subunits (Gregg et al, 1996). However there was a similarity of data gathered in hDAT when comparing it to data gathered under the exact same conditions by Harris et al, while not using the α2δ or 3 subunits. The experiments with CaV 1.2α1 subunit, the 3 subunit, and the α2δ subunit, provided nearly the exact same results as Harris et al. This runs counter to the minimum requirements for functional calcium channels as suggested by Gregg et al. By comparing both data sets it was demonstrated that, for a reason yet to be discovered, the and α2δ subunits are not necessary for 'normal function' of at least the CaV 1.2α1 subtype. To further explore the mechanism behind this discrepancy studies evaluating differing calcium channel subtypes in different cell cultures should be conducted without overexpression of the calcium channels as done by Gregg et al. This overexpression may have led to altered activity of the calcium channels, leading the experimenters to incorrectly assert the necessity of the and α2δ subunits.

Minor technical differences aside, this novel calcium screening assay that co-expresses calcium channels and monoamine transporters, has allowed for the rapid & predictive profiling

of novel compounds. The assay has allowed for differentiation of substrates, blockers, and now hybrid compound pharmacology. With this profiling it is also possible to estimate therapeutic and abuse liability implications of the novel compounds. For example the S(+)-N-Methyl enantiomer exhibited a pharmacological profile notably similar to MDMA, it had significant potency in all three MATs but maintained a low dopaminergic (DAT): serotonergic (SERT) ratio. Meanwhile the R(-)-N-Methyl enantiomer displayed remarkably selective substrate at hNET, almost 20 times more potent at hNET than hDAT or hSERT. Among many amphetamine compounds it is unique to identify those that are primarily selective releasers in NET and with the observations here R(-)-N-Methyl 4-MA could potentially be a good candidate for clinical drug trials that require that degree of selectivity. The similarity of the profile of S(+)-N-Methyl 4-MA to MDMA also highlights it as a potential non-scheduled substance to be used in place of MDMA in clinical trials for PTSD therapy (Sessa, 2017). Meanwhile the profile of R(-)-N-Methyl 4-MA makes it a potential good candidate for an attention deficit disorder (ADD) and attention deficit/hyperactivity disorder (ADHD) medication with lower abuse liability, and more adrenergic selectivity due it to its slight stimulant effects and strong adrenergic component.

The stereospecificity alone does not dictate the activity of the drug, it is also important to understand which class of drug each compound falls under. As discussed previously each drug typically falls within two classes. The first class being those termed ‘inhibitors’ that bind the transporter to block neurotransmitter uptake. The second type termed ‘substrates’ bind to the transporter, are subsequently transported into cells, and typically evoke release of neurotransmitters by reverse transport of compounds that acted on the MATs (Kahlig et al, 2005; Khoshbouei et al, 2003; Rothman and Baumann, 2006; Scholze et al, 2000) . Recent experiments have evidenced that there is a third class of compounds. This third class of compound displays

hybrid activity, acting as “full” or “partial” substrate in one or more MATs while acting as blocker in the others (Blough et al., 2014). This new classification of drug adds another layer of complexity and flexibility to the therapeutic capacity of amphetamine like compounds and potentially other monoamine transporter agonists and antagonists.

It is not until the N-Ethyl compounds that we begin to observe the hybrid activity of these compounds. The N-Ethyl enantiomers are of great interest not only due to its hybrid activity, seen in both enantiomers, but also due their selectivity for hSERT and their extremely weak effect at hDAT. While it may seem that they are selective for hNET and hSERT, by examining the efficacy at each transporter it should be noted the N-Ethyl 4-MA enantiomers are at least 20% less efficacious in hNET (**Table 11.2**). The S(+)-N-Propyl enantiomer also exhibits similar activity with a remarkable favoritism towards SERT as a substrate, taking into account that it work as a weak blocker in hDAT and hNET. These drug profiles appear to be unique and not directly comparable to any known compound outside those analyzed in the experiments done on the N-Alkyl 4-MA analogs.

While no current drug profile fits exactly, the selective serotonergic releasing and weak dopaminergic blocking activity seen by both of the N-ethyl enantiomers and the S(+)-N-propyl enantiomer is similar to the profile of a drug named Fenfluramine. Fenfluramine blocks the reuptake of 5HT while simultaneously increasing transport mediated release. It was initially prescribed as an anorectic to help manage exogenous obesity. However it was withdrawn from the public market in 1997 in the United States of America, after reports of heart valve disease, pulmonary hypertension, and cardiac fibrosis due to its interaction with a serotonergic transporter subtype found in the heart (NCBIPC, 2017; Connolly et al., 1997; Abenheim et al., 1996). The molecular mechanism behind why fenfluramine was causing such widespread issue

was not discovered until a few years later. It was discovered the impaired function of the 5-HT2B receptor subtype lead to cardiomyopathy, hypertrophy, and disruption of the intercalated disks in recombinant mice when compared to wild type mice (Nebigil et al., 2001). Ten years later it was discovered that the 5-HT2B receptor subtype was required for the anorectic effects of Fenfluramine by using wild-type and knockout mice studies (Banas et al., 2011). Due to the similarity of the compounds (**Figure 12.2**) The S(+)-N-Ethyl or S(+)-N-Propyl enantiomer may be utilized as possibly safer anorectic, if further experiments demonstrate lower non-specific effect of these drugs on the 5HT2B receptor.

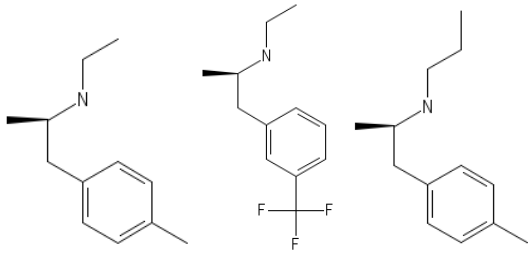


Figure 12.2 Structural similarities of S(+)-N-Ethyl 4-MA, S(+)-Fenfluramine, S(+)-N-Propyl 4-MA

It is also possible that all three S(+)-N-Alkyl 4-MA analogs could potentially be used as part of a replacement therapy protocol for amphetamines, amphetamine like compounds, or other monoamine effector drugs. The shift of drugs from substrate (agonist) to blocker (antagonist) allows for a much more well rounded approach to replacement therapy based on agonist and antagonist advantages in stimulant disorders as summarized by Grabowski et al (Grabowski, Shearer, Merrill & Negus, 2004).

Agonist	**Antagonist**
Decrease drug use+	Decrease drug use+
Diminish use related risks+	Diminish use related risks+
Patient acceptance good+	Patient acceptance poor−
Potentially enhanced compliance+	Noncompliance common−
Familiarity with effects/side effects+	Potential significant side effects−

Table 12.1 Summary of advantages (+) and disadvantages (−) of agonist and antagonist approaches in stimulant disorders (Grabowski, Shearer, Merrill & Negus, 2004)

Based upon the trend of the drugs established by the calcium screening assay (**Table 11.1**) and the relationship between the DAT:SERT ratio and abuse liability established by Bonano et al., a protocol proceeding towards a less addictive replacement drug could be established (Bonano et al, 2014). The protocol could initially introduce the S(+)-Methyl analog and progress towards the S(+)-Propyl analog by first reducing the amount of drug and then switching to the longer chain analog until dependency is abolished.

More data is required to make definitive assertions about the compounds tested. An ideal next step would be to analyze compound activity *in vivo* through microdialysis studies. However there are already many strong therapeutic and experimental possibilities opened up by preliminary analysis of these compounds.

VI. Conclusion

- S(+) enantiomers were consistently more potent than the R(-) enantiomers, with the exception of the N-Methyl analogs in hNET. This clearly further established importance of the drug stereospecificity in amphetamine like compounds.
- There was a negative correlation between chain length and potency implying that the N-alkyl chains were necessary to interact with binding pocket or at least allosterically regulate binding.
- There was a negative correlation between chain length and dopaminergic activity implying that increased N-alkyl chain length could be used to attenuate dopaminergic component of other amphetamine like compounds.

VII. References

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