universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Phosphorylation of Threonine 48 in the N-terminus of the Human Dopamine Transporter by Okadaic Acid."

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (Msc)

Vienna, 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Molekulare Biologie
Betreut von / Supervisor:	Univ.-Prof.Dr. Harald H. Sitte

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Wien, am 5.4.2016

Dedication & Acknowledgements

I dedicate this work to my brother. May you find your ways as I have. Discover and unleash your passion for sweet little molecules!

I thank Prof. Harald Sitte and Dr Jae-Won Yang for supporting me in completing this Master's thesis and assisting me in a self-reliant work ethic.

Furthermore, I thank my colleagues at the lab, especially Tina, Sonja, Marion and Kathrin, but also all the others. All my colleagues supported me in any way they could, for which I am deeply grateful.

Last but not least, I thank my family for their understanding and support in dire times.

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Abbreviations

[^{3}H]DA	tritium labelled dopamine
5HT	5-hydroxytryptamine, serotonin
8-Br cAMP	8-bromoadenosine 3',5'-cyclic monophosphate
aa	amino acids
Ab	antibody
Akt	protein kinase B; also PKB
Ala, A	alanine
AM	anisomycin
AMPH	D-amphetamine
ANOVA	analysis of variance
Asp, D	aspartate
AUC	area under the curve
Beta-PMA	phorbol-12-myristat-13-acetate
CA	calyculin A
CamKII	Ca^{2+}/Calmodulin-dependent protein kinase II
Cdk5	cyclin-dependent kinase 5
cDNA	complementary DNA
cGPK	cGMP-dependent protein kinases
CIP2A	Cancerous inhibitior of PP2A
CKI/II	casein kinase I and/or II
CMV	cytomegalovirus
COMT	catechol-O-methyltransferase
cpm	counts per minute
DA	dopamine
DAT	dopamine transporter
DMEM	Dulbecco's Modified Eagle's Medium
ERK	extracellular signal-regulated kinase
FCS / FBS	fetal calf serum / fetal bovine serum
GABA	γ-Aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glu, E	glutamate
HEK293	human embryonic kidney cells
hSERT	human serotonin transporter
HVA	homovanillic acid

JNK	c-Jun-N-terminal kinase
kDA	kilo Dalton (a unit for mass weight of proteins)
KHB	Krebs-HEPES buffer
K_m	Michaelis-Menten constant
KO	knock-out
L-DOPA	L-3,4-Dihydroxyphenylalanine
LC	liquid chromatography
Leu, L	leucine
LLC-PK_1 cells	Lewis Lung Carcinoma-Porcine Kidney cells (mature epithelial cells)
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
Maz	mazindol
MCLR	microcystin-LR
Mg^{2+}	magnesium ion
Mn^{2+}	manganese ion
MQ-H_2O	Millipore Milli-Q treated water (deionised)
MS	mass spectrometry
NE	norepinephrine
NET	norepinephrine transporter
OA	okadaic acid
OD	optical density
PBS	phosphate buffered saline
PCA	para-chloroamphetamine
PDZ domain	Domain name is an acronym of Drosophila disc large tumor suppressor (Dlg1) and zona occludens 1 (ZO-1)
PhD	Doctor of philosophy
PICK1	Protein interacting with PKCα 1
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PME-1	phosphatase methylase-1
PolyA signal	polyadenosine signal
PP1	protein phosphatase 1
PP2A	Protein phosphatase 2A
PPM family	metal-dependent protein phosphatase family
PPP family	Ser/Thr phosphoprotein phosphatase family
PTK	protein tyrosine kinase

PVDF membrane	polyvinylidene difluoride blotting membrane
rDAT	rat DAT
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis with SDS as detergent
SEM	standard error of the mean
Ser, S	serine
SERT	serotonin transporter
SLC6 family	solute carrier 6 family
Thr, T	threonine
TIP	type 2A-interacting protein
UPT	uptake
VMAT2	vesicular monoamine transporter 2
V_{max}	maximum velocity = transport capacity
VTA	ventral tegmental area
WB	Western blot
WT	wild type
YFP	yellow fluorescent protein
Zn^{2+}	zinc ion
Δ (delta)	change

Introduction

In the mammal brain several neurotransmission systems can be found. Besides GABAergic and glutaminergic interneurons, which make up a vast number in the brain, monoaminergic neurons organise body function, such as hunger, sleep, arousal, memory, induce locomotion and planning ahead. Monoamines, such as the neurotransmitters serotonin (5-hydroxytryptamine; 5HT), dopamine (DA) and norepinephrine (NE), are chemical structures consisting of an aromatic ring and a two-carbon chain connecting an amino residue to the aromatic ring.

The serotonergic system comprises only about 250,000 neurons in the human brain [Artigas 2013]. Most serotonergic cell bodies are localised in the brain stem and the Raphe nuclei in the midbrain. Interestingly, a noteable amount of serotonergic neurons can be found outside the Raphe nuclei and the majority of neurons in the Raphe nuclei are not 5HT neurons. Due to substantial branching of axons and dendrites, serotonergic neurons innervate almost the entire brain; main projections reach hippocampus, basal ganglia, cingulum and cortical areas [Siegel 2011]. Those are brain regions involved in emotional responses, empathy, hunger, sleep and stress-related behaviour. [Webster 2001; Nestler 2009; Cooper 2002; Siegel 2011]. NE is produced as a neurotransmitter by norepinephrinergic neurons situated in the locus coeruleus and the lateral tegmental nuclei of the brain stem. Similar to 5HT neurons, the axons and dentrites branch extensively and spread far through the brain. Neurons originating from the locus coeruleus innervate the frontal cortex, hippocampus and olfactory bulb, whereas the majority of the hypothalamic nuclei receive signals from the lateral tegmentum. However, both NE loci send controlling signals to cerebellum, thalamus and amygdala. The noradrenergic system regulates arousal, attention, vigilance and memory. This neurotransmission system is also involved in modulating incoming pain signals. Agents blocking β-adrenergic receptors can inflict memory loss when the memory has been acquired in a situation of emotional stress and trauma [Webster 2001; Nestler 2009].

The dopamine system

The dopaminergic system on the other hand is linked to executive functions, such as motor control, motivation, arousal, memory and learning, reinforcement and reward in adaptive stereotypic behaviours such as drug addiction. It has been established that the dopaminergic system is targeted by various psychotropic substances, for example amphetamines, cocaine and haloperidol [Webster 2001; Nestler 2009; Purves 2008; Sotnikova 2006]. Via the pathway projection to the pituitary gland DA signalling also controls prolactin secretion (lactation), sexual gratification and is involved in nausea [Webster 2001; Nestler 2009; Cour 2013]. DA is derived from tyrosine: The enzyme tyrosine hydroxylase transfers an hydroxyl group to tyrosine, thus producing L-DOPA. The intermediate is then processed by the aromatic L-amino acid decarboxylase to dopamine. DA is stored in axonal vesicles upon release into the synaptic cleft. After neurotransmitter exocytosis DA can be recycled.Once neurotransmitter molecules are not reused, DA is metabolised by monoamine oxidases B (MAO-B) and catechol-O-methyltransferase (COMT) to homovanillic acid (Figure 1). Enzymatic degradation with COMT

takes place in neuroglia. In noradrenergic neurons DA is further transformed to NE. Due to the similarity of DA and NE, DAT and NET can perform uptake for both molecules, with different pharmacokinetic properties *in-vitro* [Gu 1994].

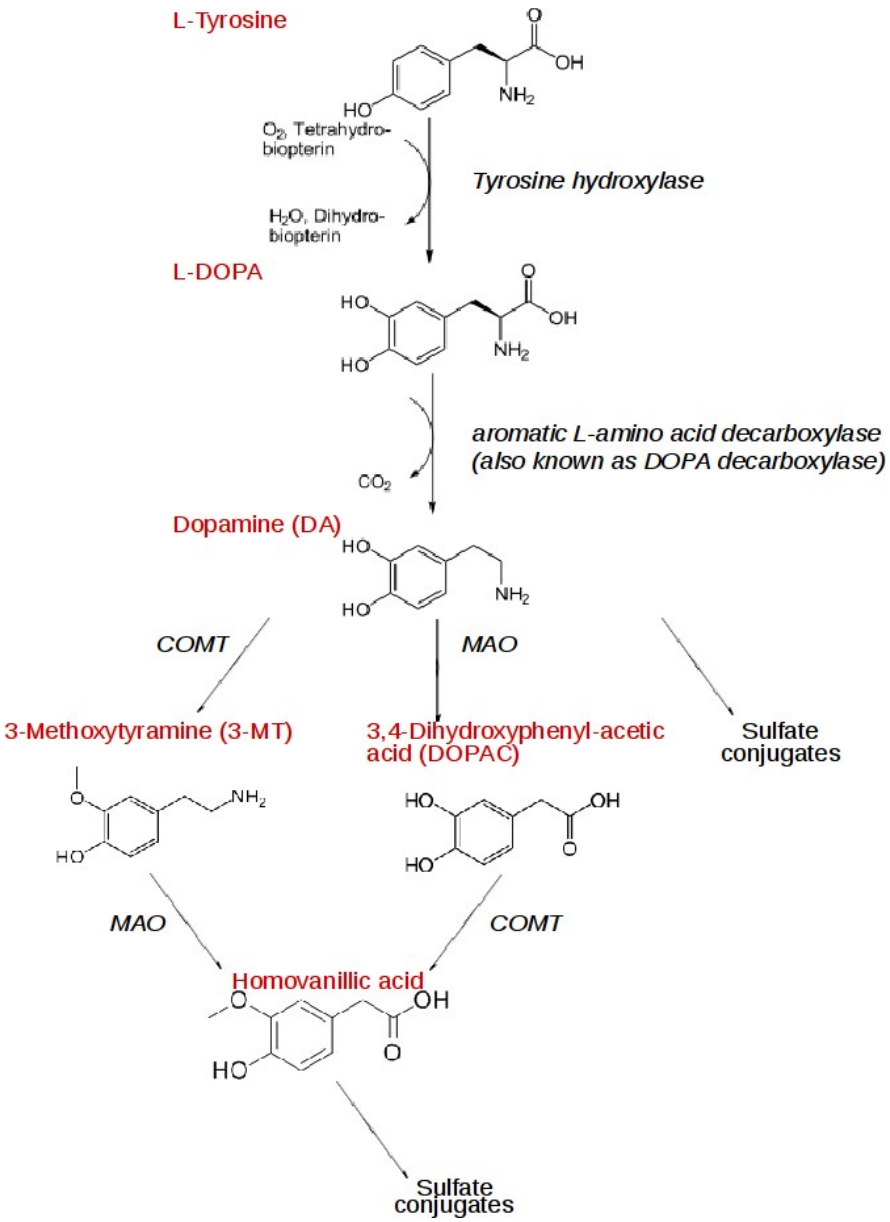

Figure 1. Biosynthesis and degradation pathways for dopamine. *DA is synthesised from Tyrosine with an intermediate, L-DOPA. In noradrenergic neurons DA is further transformed into NE. DA when not recycled is being degraded by either COMT or MAO to homovanillic acid and sulfate conjugates.*

Only a small number of neurons in the human brain (about 400,000) produce DA for signalling [Schultz 2007]. The perikarya of these neurons are restricted to certain areas. However, dopaminergic axons project to various regions of the brain [Björklund 2007]. Groups of dopaminergic cell bodies were mapped in 1964 by Annica Dahlström and Kjell Fuxe, assigning labels starting with the letter "A" (for "aminergic") to the individual groups [Dahlstroem 1964]. However, their system is not limited to dopaminergic neurons; also noradrenergic neurons are described (A1 to A7). Areas labelled A8 to A14 characterise DA expressing neurons. The

substantia nigra contains dopaminergic groups A8 and A9; the ventral tegmental area is listed as A10, the posterior hypothalamus as group 11, the arcuate nucleus as group 12, the zona incerta as group 13 and the periventricular nucleus as group 14. The dopaminergic system is organised in four projection pathways: the mesostriatal, the mesolimbic, the mesocortical and the tuberoinfundibular system (Figure 2) [Nestler 2009].

- The mesostriatal pathway originates in the Substantia nigra pars compacta, a part of the midbrain. Neuronal axons spread from there to the basal ganglia (Nucleus caudatus and putamen). Basal ganglia are known to be the hub for coordination of movement. In neurodegenerative diseases that target this system, Parkinson's Disease respectively, the patient looses the ability to control movements of his or her body.
- The mesolimbic projection system emanates from the ventral tegmental area (VTA) of the mesencephalon to the limbic system (Hippocampus, Fornix, Amygdala, Nucleus accumbens). The mentioned pathways are associated with learning and memory.
- Neurons that spread from the VTA to the cortex form the mesocortial projection system. The cortical regions in the frontal lobe being innervated, namely the prefrontal cortex, are associated with decision making in humans. Mesolimbic and mesocortial projection systems are not quite distinct, they are both involved in motivation and learning of adaptive behaviours. Such a behaviour is reinforcement in addiction. The reward pathway activated in addiction is a loop system involving VTA, Nucleus accumbens and cortex, also affecting the amygdala. Most common drugs which affect the DA systems are cocaine and amphetamines.
- The tuberoinfundibular pathway controls the secretion of prolactin in the anterior pituitary and is thus involved in the regulation of food intake and sexual behaviour. Neurons of this projection emanate from the Nucleus infundibularis and lead over to the eminentia mediana of the hypothalamus.

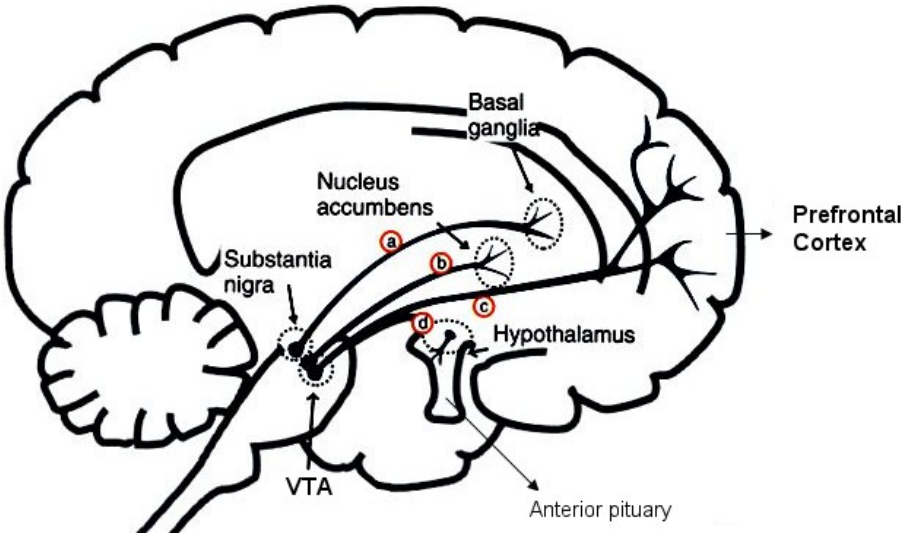

Figure 2. Anatomical locations of dopaminergic projection systems in the human brain *(adapted from Leuner K, Müller WE (2006). The complexity of the dopaminergic synapses and their modulation by antipsychotics. Pharmacopsychiatry 39(Suppl.1):S15-S20) a. Nigrostriatal pathway. b. Mesolimbic system. c. Mesocortical pathway. d. Tuberoinfundibular pathway.*

Termination of synaptic transmission in dopaminergic neurons: The dopamine transporter

As illustrated in Figure 3, DA is synthesised in the neuron and consecutively stored in vesicles via the vesicular membrane transporter 2 (VMAT2) [Fon 1997; Sulzer 2005; Sulzer 2011; German 2015; Isingrini 2015]. Upon a Ca_V-channel mediated trigger, vesicles fuse with the presynaptic membrane and subsequently release the stored DA into the synaptic cleft. When DA binds to post-synaptic dopamine receptors, the neurotransmission is carried on in the next neuron. The next step in neurotransmission for the presynaptic neuron is resetting: The DA is reuptaken into the presynaptic neuron via transporter proteins with specific affinity to DA[1], the dopamine transporter (DAT). Instantaneously after DA reaching the cytosol, it is taken up into vesicles via the VMAT2 and the cycle can start again [Sulzer 2005; Sulzer 2011]. The presynaptic neuron can be elicited several times before the DA storage is depleted. Removal of DA from the synapse is terminating neurotransmission entirely.

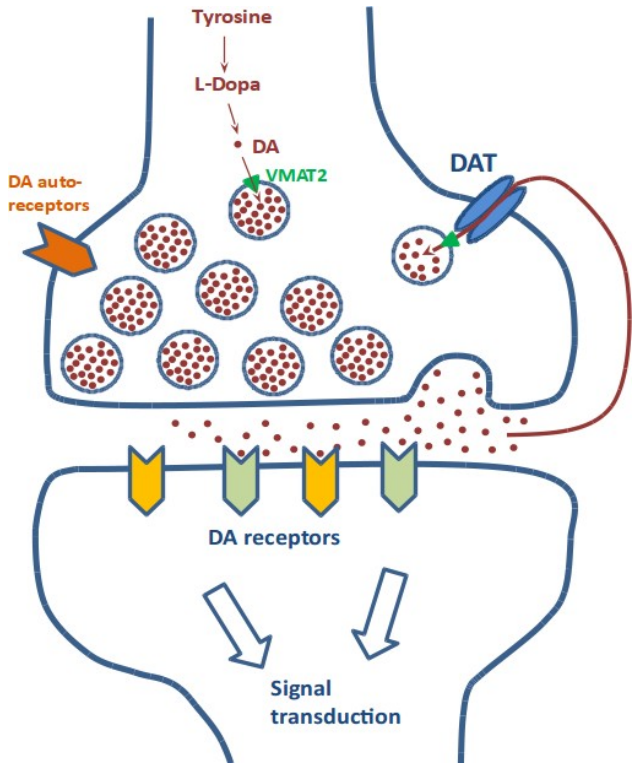

Figure 3. Neurotransmission at a dopaminergic synapse. *After elliciting an action potential in the post-synaptic neuron, the transmission is terminated. Dopamine is reuptaken into the presynaptic neuron and being recycled. The protein responsible to clear the synaptic cleft from DA is DAT .*

The DAT and neurological diseases

Various neurological disorders have been linked with the DAT so far. For instance, the group of movement disorders, leading to bradykinesia or dyskinesia [Bhatia 1994] involve attention deficit hyperactivity disorder, Parkinson's Disease and forms of parkinsonism [Lee 2000]. Disorders not classified as movement disorders, but linked to the DA system, are depression,

1 It has furthermore been shown that NET can also take up DA effectively, but not 5-HT [Moron 2002].

addiction, schizophrenia and Tourette syndrome [Sulzer 2011; Sotnikova 2006]. To study mentioned neurological diseases and DAT involvement several animal models and DAT mutants were generated and tested [Gainetinov 2008].

Structure of the DAT

The DAT is a member of the solute carrier (SLC) superfamily. SLC6 family members are sodium:neurotransmitter symporters which import neurotransmitters along with sodium and chloride ions. DAT (SLC6A3) imports two sodium ions and one chloride ion along with one DA molecule as the ion gradient is the driving force for DA import [Torres 2003b]. The 620 amino acids (aa) span across the cellular membrane as twelve domains, connected with shorter or longer loops. The amino- and carboxyl termini of DAT situated intracellularly are long and unstructured. The C-terminus (42aa) is important for ER export and protein integration into the membrane [Madsen 2012; Rickhag 2013a]. In contrast, the N-terminus (68aa) plays a major role regarding functional regulation of DAT (as presented in section “Regulation of DAT function”).

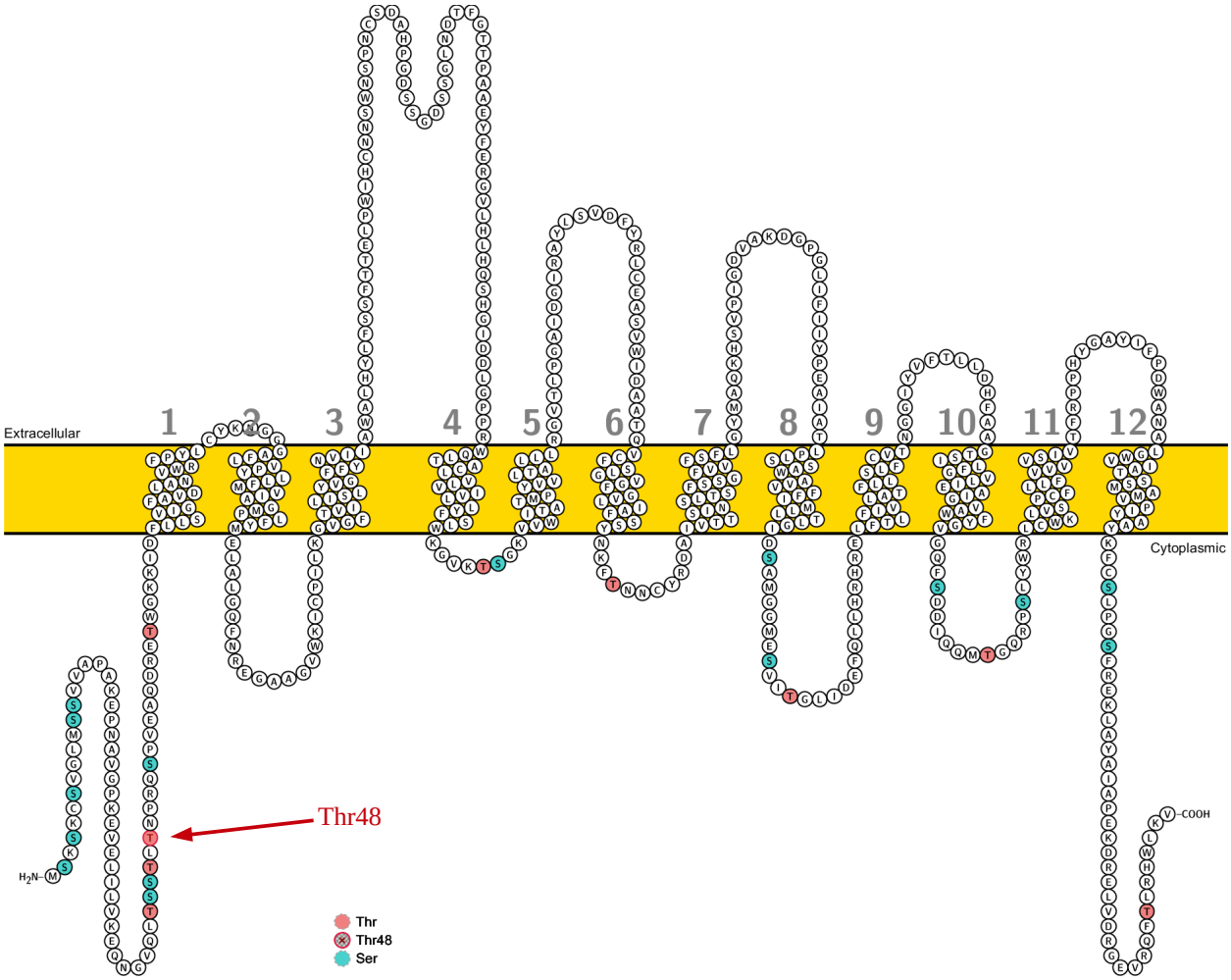

Figure 4. Simplified structure of the human dopamine transporter. *The DAT has twelve membrane spanning domains, interconnected with loops. The protein's termini are localised in the cytosol. Post-translational modifications have shown to alter protein function or surface expression. Intracellular phosphorylation at serine (S, blue) or threonine (T, red) residues may be targeted by kinases and phosphatases, suggesting phosphorylation and involvement in protein regulation. To predict possible phosphorylation sites kinase and phosphatase binding domains must be compared to the cytosolic amino acids. Figure arranged with Protter Webtool (http://wlab.ethz.ch/protter/start/).*

Regulation of DAT function

The regulation of monoamine transporters is highly complex: One the one hand, many pathways leading to post-translational modifications have shown to modulate transporter function. On the other hand, also protein-protein interactions influence transporter function or localisation on the cell surface. Some functions are controlled by more than one pathway and some pathways are dictating more than one function [Vaughan 2013].

Protein-protein interactions

DAT oligomers

The presence of DAT tetramers has been observed in several *in-vitro* studies. Interestingly, these tetramers are formed within the ER and remain a single entity through-out all DAT cycling processes. However, the situation *in-vivo* remains unclear [Milner 1994; Hastrup 2001; Seidel 2005; Sorkina 2003; Torres 2003a]. Torres et al. reported in 2003 that some mutants localised in an oligomer have a dominant-negative effect on other, non-mutated DAT protomers in that oligomer [Torres 2003a]. These negative effects are either reduction of transporter function or affected transporter localisation to the cell surface. Zhen et al. observed single DAT units affecting other surrounding DAT protomers in matters of conformational state under particular experimental conditions. The thereby induced conformation is characterised by altered transport activity and sensitivity to reuptake inhibitors. Occurence of DAT tetramers *in-vivo* in certain DA brain areas might explain the intense sensitivity to stimulants [Zhen 2015].

Interaction with DA autoreceptors

A direct connection of DA D_2 autoreceptors and DAT promotes the recruitment of further DATs to the cellular surface [Lee 2007]. A mouse model in which this interaction was disrupted presents a hyperactive phenotype. The underlying mechanism suggests impaired DA uptake and/or D_2 receptor signalling [Lee 2007].

Interaction with scaffolding proteins and transporter localisation

Protein interacting with PKCα 1 (PICK1) and the DAT C-terminus connect via their so called PDZ domain. Binding may serve as compartment-specific anchor, holding DAT in endocytic compartments [Madsen 2012]. The phenotype of a mouse model with disrupted PDZ domains in DAT shows strongly decreased expression of DAT in the striatum [Rickhag 2013a]. In general, C-terminal interactions via the PDZ domain play a major role in DAT localisation. The surface form of DAT is highly motile and equally distributed in lipid rafts as well as in non-raft domains. DAT presence in lipid rafts dictates interaction partners [Adkins 2007; Eriksen 2009; Foster 2008]. Association of Flotillin-1 to DAT is essential for it's integration into lipid rafts as well as for PKC-mediated endocytosis [Cremona 2011]. Interaction of DAT with α-synuclein, a protein associated with Parkinson's Disease when overexpressed, increases DAT membrane expression and thereby DA uptake. α-Synuclein stabilises the surface form of DAT under basal conditions [Fountaine 2007]. In a physiologic environment DAT is expressed perisynaptically and

internalised constitutively. Endocytosis of DAT is tied to clathrin and dynamin and may be induced by PKC activation. After clathrin-mediated internalisation the majority of DAT is being sorted for degradation, but DAT may also be re-established in the plasma membrane [Loder 2003; Eriksen 2010a; Mortensen 2008; Rao 2011; Sorkina 2005].

Post-translational modifications

Amino acids can be modified by reversible phosphorylation, glycosylation, palmitoylation and ubiquitylation. The amino acids in question of the DAT have been investigated intensely, revealing the importance of glycosylation for DAT localisation at the cell surface, palmitoylation for PKC-mediated membrane turn-over and that ubiquitylation determines whether DAT internalisation is temporary or permanent (the latter leading to degradation) [Daniels 1999; Foster 2011; Li 2004; Miranda 2005; Miranda 2007, Patel 1994; Vaughan 1996]. Phosphorylation and dephosphorylation of monoamine transporters were proven to be substancial in function regulation and transport to the cell surface, internalisation and protein dimerisation (eg. certain transcription factors; see sections below). It should be mentioned that, in general, the complexity of protein regulation increases with every accessible amino acid residue for post-translational modifications and with every possible protein interaction. In case of phosphorylation the target amino acid residues are majorily threonines (Thr, T; ~86%) and serines (Ser, S; ~12%), but also tyrosines (Tyr, Y;~2%) [Shi 2009]. However not all accessible serines and threonines may be phosphorylated at once or at all.

Influence of phosphorylation on hDAT

Phosphorylation is mediated by protein kinases, such as Protein kinase A (PKA), Protein kinase C (PKC), cGMP dependent protein kinase (PKG), Ca^{2+}/Calmodulin-dependent kinases (CamKs), Casein kinases (Cks), p38MAPK and extracellular signal-regulated kinase (ERK) for Ser and Thr. As many as about 430 genes encode Ser/Thr protein kinases in the human genome. For tyrosine phosphorylation of proteins biosystems express non-receptor protein tyrosine kinases (PTKs). The phosphorylation state can be reversed via protein phosphatases, such as protein phosphatase 1 and 2 (PP1, PP2). To predict possible phosphorylation sites of a protein, kinase and phosphatase binding domains must be compared to the sequence of (cytosolic) amino acids. For this purpose there are a few online tools available at the moment, for example the Phospho Motif Finder [Amanchy 2007]. A variety of binding motifs dictate which kinases may bind and phosphorylate the protein at a certain amino acid residue within the binding motif. For every intracellular Ser and Thr of hDAT at least one protein kinase consensus sequence has been predicted; in some cases multiple types of protein kinases may interact with the same amino acid.

In 1997 it was declared that PKA had no effect on DAT, but PKC which has the same binding motif as PKA [Vaughan 1997]. These findings were challenged since then and the reception of arising research is controversial [Batchelor 1998; Gorentla 2009; Page 2004; Pristupa 1998].

N-terminal Ser2, Ser4, Ser7, Ser12 and Ser13 were reported to be phosphorylated by PKC, CamKIIα and PKA [Fog 2006; Kristensen 2011; Moritz 2013]. Trunctation of the first twenty-two amino acids of hDAT in heterologous cells lead neither to insensitivity to beta-PMA, a PKC activator, nor to impairment of protein downregulation [Grånäs 2003; Khoshbouei 2004]. Deletion of the first twenty-two amino acids or mutation of mentioned serines to alanines (Ala, A) displayed the same phenotype: normal response in uptake assays, oligomerisation or PKC-mediated internalisation and dramatically reduced efflux (about -80% of WT) [Fog 2006]. Dephosphomimetic Ser2, Ser4 and Ser13 and phosphomimetic Ser7 and Ser12 restored a significant fraction of WT efflux, presenting Ser7 (phosphorylated by PKA) and Ser12 as key role players. Mutation of either Ser2, Ser4 or Ser13 (phosphorylated by CamKIIα and PKC) to aspartate (Asp, D; phosphomimetic) did not show any significant changes in efflux [Khoshbouei 2004; Moritz 2013]. Recently, Moritz et al. argued that Ser2 is not a phosphorylation site but has an effect on phosphorylation level of neighbouring serines [Moritz 2013]. Amperometry with cells expressing hDAT Ser to Asp mutations of the N-terminal serines lead to the conclusion that AMPH-independent efflux can take place and AMPH-induced efflux is mediated by CamKIIa at the C-terminus [Fog 2006]. DAT^{S7A} mutation interferes strongly with (–)-2-β-Carbomethoxy-3-β-(4-fluorophenyl)tropane (CFT, WIN 35428) binding to DAT. DAT^{WT} and DAT^{S7A} showed increased CFT binding with Zn^{2+} treatment, whereas DAT^{S7D} could not be affected by Zn^{2+}, indicating a certain function of Ser7 phosphorylation in inward-facing and outward-facing equilibria [Moritz 2013].

rDAT expressed in heterologous cells could be phosphorylated by PKCα, PKCβI, PKCβII, PKCγ, ERK1, ERK2, CaMKII, PKA catalytic subunit, PKG, casein kinase II (CKII), p38, c-Jun-N-terminal kinase (JNK), cyclin-dependent kinase 5 (Cdk5), and protein kinase B (Akt) in-vitro [Foster 2012; Gorentla 2009]. Furthermore, particularly Thr53 has been reported being phosphorylated by ERK1, JNK and p38MAPK in rDAT [Foster 2012; Gorentla 2009]. Thr53A or Thr53D mutation lead equally to dramatically reduced uptake abilities and presented virtually no efflux [Foster 2012].

Juxtamembraneous Thr62 has a cannonical consensus sequence for phosphorylation by either PKA, PKC or cGMP-dependent protein kinase (cGPK) which is also highly conserved across species. Further functional assays delivered information of the $hDAT^{T62D}$ (aspartate, phosphomimetic) mutant to be less effective in substrate uptake in regard to $hDAT^{WT}$ or $hDAT^{T62A}$, but similar in character in efflux experiments. This led to the suggestion that the phosphomimetic D mutant might have shifted the conformation equilibrium to the inward-facing state. Indeed, by adding small amounts of Zn^{2+} the protein transport efficiency could be “rescued“ [Guptaroy 2009].

Torres et al. produced a series of DAT C-terminal mutants, all of them showed impaired function, especially Ser582A. This mutation abolished transporter function entirely, because of retention of Ser582A in the cytoplasm; hence it does not reach the cell surface. In oligomeric forms compromising hDAT WT and Ser582, cell surface expression is dramatically reduced [Torres 2003a]. CamKIIα phosphorylates T613 in the C-terminus, leading to enhanced AMPH-induced efflux. When residues 612-614 were mutated (4A mutation), efflux was severely decreased and efflux could not be enhanced by CamKIIα in comparison to hDAT WT [Fog 2006]. The same research group also examined the N-terminal residues 1-27 and found PKC and CamKII to be

able to induce phosphorylation of the present serines. Moritz et al. specified this to Ser13 [Moritz 2013].

Impact of pharmacological agents upon kinase-mediated DAT phosphorylation

PKC activation via Ca^{2+}, DAG, phorbol esters, high levels of dopamine or METH results in a decrease of transport capacity and PKC-mediated endocytosis (dynamin- and clathrin-dependent; requires DAT C-terminal FREKL motif). Moreover, DAT requires Flotillin-1 for internalisation and high levels of sucrose is able to block endocytosis. However, PKC inhibitors (e.g. Staurosporine, BIM-1, Go6979, the Ca^{2+} chelator EGTA, LY279196, LY379196) work against mentioned effects. This has been reported frequently and summarised in reviews of Vaughan and Foster (2013) and German et al. (2015). Reactive oxygen species, inflammatory cytokines and anisomycin are potent p38MAPK activators. As such, they can reduce DA uptake by phosphorylation of rDAT Thr53, for example [Foster 2012; Gorentla 2009]. The p38MAPK inhibitor SB203580 in contrast, inhibited the described phenotype [Zhu 2005].

CamKIIa can be activated by raising levels of Ca^{2+}, displaying increased DAT surface levels and phosphorylation of N-terminal serines and C-terminal Thr613. CamKIIa inhibitors, such as KN62, KN-92 and the Ca^{2+} chelator BAPTA-AM, decreased AMPH- mediated DA efflux, a trait likely to be caused by a change in conformation leading to functional inactivation of DAT [Fog 2006; Gnegy 2004; Sakrikar 2012; Steinkellner 2012; Steinkellner 2014]. DAT upregulation by D2 and D3 receptors is mediated by ERK1/2 in a cell line expressing constitutively-active MEK and hDAT [Moron 2003]. Inhibition of ERK1/2 is usually accomplished by inhibiting upstream MEK1/2, resulting in reduced DA uptake [Lin 2003; Moron 2003; Owens 2012].

Inhibition of the PI3K/Akt pathway, which - in multiple ways - controls cell growth and protein synthesis, inhibits also DA uptake via attenuating transporter capacity due to DAT internalisation and in addition, it decreases DAT phosphorylation. Such inhibitors are LY294002 and ML9. Overexpression, on the other hand, emphasises enhanced DA transport capacity and surface expression [Carvelli 2002; Garcia 2005]. It is suggested that PI3K/Akt activation promotes DAT surface recruitment which is counteracting to basal, dynamin-dependent DAT endocytosis. This theory has been suppported by Speed et al. who could show that only inhibition of Akt2 reduced DAT cell surface expression [Speed 2010]. In functional studies, it has been demonstrated that inhibition of PI3K/Akt also reduces AMPH-induced DA reverse transport [Lute 2008].

Protein phosphatases and their contribution to DAT regulation

As described thoroughly above, a variety of protein Ser/Thr kinases (about 430 genes in the human genome) execute protein phosphorylation, among them monoamine transporters. Subsequent dephosphorylation is realised by only few Ser/Thr protein phosphatases (about 40 genes in the human genome). Substrate specificity is achieved by combinatorial assembly of three subunits to form a functional holoenzyme.

Ser/Thr protein phosphatases

Ser/Thr protein phosphatases are clustered in three major families: Ser/Thr phosphoprotein phosphatase family (PPPs), metal-dependent protein phosphatases (PPMs; Mn^{2+}/Mg^{2+} ions; pleckstrin (formerly PP2C) and pyruvate dehydrogenase phosphatase) and aspartate-based phosphatases FCP/SCP. All family members are highly conserved across species. Up to-date seven PPP family members were identified and recently reclustered [Shi 2009]:

- PP1,
- PP2 (formerly PP2A; designation throughout this thesis: “PP2A”),
- calcineurin (formerly PP2B, now PP3),
- PP4,
- PP5,
- PP6,
- PP7

Most abundant Ser/Thr protein phosphatases are PP1 and PP2A which catalyse about 90% of all protein dephosphorylations in most tissues and cells [Lin 1998]. It has been determined that other PPP family members have an extremely low basal expression level in cell models [Zhang 2012]. All PPP family members form a trimeric holoenzyme, consisting of a substrate-specific (A) subunit, a catalytic (C) subunit and a regulatory (B) subunit. Combination of different isoforms of these subunits create a variety of more than 70 different possible holoenzymes for PP2A alone, for example.

Table 1. Overview of PP2A subunits. *Variation of the subunits and their isoforms can form over 70 different holoenzymes, specifically targeting certain proteins and specific domains within.*

Subunit	Isoforms	Function
A	α, β	Structural subunit, responsible for substrate recognition (scaffolding of the holoenzyme)
B (B55, PR55) B' (B56, PR61) B" (PR48/PR72/PR139) B"' (PR93/PR110)	α, β, γ, δ α, β, γ, δ, ε	Regulatory subunit, target for accessory proteins
C	α, β	Catalytic subunit, effectively dephosphorylates amino acid residues

The protein phosphatase 2A

The PP2A is a trimeric protein with 3 subunits as described above and in detail in Table 1. Certain B subunits have up to five isoforms. The B subunits B, B', B" and B"' are structurally distinct, as illustrated in Figure 5. Hence, the A subunit provides quite a conformational flexibility [Shi 2009; Zhang 2012].

PP2A subunit isoforms can form over seventy different holoenzymes, all of which playing a

major role in various cellular processes involving development, cell proliferation and apoptosis, cell cycle regulation, cytoskeleton dynamics, numerous signal transduction pathways, and PP2A seems to be an important tumour suppressor [Janssens 2001; Janssens 2005; Mumby 2007; Ogris 1999; Westermarck 2008]. In the progress of disease onset, including Alzheimer's and various forms of cancer, impaired PP2A function has been reported [Shi 2009; Westermarck 2008; Zhang 2012]. However, not only aberrations in the genetic material or depletion of the presence of (functional) binding partners impede catalytic activity of PP2A, but also the absence of metal ions (Mn^{2+}/Mg^{2+}) which are necessary for the enzymatic reaction [Shi 2009]. Missense mutations in the scaffolding A subunit (both isoforms) lead to incomplete association with the C subunit [Ruediger 2001a,b; Wang 1998]. Moreover, methylation of the Leu309 (C-terminal) in the C subunit of the PP2Aac core enzyme may promote recruitment of B-B" to the other subunits [Bryant 1999; Gentry 2005; Kloeker 1997; Longin 2007; Tolstykh 2000; Wei 2001; Wu 2000; Yu 2001], however lack of 14 C-terminal amino acids did not prevent holoenzyme formation [Ikehara 2007; Xu 2006; Xu 2008; Yu 2001]. Furthermore, if the C subunit is rendered inactive, the assembly of the holoenzyme is hindered. For example binding of the inhibitor okadaic acid near the active site in the C subunit is inactivating the catalytic subunit of PP2A [Xing 2006].

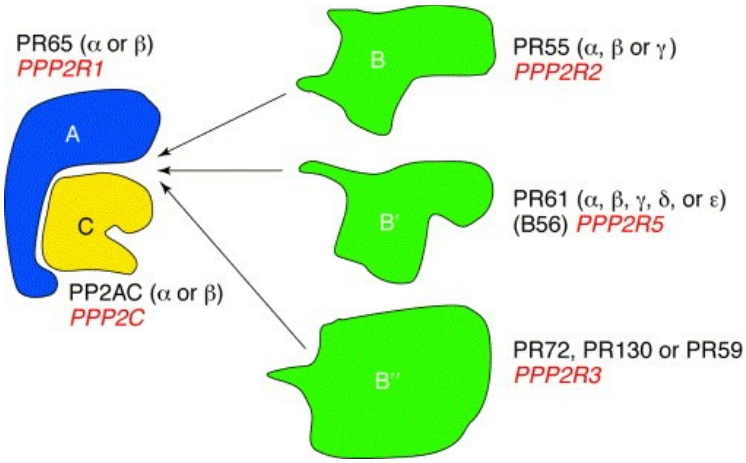

Figure 5. Structural assembly of the PP2A holoenzyme with different B subunits. *The scaffolding A subunit forms a nutshell, interacting with the C and one of the B subunits this way. The B subunits B, B', B'' and B''' are structurally distinct. Hence, the A subunit provides quite a conformational flexibility. Taken from Millward et al. (1999) Regulation of protein kinase cascades by protein phosphatase 2A. Cell 24(5):186-191.*

Several inhibitors of PP2A have been identified, among them okadaic acid (OA), calyculin A (CA), microcystin-LR (MCLR) and cancerous inhibitor of PP2A (CIP2A). PP2A inhibitors can be classified in three distinct groups: Toxins, endogenous PP2A inhibitors and viral oncoproteins.

- OA is a toxin produced by the marine sponges *Halichondria okadai* and *Halichondria malanodocia* and dinoflagellates (algae) *Prorocentrium spp.* The fatty acid-derived toxin causes diarrhoeal shellfish poisoning in humans. At molecular level, OA inhibits reversibly PP1 and PP2A, but activates atypical PKC isoforms. The capability of OA to inhibit PP2Ac is extremely strong (IC_{50}=0.1nM), however inhibition of PP1 is not as

effective (IC_{50}=10-15nM) due to improper binding at the PP1 binding site [Dawson 1999; Holmes 1990; Reguera 2014; Standaert 1999; Takai 1992; Westermarck 2008; Valdiglesias 2013; Xing 2006].

- Overexpression of endogenous inhibitors of PP2A have been reported to promote tumour growth, as those inhibit PP2A's tumoursuppressing actions. Proteins that fit this description are CIP2A, I2PP2A/SET, protein phosphatase methylase-1 (PME-1) and type 2A-interacting protein (TIP) [Westermarck 2008]. CIP2A inhibits PP2Ac to stabilise Myc. Association with both have been reported. Interaction of PP2A and Myc was impeded when MycS62 was mutated to alanine [Junttila 2007].
- Viral oncoproteins are produced by DNA tumour viruses, such as adenovirus, polyomavirus and simian virus (SV40; produces so called T-antigens). These viruses transform mammalian cells through the controlled dysregulation of several proteins which are involved in the control of replication and proliferation (oncogenes, tumour suppressors) by their own cancerogenic proteins [Westermarck 2008; Zhang 2012].

Thr48, a phosphorylation site not yet characterised

PP2A regulates preservation and reduction of DAT phosphoresidues via it's catalytic subunit

DAT-PP2A interaction has been reported in rat striatum [Ramamoorthy 2010; Samuvel 2008]. The specificity of the interaction was confirmed by co-immunoprecipitation with a heterologous cell line expressing hDAT and with native striata from wild type or DAT knock-out mice (conducted by Jae-Won Yang). Interaction of PP2A with monoamine transporters has been characterised, determining the subunit C as binding partner since PP2Ac inhibition by OA is disrupting this interaction [Baumann 2000; Ramamoorthy 2010; Samuvel 2008]. Therefore it is suggested that OA is binding to the catalytic subunit of PP2A as well and that OA and the transporters compete for the binding site, or the associations may be very sensitive to inhibitor-induced conformational changes [Gupta 1997].

Identification and confirmation of the phosphorylation site at Thr48 in hDAT

It has been shown that mutations in the phosphorylation sites at the terminal end of DAT have severe functional consequences [Fog 2006; Foster 2012; Guptaroy 2009; Moritz 2013]. Consequently, hDAT Thr48 was confirmed as a phosphorylation site in a heterologous LLC-PK_1 cell model by means of immunprecipitation of DAT and further mass spectrometry (MS) analysis (conducted by Jae-Won Yang). His findings clearly showed enhanced phosphorylation upon OA treatment at Thr48, whereas Ser7 and Ser53 showed far less response to the PP2A inhibitor. Further, the PKC activator beta-PMA, a PKA activator (8-Br cAMP) and anisomycin for p38MAPK activation were tested whether they could induce enhanced phosphorylation at Thr48. These substances could not induce a phosphorylated state at Thr48 in hDAT. To quantify the increase of phosphorylation at Thr48 by OA, he performed Stable Isotope Labeling by Amino acids in Cell culture (SILAC). Functional assays to determine the role of phosphorylation at Thr48 in hDAT regulation are carried out as the present master thesis.

Materials and Methods

Cell culture

LLC-PK$_1$, short for Lewis Lung Carcinoma-Porcine Kidney, cells are mature epithelial cells and have been widely used for phosphorylation studies [Bauman 2000; Foster 2012; Gorentla 2009; Moritz 2013]. For conducted experiments stable cell lines expressing hDAT wild type and mutant have been utilised, provided by Prof. James D. Foster (Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, USA).

LLC-PK$_1$ cells were stably transfected with the pcDNA3 vector (Invitrogen), harbouring a CMV promoter, a T7 promoter, a bGH polyA signal, an ampicillin cassette, a neomycin cassette and hDAT cDNA as shown in Figure 6. The hDAT cDNA insert is flanked by restriction sites for the restriction enzymes KpnI and EcoRI.

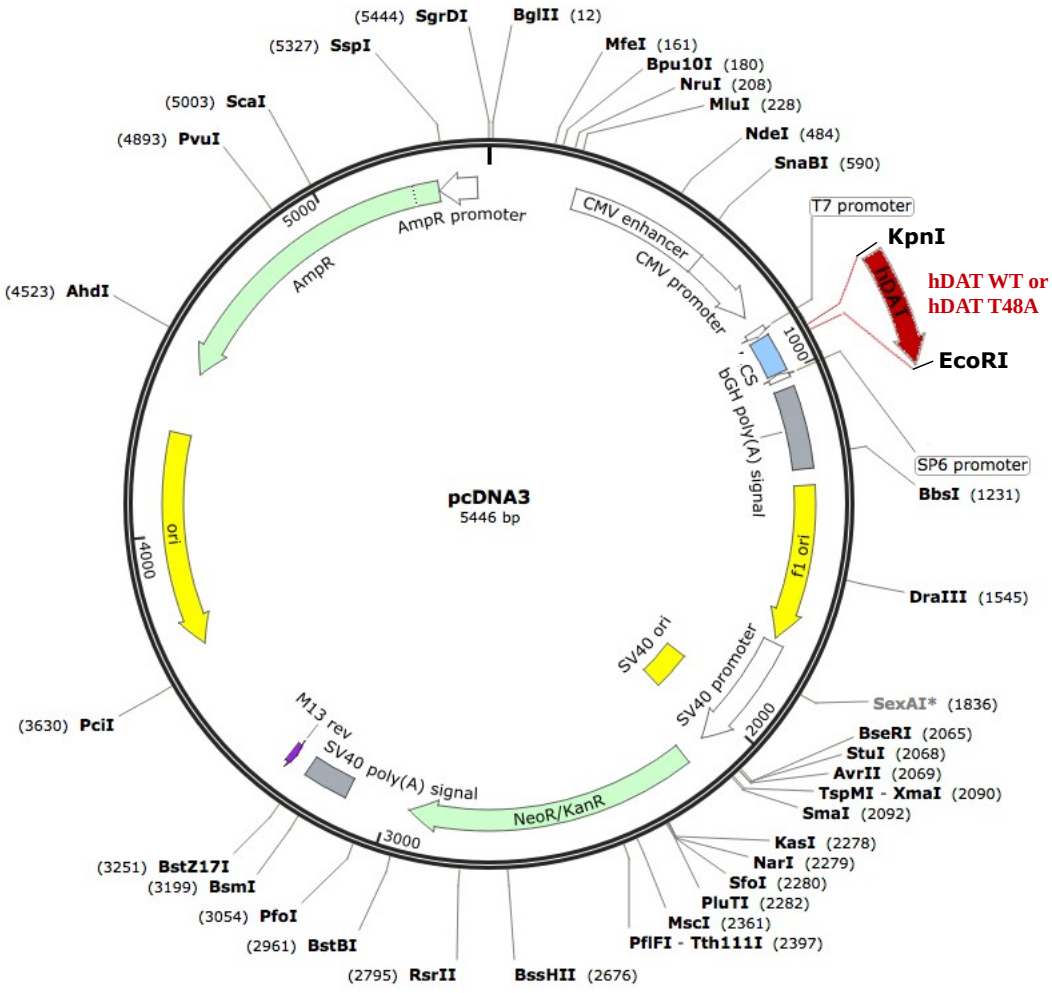

Figure 6. The plain pcDNA3 vector with marked insertion sites for hDAT WT and T48A cDNA. *The pcDNA3 vector harbours a CMV promoter, a BGH polyA signal, an ampicillin cassette, a neomycin cassette and hDAT cDNA. The hDAT cDNA insert is flanked by restriction sites for the restriction enzymes KpnI and EcoRI. The figure*

was generated with SnapGene 3.1 with the vector pcDNA3 provided by Invitrogen and cDNA of hDAT.
Cells have been cultivated with Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (9 g/L glucose) with L-glutamine (Sigma-Aldrich; Product-ID D5796-500ML), further supplemented with 10% fetal calf serum (FCS, Sigma) and 5 mL penicillin/streptomycin solution (10000 u penicillin and 10 mg streptomycin per mL, Sigma) in a humidified incubation chamber gassed with 5% CO_2 at 37 °C. To maintain transfection stability via antibiotic resistance selection constraint the cells have been treated with G418 (Geneticin[2]; final conc.: 250 µg/mL; Gibco) after every passage.

Uptake assay

The cells were seeded in a density of 80,000 cells in 0.5 mL in a 48-well plate (confluence 80-90%) 24 h before an uptake assay was performed.

For a detailed protocol see Addendum I.

hDAT WT and hDAT T48A expressing cells were preincubated with OA for 30 min. in Krebs-HEPES buffer (KHB; pH = 7.3; *recipe see Addendum II*).

Tritium labelled DA solutions (in KHB) were applied immediately after preincubation for 60 s. Applied [^{3}H]DA solutions were concentrated 0.2/1/3/10/30/45/60/100 µM (of which 0.1 µM [^{3}H]DA). During each experiment each concentration was applied in triplicates or hexaplicates. To stop the uptake, the DA containing solution was removed and cells washed with ice cold KHB twice. Non-specific uptake was taken into account (10 µM mazindol; 10min pre-incubation). Treated cells were lysed with 1% SDS and transferred into scintillation tubes containing 2 mL scintillation cocktail (Rotiscint® eco plus, Carl Roth Germany). The beta radiation produced by the [^{3}H]DA was detected as light impulses with a beta-counter (Packard Tricarb 2300 TR).

A step-by-step protocol can be found as Addendum III.

Superfusion experiments

An substrate efflux assay using a superfusion apparatus shows the DAT's reaction to applied substances when the transport direction is reversed by D-amphetamine (AMPH). The cells were seeded in a 96-well plate in a density of 40,000 cells on previously inserted coverslips (0.5 cm in diameter).

Detailed protocol see Addendum IV.

Beforehand, the superfusion apparatus was cleaned with isopropanol and H_2O and readied for the cells by washing with KHB, supplemented with 100 µM pargyline (Sigma) and 100 µM L(+)-ascorbic acid (VWR International PROLABO), for 15 min. hDAT expressing cells were exposed

2 G418 blocks polypeptide synthesis by inhibiting the elongation in prokaryotic and eukaryotic cells. Resistance to G418 is encoded by the Neomycin resistance gene (neo).

to a [³H]DA solution (0.2 µM) in KHB for 20 min. After the cells incorporated the applied [³H]DA, the coverslips were inserted into the superfusion chambers (12 chambers, to measure conditions simultaneously and individually). Subsequently, the cells were constantly superfused with KHB or solutions additionally containing OA and/or AMPH in a one-way manner. At first, excess tritium-labelled substrate was washed out with KHB for 45 min at 25 °C at a perfusion rate of 0.7 mL/min. Liquid passing the cells was collected in 2min fractions (supplemented with 2mL scintillation cocktail) after the washout phase. Fractions of basal conditions have been collected (buffers without AMPH; min 0-14), followed by fractions of the rising phase (directly after application of AMPH) and plateau phase (min 20, 22, 24, 26). After collection of the last fraction, the cells were lysed with 1% SDS and collected to determine the amount of residual [³H]DA. The beta radiation produced by the tritium was translated into light impulses with a scintillation cocktail (Rotiscint® eco plus, Carl Roth Germany) and measured with a beta-counter (Packard Tricarb 2300 TR) as counts per minute (cpm).

A step-by-step protocol can be found as Addendum V.

Determination of protein expression and Western blot

To compare protein expression of the WT and the T48A mutant, two million cells per 6 cm dish, containing 6 mL culture medium, were seeded. Culture medium was removed 24 h later and washed with phosphate buffered saline (PBS). Cells were lysed with RIPA buffer containing protease inhibitors (Protease inhibitor cocktail, Roche).

Subsequently, soluble fractions were extracted by centrifugation from the cell lysate for 10 min. at 15700 xg at 4 °C. The supernatant was transferred into a new sample tube and protein concentration was measured with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Protein standards (Bovine Serum Albumine Standard, concentrated 2 mg/mL, Thermo-Fisher Scientific) were concentrated 0/0.1/0.25/0.5/1/1.25/1.75 mg/mL (linear proportions confirmed by calculation of r^2; r^2 = ~0.99).

WT and T48A cell lysate samples with protein concentrations of 1/5/10/20/50 µg were loaded onto a 10% polyacrylamide running gel combined with a 5% polyacrylamide cover gel.

The solubilised proteins were separated by size with a polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride blotting membrane (PVDF membrane). Subsequent immunolabeling with DAT- or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific and horseradish peroxidase-conjugated antibodies (details see Table 2) and visualised on a film (Amersham Hyperfilm™ ECL High performance chemiluminescence film, =“hyperfilm”, GE Healthcare) with either SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) or Amersham™ ECL™ Prime/Select Western Blotting Detection Reagent (GE Healthcare).

A step-by-step protocol can be found as part of Addendum VI.

Table 2. Antibodies used for immunolabeling of DAT and GAPDH immobilised on a PVDF membrane.

DAT	primary antibody	Goat Anti-DAT sc-1433 (C-terminal epitope, concentrated 200µg/mL; used dilution 1:800 in 0.1% TBST, Santa Cruz)
	secondary antibody (horseradish peroxidase-conjugated)	Donkey Anti-Goat IgG-HRP sc-2020 (concentrated 100µg/ml, used dilution 1:5000 in 0.1% TBST, Santa Cruz)
GAPDH	primary antibody	Chicken Anti-GAPDH AB2302 (concentrated 1mg/mL, used dilution 1:5000 in 0.1% TBST, Millipore)
	secondary antibody (horseradish peroxidase-conjugated)	Donkey Anti-Chicken IgY-HRP SA1-300 (concentrated 32µg/ml, used dilution 1:5000 in 0.1% TBST, Pierce)

Cell surface biotinylation assay

For cell surface biotinylation assays two million cells were grown on 6cm dishes for 24 h under standard conditions. The cell confluence at the time of the assay was about 80%. After removal of culture medium the cells were washed once with 3 mL KHB, preincubated with 0.5 µM OA in KHB for 30 min at room temperature, followed by three washing steps with 3 mL PBS^{2+} before incubating with 2 mL Sulfo-NHS-SS-biotin (not membrane-permeable, Thermo-Fisher Scientific) concentrated 1 mg/mL for 15min at 4 °C twice. To wash off unbound biotin the cells were washed with PBS^{2+} three times on ice. To capture residual biotin molecules 2 mL of 100 mM glycine (in PBS^{2+}) was added for 15 min at 4 °C. Washing with PBS^{2+} and glycine solution was performed twice. After removal of the glycine solution, cells were washed with PBS^{2+} again three times on ice. Biotinylated cells were incubated with 500 µL RIPA buffer for 30 min at 4 °C for lysis and subsequently scraped off. The cell lysate was transferred into a 1.5 mL tube and centrifuged at 15700 xg for 10 min at 4 °C. The supernatant (further referred to as “cell lysate”) was transferred into a new tube and the protein concentration was measured using the BCA method mentioned above. SDS-PAGE, Western blotting and immunodetection have been performed as described in the section above.

After the protein contration of the cell lysates have been determined, 75 µL were transferred into a new 1.5 mL tube and set aside. 500 µL RIPA buffer and 60 µL streptavidin agarose beads (Pierce™ High Capacity Streptavidin Agarose) were added to the residual cell lysate over night at 4 °C on a mild shaking device for protein pull-down. Tubes containing cell lysates were handled the same way. Consequently, the solutions with streptavidin agarose beads were centrifuged at 6,500 xg at 4 °C for 5 min. The supernatant was removed and discarded. New RIPA buffer with protease inhibitor cocktail was added and centrifuged again. The buffer exchange step and the centrifugation step were repeated twice to wash unbound proteins off the beads. The supernatant was removed again and subsequently 60 µL of 100 mM dithiothreitol

(DTT) added. To elute the biotinylated proteins tubes containing the beads in DTT were incubated at 37 °C on a Eppendorf thermomixer (800rpm) for 30min. The tubes containing cell lysates were treated the same way: 20 µL 100 mM DTT were added to the solution and placed on the thermomixer as well. After the incubation time the supernatant of the tubes containing the beads was transferred into a new tube. The prepared samples were stored at -20 °C until they were required for Western blot.

A step-by-step protocol can be found as part of Addendum VI.

Data Analysis

The initial data in counts per minute (cpm) was compiled in Excel 2010 and arranged in Graphpad Prism 5. Statistical analysis was performed with Prism only (Student's *t* test, one-way ANOVA with Tukey's multiple comparison test).

For uptake experiments the data of single point measurements (DA concentrations) were plotted as an X/Y dot plot. A constant cell number expressing a constant amount of hDAT can process its substrate in increasing concentrations with less efficiency, producing an exponential phase and a plateau phase, ideally forming a monoexponential hyperbola (Michaelis-Menten kinetics) due to transport saturation. Thus, values obtained during uptake assays are fitted to a monoexponential hyperbola to calculate the kinetic parameters K_m (transport efficiency) and V_{max} (transport capacity). Data is presented as means±SEM.

Compiled data of efflux experiments were plotted on a X/Y dot plot illustrating the cpm of the tritium beta emission for each fraction transformed into data points as percentage of the total efflux (all fractions summed; 100%). The area under the curve (AUC), summing up the AMPH-mediated efflux (background noise subtracted), has been calculated for each test condition and subjected to statistical analysis. Data is presented as means±SEM.

Results obtained from Western blotting were discussed by the presence or absence of a signal of a certain size (in kDa), and signal density (black on light grey background). Signal density was determined of a scan of the hyperfilm via ImageJ. Certain areas were selected and optical density computed. Unspecific background signal was subtracted from computed optical density values. Adjusted measurements were plotted in a bar blot as means±SD.

Appropriate statistical analysis of the data obtained was performed as indicated in the figure legends. Briefly summarised, Student's *t* test (unpaired) was applied to test for significance between two experimental conditions (untreated, treated). For comparing more than two conditions (untreated, treated) a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test (a post hoc *t* test; two-tailed), was utilised. Significant differences are highlighted with at least one asterisk (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

Results

DA uptake assays reveal a tendency of uptake reduction when Thr48 is phosphorylated

Transport proteins are characterised by their transport capability of their substrate via Michaelis-Menten-kinetics. To gain information about transport capacity and efficiency of DA transport under the influence of OA, uptake assays were performed without or with treatment of 0.5 µM OA for 30 min in a heterologous LLC-PK_1 cell line expressing hDAT. Experimental data was fitted to a monoexponential hyperbola after assays were performed six times each (Figure 7). The OA-treated WT displayed a reduced transport capacity of 44.01±4.891 pmol/10^6 cells/min, a reduction by 40% of the mean in regard to the untreated WT (73.22±8.1359). However, due to the data scattering, the difference did not reach statistical significance (p=0.1964). Kinetic parameters were also determined for $hDAT^{T48A}$ in the presence and absence of OA. In this case, the probability of difference was even lower when a t-test was applied (p=0.5339). V_{max} of $hDAT^{T48A}$ was 253.5±30.2 and reached 197.9±19.95 after OA treatment. K_m values did not differ significantly between treated and untreated conditions (Table 3). The affinity of DAT to it's substrate was about 2.6 µM for $hDAT^{WT}$ and about 2.7 µM for pThr48 $hDAT^{WT}$. The T48A mutant presented less affinity to its substrate: About 4.3 µM for the untreated and 4.6 µM for the treated condition.

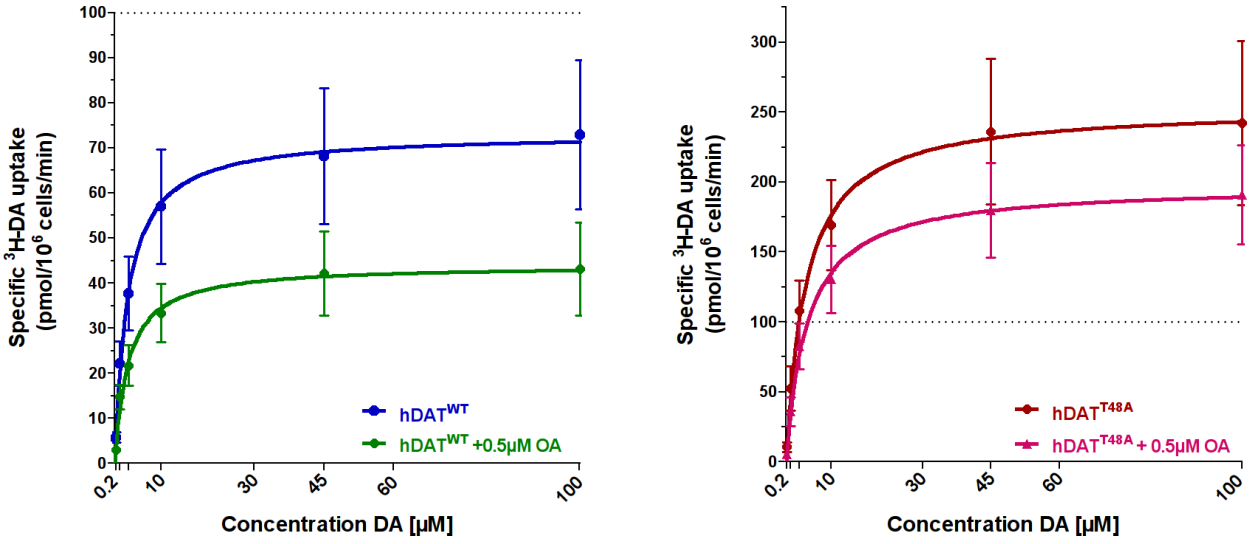

Figure 7. Uptake kinetics of [³H]DA in cells expressing $hDAT^{WT}$ and $hDAT^{T48A}$. *[³H]DA uptake was measured at indicated DA concentrations and kinetic analysis was performed as described in "Materials and methods" in $hDAT^{WT}$ (●), $hDAT^{WT}$ with OA (●), $hDAT^{T48A}$ (●) and $hDAT^{T48A}$ with OA (●). Each experiment was performed 6 times in triplicates or hexaplicates. Preincubation with 0.5 µM OA for 30 min reduced uptake capacity in the wild type as well as in the mutant, however only the computed p-value of the applied t-test for $hDAT^{WT}$ treated and untreated showed a high probability to be different. Cell lines show high data scattering, likely due to varying protein surface expression levels, therefore data could not be compared directly. Y=100 pmol/10^6 cells/min is indicated with a dotted line in each graph. V_{max} and K_m values are evaluated in detail in Table 3.*

Table 3. Important statistical parameters and Michaelis-Menten kinetics on hDAT

hDAT WT n = 6	**kinetics:**	**V_{max} (pmol/10^6cells/min; mean±SEM)**	**K_m (µM; mean±SEM)**
	WT not treated	**73.22 ± 8.14**	**2.645 ± 1.34**
	WT +OA	**44.01 ± 4.89**	**2.745 ± 1.42**
	t-test: p = 0.1964, difference not significant		
hDAT T48A n = 6	**kinetics:**	**V_{max} (pmol/10^6cells/min; mean±SEM)**	**K_m (µM; mean±SEM)**
	T48A not treated	**253.5 ± 30.20**	**4.315 ± 2.25**
	T48A +OA	**197.9 ± 19.95**	**4.597 ± 2.01**
	t-test: p = 0.5339, difference not significant		

hDAT expression patterns and surface expression

To analyse hDAT expression patterns in LLC-PK_1 cells two million cells were cultivated, lysed and proteins were resuspended in RIPA buffer containing protease inhibitors. After extracting membrane fragments from the protein suspension by centrifugation, the total protein concentration was determined. 15 µg of cell lysates were loaded onto a SDS-polyacrylamide gel, and subsequently Western blotting was performed. The glycosylated and the non-glycosylated T48A mutant was found to co-migrate along the glycosylated $hDAT^{WT}$ at 70-100kDa and 55kDa after development on hyperfilm with ECL solution [Kurian 2009]. The data was further digitalised and analysed for optical density (OD) with ImageJ (Figure 8A,B). The data is shown normalised to $hDAT^{WT}$ expression or $hDAT^{T48A}$ expression in a bar chart ($hDAT^{WT}$ n=12; $hDAT^{T48A}$ n=9). Cells pretreated with OA did not show significant difference (WT p=0.8939; T48A p=0.2279) to untreated cells in total protein lysates when a Student's t-test was applied. To determine specifically hDAT surface expression levels for all tested conditions biotinylation assays were performed (Figure 8C,D). Samples of protein surface biotinylation assays were found to be contaminated with intracellular proteins, such as the non-glycosylated DAT form and GAPDH, highlighted in red.

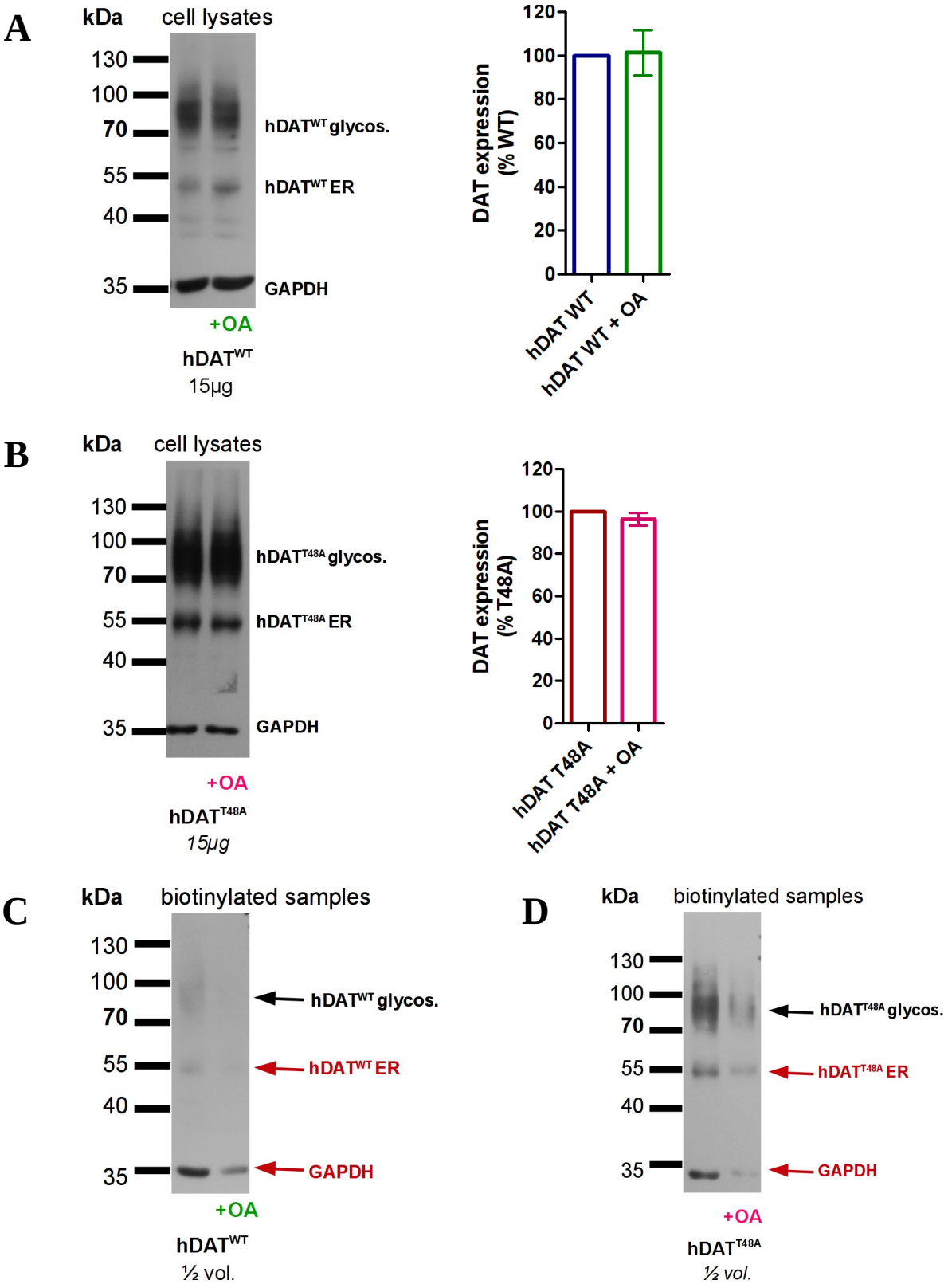

Figure 8. Representative Western blot of cell lysates and biotinylation assays. *For each condition one sample was chosen to be displayed in a representative Western blot. A. illustrates the hDAT^{WT},expression (left panel) and quantitative comparison of untreated versus OA-treated cells (right panel). B. represents the corresponding results for the T48A mutant. Expression patterns before and after treatment with OA do not differ (WT+OA mean 101.4±10.4, p=0.8939, n=12; T48A+OA mean 96.37±2.89, p=0.2279, n=9). Western blots performed after the biotinylation assays displayed unreliable data and contamination (marked in red) with intracellular proteins (C,D).*

Phosphorylated Thr48 and the dephosphomimetic mutant T48A do not exert different characteristics in efflux assays than the hDAT^{WT}

To gain information whether phosphorylation at Thr48 in hDAT affects AMPH-induced DA reverse transport (efflux), using a superfusion apparatus, was evaluated. For each experiment either the phosphomimetic WT or the dephosphomimetic T48A mutant were tested against the untreated WT (6 traces/condition/superfusion). After performance of three superfusions testing paired conditions, a trace graph was designed and area under the curve (AUC) was computed. Initial data are counts per minute (cpm) as the tritium beta emission for each fraction were transformed into light impulses. Resulting corrected data points are shown as percentage of the total efflux (all fractions summed; 100%) and the data is thus independent of protein surface expression. Mean±SEM of all efflux traces are presented in Figure 9 left panel.

After performance of three superfusions, evaluation with a one-way ANOVA and Tukey's multiple comparison test of the AUC of each condition followed (Figure 9 right panel). Arbitrary AUC values calculated were as follows: hDAT^{WT} 131.9±11.03, hDAT^{WT} pThr48 124.3±13.98, hDAT^{T48A} 139.7±22.21. Basal efflux was already determined and subtracted (hDAT^{WT} 4.32%·min^{-1}, hDAT^{WT} pThr48 3.77%·min^{-1}, hDAT^{T48A} 5.62%·min^{-1}). Statistical analysis negated significant difference in the ability of producing efflux between hDAT^{WT}, hDAT^{WT} pThr48 and hDAT^{T48A} (p=0.6318; Figure 9 right panel). Unphosphorylated hDAT^{WT} and the dephosphomimetic T48A mutant exert similar AUC values.

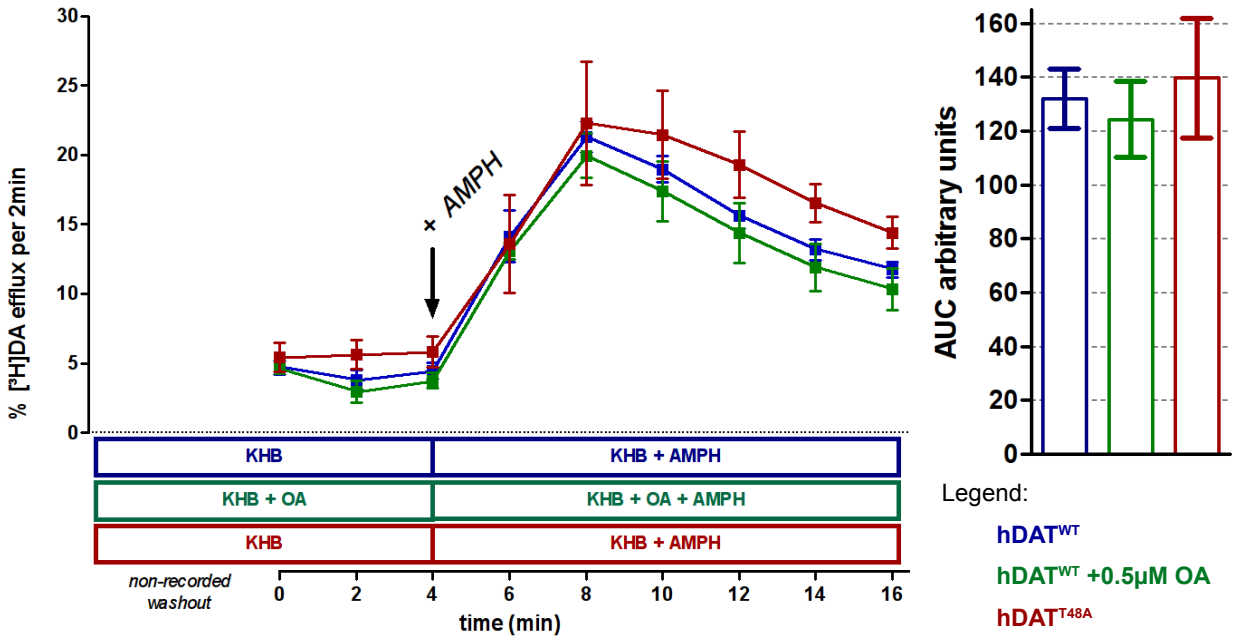

Figure 9. AMPH-stimulated reverse transport of [³H]DA by hDAT^{WT} (■), hDAT^{WT} pThr48 (■) and hDAT^{T48A} (■). *Stably transfected LLC-PK1 cells expressing hDAT^{WT} and hDAT^{T48A} were preloaded with [³H]DA at 37°C and subsequently superfused (as described in "Materials and Methods"). After the washout phase basal efflux was defined (2min fractions; min0-4): hDAT^{WT} 4.32%·min^{-1}, hDAT^{WT} pThr48 3.77%·min^{-1}, hDAT^{T48A} 5.62%·min^{-1}. Addition of 10µM AMPH to the superfusion buffer (min6-16; 6 fractions à 2min) did not exert different observations between test conditions (AUC±SEM): hDAT^{WT} 131.9±11.03, hDAT^{WT} pThr48 124.3±13.98, hDAT^{T48A} 139.7±22.21. Experiments were performed 3 times on different days in a bulk of either hDAT^{WT} and hDAT^{WT} pThr48 or hDAT^{WT} and hDAT^{T48A} and the resulting traces of the efflux assay were pooled according to the test groups. A performed one-way ANOVA and Tukey's multiple comparison test of the AUC of each condition shows that there is no significant difference of the three test conditions (ANOVA p=0.6318).*

Discussion

Uptake assays tend to result in reduced substrate uptake after OA treatment

Uptake assays show reduced DA uptake capacity, both in the WT and the T48A mutant by OA. However, due to data scattering no significance could be detected for either WT+OA versus untreated WT ($p=0.1964$) or T48A+OA versus T48A ($p=0.5339$). Furthermore, the cell lines could not be compared directly as they exhibited different expression levels, the V_{max} of T48A was 3.5 times higher than the V_{max} of WT. Due to data scattering and different protein expression levels of the present cell lines the experimental data required normalisation to DAT surface expression. To determine hDAT surface expression biotinylation assays were performed with a membrane impermeable biotin conjugate. However, persistent contamination with intracellular proteins, such as unglycosylated forms of hDAT and GAPDH, lead to inconclusive results. Therefore we examined hDAT expression patterns of the cell lysates with and without pretreatment of OA (Fig. 8). Applied Student's t-tests on $hDAT^{WT}$ and $hDAT^{T48A}$ negated difference between OA-treated and untreated cells. Thus suggests that OA does not affect DAT protein expression.

Reverse transport is not affected by either OA or the alanine substitution at Thr48

Analysis of AMPH-mediated efflux by $hDAT^{WT}$, $hDAT^{WT}$+OA and $hDAT^{T48A}$ did not show statistical differences. Thus it is concluded that phosphorylation at Thr48 does not affect reverse transport. It might be suggested that impaired uptake capacity may distort the superfusion results. However, due to 20 minutes uptake time before the experiment was started the cells are considered equally saturated with [^{3}H]DA. Mutations of DAT could lead to a DA leak represented by a considerable high basal efflux. Subtle differences of the basal efflux have been observed for the T48A mutant, however without statistical significance.

Several already examined DAT mutants presented specific characteristics. For example, a Δ1-60 truncation mutant could not be exported to the cell surface, but remained retained intracellularly [Torres 2003a]. Single point mutations (A or D) at Thr53 in rDAT lead to a reduced uptake by half and to virtually no efflux [Foster 2012]. An alanine mutation at Thr62 in hDAT is affecting DA uptake by about 20%, however efflux has not been shown in the article. The corresponding phosphomimetic D mutant lacked efflux and uptake almost entirely. However, efflux could be rescued to Thr62 levels due to administration of zinc in the superfusion buffer [Guptaroy 2009]. It was established that this kind of mutant was preferably inward-facing. Thr62 corresponds to Thr81 in the closely related hSERT. Alanine and aspartate mutants displayed a dramatic decrease uptake and almost no efflux [Sucic 2011]. Analysis of Thr48 in hDAT does not conform with any of the findings of the mentioned mutants and presents itself unique. However, efflux data for the T62A has not been shown and T48D or T48E mutants have not been tested yet. It is possible that these mutants might show strong similarities, inward-facing conformations for example, after extensive investigation.

All isoforms of PP1 and PP2A are inhibited with different affinities by OA within the viable cell. However, it is not yet clear which pathways might have been altered or alternatively activated during incubation times of 30 min. during uptake and of 46 min. during efflux assays. Both, PP1 and PP2A, have been reported to associate with DAT and blocking lead to hyper-phosphorylation of DAT as well as *ex-vivo* and *in-vitro* [Bauman 2000; Foster 2003]. PP2A alone has about 70 different isoforms [Shi 2009]. It is not yet determined which isoforms of both PPP family members are dephosphorylating DAT and where specifically with which affinity. Hence, it is not surprising that treatment with protein phosphatase inhibitors like OA also affects rDAT although rDAT is lacking a Thr48. It has been shown by Jae-Won Yang that in hDAT OA specifies between Ser7, Thr48 and Ser53, but it remains unclear which other phosphorylation sites might be affected at the same time as Thr48. Thus, the results obtained could possibly arise from pThr48 alone, or in combination of one or many other phosphorylated residues in hDAT. To manage to distinguish between PP1 and PP2A effects more specific inhibitors, such as the endogenous PP1 inhibitors 1 or 2 or PP2A inhibitor CIP2A. These inhibitors have been mentioned in other publications before [Foster 2003; Eto 2012]. To conclude, only D and E substitutions at Thr48 might simulate specific phosphorylation more accurately.

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Zusammenfassung

Einleitung. Neurotransmitter transporter der SLC6 Familie haben lange intrazelluläre Termini. Unter normalen Bedinungen und bei Behandlung mit bestimmten Pharmaka werden Monoamintransporter vor allem druch Ser/Thr (De)Phosphorylierungen reguliert. Während Ser/Thr Kinasen Phosphatreste an Ser/Thr anhängen, sind Phosphatasen für deren Entfernung zuständig. Phosphorylierung von einzelnen oder von Gruppen von Ser/Thr beeinflussen stets bestimmte Funktionen oder die Struktur des Proteins, und somit messbare Parameter.

Die N-terminal situierten Thr53 im rDAT und Thr62 im hDAT haben sich als sehr wichtig für die Modulation von DAT gezeigt. Obwohl Thr48 nicht zwischen vielen Spezies konserviert ist und nur im hDAT aufzufindent ist, haben wir dieses als Phosphorylierungstelle identifiziert, was darauf schließen lässt, dass Thr48 bei regulativen Prozessen eine Rolle spielen könnte. Unter normalen Bedingungen liegt Thr48 in heterologen Zellsystemen unphosphoryliert vor. Die Inhibierung von PP2A mit Okadarsäure (OA) führt auf äußerst spezifische Weise zur Anhäufung von pThr48, jedoch nicht von Ser7 oder Ser53. Die Interaktion von hDAT mit PP2A wurde durch Immunopräzipitation und anschließender Massenspektrometrie bestätigt.

Ziel dieser Arbeit. Ziel dieser Arbeit ist es, mit *In-vitro*-Methoden herauszufinden ob hDAT pThr48 bei der Neurotransmission mit DA beim Menschen eine Rolle bei der Transporter-regulation spielt.

Methodik. Heterologe LLC-PK_1 Zelllinien, die $hDAT^{WT}$ und $hDAT^{T48A}$ exprimieren, werden sowohl sogenannten Uptake- und Efflux-Assays unterzogen als auch deren Oberflächen-expression durch den Biotinylierungsassay bestimmt.

Ergebnisse. Die Aufnahme von DA über den hDAT in die Zelle ist sowohl beim WT als auch bei der T/A muante bei Behandlung mit 0,5µL OA für 30min erniedrigt. Experimente, die den Efflux von DA erfassen, zeigen bei keiner Testbedingung eine Verändung zum unbehandelten Wildtyp. Die Proteinexpression von hDAT ist im Allgemeinen bei den LLC-PK_1-Zellen sehr bescheiden. Die Oberflächenbiotinylierung hat nicht die Ergebnisse geliefert, die erwartet wurden: Die Proben der Protein-Pulldowns war maßgeblich mit dem zytosolischen Protein GAPDH kontaminiert, was auf ein Problem der Methode hindeutet.

Diskussion. Um eine vernüftige Diskussion über den Bezug von sowohl $hDAT^{WT}$ und $hDAT^{T48A}$ zueinander, mit und ohne Behanldung mit dem Phosphataseinhibitor OA, als auch über deren Einfluss auf die Regulation von hDAT führen zu können, muss die Überexpression beider hDAT-Formen ermittelt werden, anstatt sich auf das Verhältnis von Behandelten zu unbehandelten Zellen zu beziehen. Derzeit hat es den Anschein, dass pThr48 keine Auswirkung auf die Fähigkeit des hDAT-Proteins bei der AMPH-induzierten DA-Ausschüttung hat. Schluss-folgerungen über die Uptake-Versuche können erst nach Normalisierung der Daten auf die oberflächenexprimierte Proteinmenge gezogen werden; bei der derzeitigen Lage könnte man schnell zu dem voreiligen Schluss kommen, dass pThr48 keine Auswirkungen auf hDAT beim Uptake hat. Andere bereits untersuchte DAT-Mutanten helfen die Daten und Rolle von Thr48 zu interpretieren.

Abstract

Introduction. Neurotransmitter transporters of the SLC6 family expose long termini to the cytosol. Under basal conditions and after administration of certain drugs, the monoamine transporters are mainly regulated via Ser/Thr (de)phosphorylations. Whereas Ser/Thr protein kinases manage the addition of a phosphates to the amino acids, Ser/Thr phosphatase catalyse the removal of those. Phosphorylation at each or a group of Ser/Thr alter distinct functions or the structure of the protein and thus measurable parameters.

N-terminal Thr53 in rDAT and Thr62 in hDAT have shown to be important in DAT modulation. Although Thr48 is not conserved across the species and only expressed in hDAT, our lab has identified it as a phosphorylation site and might be important in regulatory processes. Under basal condition in heterologous cell lines Thr48 presented itself non-phosphorylated. Inhibition of PP2A with okadaic acid (OA) lead specifically to accumulation of pThr48, but not in Ser7 or Ser53. The interaction of hDAT with PP2A was demonstrated via immunoprecipitation and ensuing mass spectrometry.

Aim of the study. To check for implications of hDAT pThr48 in regulation of human dopamine transmission using *in-vitro* techniques.

Methods. A heterologous LLC-PK_1 cell line expressing hDAT WT and T48A were subjected to uptake assays, efflux experiments and surface biotinylation.

Results. Uptake of dopamine into the cell via hDAT was decreased in the WT as well in the T/A mutant upon treatment with 0.5µM OA for 30min., however there was no significant difference detected. Efflux experiments show no impairment of amphetamine-mediated efflux in neither condition: T48A compared to WT, WT+OA compared to WT, T48A+OA compared to T48A. Protein expression levels were different between T48A (100%) and WT (10%), and protein pull-down did not work properly (contamination with notable amounts of control protein GAPDH).

Discussion. To enable a discussion about the relationship of $hDAT^{WT}$ and $hDAT^{T48A}$ and comparison of both conditions to those treated with OA in functional assays, protein surface expression must be determined and data normalised to this, instead of normalisation to 100µM ligand. At the present, for uptake assays tested conditions do not differ significantly from each other which could possibly misinterpreted that pThr48 in hDAT has no function. Regarding efflux assays, there is virtually no difference in either condition. pThr48 does obviously not alter the ability of hDAT to react upon AMPH treatment like the untreated wildtype. Similarities and dissimilarities to other investigated DAT mutants help to discuss present data.

Appendix

Addendum I:

Cell seeding for uptakes

have: want: seeding for day: ….....

10cm/10mL culture dish → 48well culture dish (0.5mL each) ….....

cell concentration 70,000/0.5mL

seeding date: ….....

<u>determine cell count in your 10cm dish:</u>

- suck off culture media
- wash with PBS (to get rid of serum), suck off PBS
- trypsinise the cells: add 1mL trypsin, wait shortly
- add media to resuspend cells: 10mL (except: only 40% of 10cm-dish full of cells)
- transfer all cells in suspension into a 50mL-falcon tube
- remove ~20µL to count cells in a cell counting chamber
- count cells (both sides of the Neubauer double cell counting chamber)
- calculate mean
- divide by 100 (to get x 10^6 cells per mL) → *to get absolute amount of cells multiplicate with added trypsin+media*
- divide by 0.14 (=factor; 0.07*10^6 cells/mL in 0.5mL wanted → 0.07/0.5=0.14) → result = dilution factor
- calculate final vol. of your seeding suspension (amount of wells*0.5mL=.....mL + extra)
- divide final vol. by the dilution factor calculated → vol. of trypsin-cell suspension required
- subtract vol. Of trypsin-cell suspension from final vol. → vol. of new media required
- mix seeding suspension

<u>seed:</u>

- re-suspend cells well (vortex shortly, if required)
- use multi-stepper (12.5mL tip)
- 0.5mL cell suspension into each well

cell count:

mean:

…..... / 100 = …..... / 0.14 = …..... (dilution factor)

end volume: ….....mL

…..... / ….....= ….....mL of trypsin-cell suspension (if you seed for the day after tomorrow /2)

..…... - ….....= ….....mL fresh media (if you seed for the day after tomorrow add ….....mL more media)

Addendum II:

Recipe Krebs-HEPES Buffer

1. Take adequate plastic beaker, label it (content, initals, date) and place it on the scale and tare
2. weigh in:

substance	manufacturer & product ID	mass weight	final concen-tration	grams required for 1 liter solution	5 liters
HEPES	Sigma H-3375	238.3 g/mol	10 mM	2.383 g	14.1 g
NaCl	Sigma S-9625	58.44 g/mol	120 mM	7 g	35 g
KCl	Merck 1.04936.500	74.56 g/mol	3 mM	0.224 g	1.12 g
$CaCl_2 \cdot 2\ H_2O$	Merck 2382.1000	147.02 g/mol	2 mM	0.3 g	1.5 g
$MgCl_2 \cdot 6\ H_2O$	Merck 5833.250	203.3 g/mol	2 mM	0.4 g	2 g
Fluka D-(+)-glucose monohydrate	Sigma 49159	198.17 g/mol	20 mM	3.96 g	17.62 g

3. fill beaker up to scale (1 liter or 5 liter) with MQ-H_2O (extremely filtered, deionised tap water)
4. add magnetic stirer and stir until there are no crystals visible any more
5. adjust pH to 7.300 with either NaOH or HCl with pH meter
6. cover the beaker with metal foil

storage: max. 2-3 weeks in the fridge (4°C), if stored right away after use

→ always check stored glucose-containing KHB for contamination before usage !

Addendum III:

Uptake with [^{3}H]DA

with LLC-PK$_1$ hDAT WT / T48A cells & **Okadaic Acid**

Preparation:

1. have a look at your cells → should be on 48-well-plate, cell density should be ok!

2. bring ice box (for cooling KHP wash buffer)

3. prepare solutions ***(per uptake with 60 wells)***:

- ***Okadaic Acid*** (OA) stock solution (500µM): provided by Jae-Won

- KHP: 150mL KHP

  25mL KHP + OA (0.5µM final conc., take 25µL of 500µM stock solution)

- [^{3}H]DA: stock: µM, final conc.: 0.1µM 9* 35µL + 10µL TA + extra vol. → 330µL KHP -/+ 9.5µL stock solution

  → keep 10µL for total activity (TA) !

- DA: aliquot from -80°C freezer (10mM) → make dilutions: 1mM, 100µM, 10µM

  1mM: 150µL → 135µL KHP + 15µL DA 10mM stock

  100µM: 100µL → 90µL KHP + 10µL DA 1mM stock

  10µM: 100µL → 90µL KHP + 10µL DA 100µM stock

- working solutions: prepare 2x (**1x with KHP**, **1x with KHP+OA**!)

		Final conc. ^{3}H-DA [µM]	**End volume [µL]**				**Final conc. MAZ [µM]**
		0.1	350				10
Eppi#	**Final conc. [µM]**	**[^{3}H]DA conc. (10x)**	**DA [10µM]**	**DA [100µM]**	**DA [1mM]**	**KHB**	**Bk/MAZ [µM]**
		1	10	100	1000		1000
1	0.2	35	7.0	xxx	xxx	308	xxx
2	1	35	35.0	xxx	xxx	280	xxx
3	3	35	xxx	10.5	xxx	305	xxx
4	10	35	xxx	35	xxx	280	xxx
5	30	35	xxx	xxx	11	305	xxx
6	45	35	xxx	xxx	15.75	299	xxx
7	60	35	xxx	xxx	21	294	xxx
8	100	35	xxx	xxx	35	280	xxx
Bk	100	35	xxx	xxx	35	277	3.5
Prä	xxx	xxx	xxx	xxx	xxx	347	3.5

➔ Vortex & spin down before use!

➔ label 1,5mL tubes accordingly (1-8, BL, Prä)

4. liquid waste flask available?

5. place 48-well plate with cells on the “Geckopad”, cut edges to the left side

Initiate procedure:

6. remove cell culture media, add 500µL KHP (always without KHP) @RT with the multistepper (be gentle, slowly) + tilted angle!

7. add markings to the bottom surface, if necessary “blank” wells, wells with cells to be counted

Uptake:

1. **process the actual "uptake" wells:**

control:

- remove KHP, add 100µL of uptake solution (cave: diff. conc.! start with tube "1"), wait 1min for each well
- suck off solution
- wash 2x with ice cold KHP (750µL)
- suck off KHP
- → 10s steps → use the timer!
- add 500µL SDS (1%) to lyse cells
- discard the pipet tip

Okadaic Acid

- remove KHP
- add 500µL KHP+**OA**, wait **45min** for each well
- suck off solution, add 100µL of uptake solution (cave: diff. conc.! start with tube "1"), wait 1min for each well
- suck off solution
- wash 2x with ice cold KHP (750µL)
- suck off KHP
- → 10s steps → use the timer!
- add 500µL SDS (1%) to lyse cells
- discard the pipet tip

2. **treat the "blank" wells:**
   - remove KHP
   - (use new pipet tip)
   - add 100µL "Prä" solution (containing paroxetine), wait 5min to block SERT
   - suck off "Prä" solution, add "blank" solution, wait 1min for each well
   - suck off solution
   - wash 2x with ice cold KHP (750µL)
   - suck off KHP
   - → 10s steps → use the timer!
   - add 500µL SDS (1%) to lyse cells
   - discard the pipet tip

3. **process the wells that are going to be counted (cells):**
   - remove KHP
   - (use new pipet tip)
   - wash 2x with ice cold KHP (750µL)
   - suck off KHP
   - add 200µL Trypsine
   - discard the pipet tip
   - count the cells of each well 2x and note amount counted *2 *(because you added 200µL Trypsine; analysis Excel sheet is standardised to 100µL)*

4. **prepare scintillation vials:**
   - use 6mL vials
   - add 2mL scintillation cocktail
   - add (well suspended) cell lysate completely (try not to get foam)
   - close lids (press hard)
   - mark the lids
   - 1 vial extra: total activity (TA): 2mL scintillation cocktail + 10µL [^{3}H]5-HT solution 0.1µM

5. **Put vials into the β-counter:**
   - Use small racks (without black inlay!)
   - Take “your” flag

6. **Program the β-counter:**
   - insert all racks
   - insert protocol flag (flag must be on the left side of the rack!)
   - press F1 (protocol overview)
   - enter protocol number and press enter
   - enter your heading: yymmdd + first letter of your name + number
   - change settings if necessary: to be counted 3H, 1 cycle, 2.5min
   - save data: yes → to: C:/…/Packard…/ → file name: yymmdd+letter+number.001
   - press F1, F1 and if necessary F2 (to start counting)
   - counting…..will take a while

7. **getting the data off the β-counter (after counting is complete):**
   - enter floppy disk
   - go to main screen (F1)
   - press F10, then F3
   - program = “trans”
   - command string = “yymmdd + first letter of your name + number” (do not enter .001)
   - press enter
   - press F3 (=run program = transfer data to floppy disk)
   - press any key
   - press any key
   - press F1 to go to main status page

8. **analyse the data on the PC (+Excel)**
   - open Excel
   - press “open file”
   - set “all files” to see your file
   - use your yymmdd + first letter of your name + number.001 file
   - Excel asks you then to convert the format into something usable:
     1. a window pops up press next
     2. untick “tab” and tick “comma” press next
     3. click on “more settings…” button
     4. decimal = **.** and 1000 mark = **,** press OK
     5. press “finish”
   - use columns A to D (D = CPMA): copy entries (first TA only, then the rest)
   - open template “AA_VORLAGE_Sättigung EASYCOPY 8 Blöcke.xls“ (or similar name)
   - go to tab “daten”
   - insert CPMA data (TA first!)
   - save file as..
   - go to tab “cpm” and check the following: conc. 3H [µM], µL, Endkonz. [µM], insert cell count
   - check blank – outliers should be marked and exluded at cell “bk cpm”
   - copy orange cells “pmol/Mio/min”
   - open Graphpad Prism 5/6 and paste into data table
     - template should be available
     - exclude single outliers, if necessary
     - adapt headlines, descriptions…
   - save the file as…

Addendum IV:

Cell seeding for superfusions

have: want: seeding for day: ….....

10cm/10mL culture dish → 96 well culture dish (0.2mL each) ….....

cell concentration 30,000/0.2mL

seeding date: ….....

<u>determine cell count in your 10cm dish:</u>

- suck off culture media
- wash with PBS (to get rid of serum), suck off PBS
- trypsinise the cells: add 1mL trypsin, wait shortly
- add media to resuspend cells: 10mL (except: only 40% of 10cm-dish full of cells)
- transfer all cells in suspension into a 50mL-falcon tube
- remove ~20µL to count cells in a cell counting chamber
- count cells (both sides of the Neubauer double cell counting chamber)
- calculate mean
- divide by 100 (to get x 10^6 cells per mL) → *to get absolute amount of cells multiplicate with added trypsin+media*
- divide by 0.15 (=factor; 0.03*10^6 cells/mL in 0.2mL wanted → 0.03/0.2=0.15) → result = dilution factor
- calculate final vol. of your seeding suspension (amount of wells*0.2mL=.....mL + extra)
- divide final vol. by the dilution factor calculated → vol. of trypsin-cell suspension required
- subtract vol. Of trypsin-cell suspension from final vol. → vol. of new media required
- mix seeding suspension

<u>seed:</u>

- re-suspend cells well (vortex shortly, if required)
- use multi-stepper (5mL tip)
- 0.2mL cell suspension into each well

cell count:

mean:

…..... / 100 = …..... / 0.2 = …..... (dilution factor)

end volume: ….....mL

…..... / ….....= ….....mL of trypsin-cell suspension (if you seed for the day after tomorrow /2)

...….. - ….....= ….....mL fresh media (if you seed for the day after tomorrow add ….....mL more media)

Addendum V:

Superfusion experiment

with LLC-PK$_1$ hDAT WT / T48A

& Okadaic Acid

cell seeding:

- According to separate protocol for cell seeding
- Most important outlines:
  - use 96-well plate → add glass platelets → coat wells with platelets with 1x PDL
  - seed 40,000 cells / 0.2mL
  - SERT induction via *tetracyclines* – do not forget to add (otherwise discard the cells)

Experiment outline: ***Okadaic Acid:***

	Wash out 25min	**Base line 3x 2min**	**Base line 5x 2min**	**+ PCA 5x 2min**	**SDS 3x 2min**
Channel 1-4	KHP	KHP	KHP	KHP	SDS
Channel 5-8	KHP + ***OA***	KHP + ***OA***	KHP + ***OA***	KHP + ***OA***	SDS
Channel 9-12	KHP	KHP	KHP + ***OA***	KHP + ***OA***	SDS
fractions / valve	*---*	***1 - 3***	***4 - 8***	***9 - 13***	***14 - 16***

Channel 1-4: control [^{3}H]DA: 0.2 µM

Channel 5-8: long-term effect of AM PCA conc.: 10 µM

Channel 9-12: short-term effect of AM Anisomycin conc.: 1µM

Okadaic Acid conc.: 0.1µM

KHP: add DMSO accordingly

Preparation:

- check cells !!!
- buffers & stock solutions:
  - KHP: use 20x stock, add α-D-Glucose Monohydrate accordingly!

    for **3L** buffer: 150mL 20x stock + 11.88g Glu monohydrate, then fill up with MQ-H_2O,

    set pH to 7.300 with NaOH or HCl
  - OA stock [500µM] in EtOH → *OA MW = 805.01 g/mol*
  - KHP + OA (0.1µM final conc.): 400mL KHP + 80µL OA [500µM]
  - PCA stock [10mM]: 1mg PCA + 485µL KHP → *PCA MW = 206.11 g/mol*
  - [^{3}H]-5HT solution [0.4µM]: 1240µL KHP +/- 14.63µL [^{3}H]-5HT from [33.9µM stock] vortex, spin down

    or 1400µL KHP +/- 16.5µL [^{3}H]-5HT from [33.9µM stock] vortex, spin down

- Sufu apparatus:
  - check syringe in the sink: must be plugged into the tube
  - turn on main power switch for the apparatus
  - fill water into the tank (mark) & turn on thermostat (25°C)
  - remove upper tubes from all chambers → open up all chambers, get rid of residual glass platelets etc. and wash with bidest. H_2O (use the wash bottle)
  - control your tube bundles, rearrange them if necessary (here: quadruplicates)
  - put all bundles into 30% isopropanol (to get rid of air in the tubings)
  - press down all the white plastic bars of the right pump
  - turn on right pump, press "run" (velocity: 34)
  - put all bundles into bidest. H_2O (5L plastic beaker) – wash for ~1h
  - check tubings: dripping?, left pump working properly?
- **put tubings into KHP in time!!!**
- load cells with [^{3}H]-5HT: suck off media (non-sterile, radioactive room)
  add 100µL of 0.4µM [^{3}H]-5HT solution / well
  put cells back into the incubator (37°C) for **20min** (incubation)
- working solutions:
  - *3L KHP (pH 7.300): should be already prepared*
  - *400mL KHP + **OA** (0.1µM final conc.): should be already prepared*
  - calculations: **0.7mL/min * min * amount of chambers * fractions (x + 3 extra) =** ….. → round up generously
  - (1) efflux 5x 2min - KHP + PCA: 50mL + 5µLEtOH + 50µL PCA [10mM]
  - (2) efflux 5x 2min - KHP + OA + PCA: 100mL + 100µL PCA [10mM]
  - SDS: 3x 2min - flask should always be filled at beginning of sufu
  - → do not forget to mix solutions!

- Insert cell platelets:
  - Set all chambers to blue
  - Put all blue tubings into the KHP buffers (the red ones remain in bidest. H_2O) = 5 min before inserting cells !
  - Stop the right pump
  - Remove upper tubings from the chambers
  - Open the chambers
  - Put in platelets and close the chambers, attach tubings correctly
  - Start right pump (level 34) → **40min wash-out**

- prepare scintillation tubes:
  - 12 rows (chambers), 16 columns + 2 TA
  - TA: 2mL scintillation cocktail + 10µL [^{3}H]-5HT working solution [0.4µM]
  - All other vials: fill in 2mL scintillation cocktail
  - Mark front vials: 3 blue, 5 red, 5 blue, 3 red – cave: liquid distributor is distributing in sinuous lines!
  - Clean the outside surface of the vials if necessary from scintillation cocktail residues
  - *About the scintillation cocktail:*
    - *it contains long-chain organic compounds*
    - *extremely toxic! (use gloves always!!)*
    - *it converts β-radioactivity into light emissions (light flashes) which are then counted via the β-counter*

- Set distributor program:
  - Press "Reset" for 2s
  - Press "Rac E" → enter
  - Press "size" → enter
  - Insert amount of fractions (>12 fractions then type in +1) → enter
  - Press "time" → enter
  - Insert "2:00" → enter
  - Press "**run**" (the gully tube/whatever is folded away, the experiment begins!)
- 3 fractions before changing the buffer, put blue/red tubings into the new buffer
- Changing the buffer: @1min 5s change the valve from blue/red to red/blue
- When you have just changed the buffer (via setting the valve to blue/red) put the unused tubings into H_2O – do not let them dry out! (uncontrollable air bubbles in the tubings are shitty in your experiment)
- When you have more than 12 fractions you need an additional rack for the vials – exchange the filled rack with the "fresh" one in time!!!
- Close all vials with lids – do not forget to mark them accordingly!
- Put them into the counting racks (slots for the flag are on the left side!), stating with TA and leave 1 slot empty after

each channel

- Program the β-counter:
  - insert all racks
  - insert protocol flag (flag must be on the left side of the rack!)
  - press F1 (protocol overview)
  - enter protocol number and press enter
  - enter your heading: yymmdd + first letter of your name + number
  - change settings if necessary: to be counted 3H, 1 cycle, 2.5min
  - save data: yes to: C:/…/Packard…/ → file name: yymmdd+letter+number.001
  - press F1, F1 and if necessary F2 (to start counting)

- counting…..will take a while

- turning off the superfusion apparatus etc. → **only if no one is using it after you!**
  - unplug the syringe in the sink → water tank will empty itself slowly
  - stop the right pump
  - turn off the main switch (power hub)
  - relax tubings in the pumps (left: turn up lever, right: press for each tubing the white button of the clamp)
  - **if sufu is not frequently used** take the tubings out of the H_2O tank and place them on the workbench & discard H_2O

- getting the data off the β-counter (after counting is complete):
  - enter floppy disk
  - go to main screen (F1)
  - press F10, then F3
  - program = "trans"
  - command string = "yymmdd + first letter of your name + number" (do not enter .001)
  - press enter
  - press F3 (=run program = transfer data to floppy disk)
  - press any key
  - press any key
  - press F1 to go to main status page

- analyse the data on the PC (+Excel)
  - open Excel
  - press "open file"
  - set "all files" to see your file
  - use your yymmdd + first letter of your name + number.001 file
  - Excel asks you then to convert the format into something usable:
    1. a window pops up press next
    2. untick "tab" and tick "comma" press next
    3. click on "more settings…" button
    4. decimal = **.** and 1000 mark = **,** press OK
    5. press "finish"
  - use columns A to D (D = CPMA): copy entries (first TA only, then the rest)
  - open template file "sufu 13+3.xls" (or similar name)
  - go to tab "daten"
  - enter copied data here: first paste TA to TA space, then paste rest of the data to the data cells
  - enter experiment settings into tab "zwischenwertung"
  - use "save as…" and save the file with the experiment date into your folder (PC or server)
  - go to tab "%ch1-12" and copy data (B6 to M18)
- open Graphpad Prism 5/6:
  - open template file "sufu 13+3.pzf" (or similar name)
  - paste data to data table
  - use "save as…" and save the file with the experiment date into your folder (PC or server)
  - adapt comments on every graph/layout
  - to get efflux bar plot:
    - go to tab "efflux" and copy values to new table (quadruplicates)
  - adapt everything you need
  - save the file
  - print whatever you need
  - **close Prism (due to licencing only 5 people at a time can open Prism)**

Addendum VI:

Biotinylation Assay

with LLC-PK$_1$ hDAT WT / T48A cells & Okadaic Acid

Biotinylation Assay

Preparations:

- cell seeding: ***>1Mio. cells in 3mL well (6-well plate)*** → like for uptakes & sufus
- 2 ice boxes (for buffers, for cell plate)
- cell scrapers (one for each condition)
- pump & flask to suck of fluids
- rocker @4°C (cold room)
- "Starving" cell culture media = DMEM without FCS or P/S
- "PBS^{2+}" = 1x PBS + 1mM $MgCl_2$, 0.1mM $CaCl_2$; stored at 4°C
- "Quench solution" = PBS^{2+} + 100mM Glycine, stored at 4°C
- "RIPA buffer" = 10mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton-X-100, 0.1% SDS, 1% Na deoxycholate; stored at 4°C
- "RIPA buffer + protease inhibitors" = 1 tablet Roche protease inhibitor cocktail in 10mL RIPA buffer; stored @4°C
- Sulfo-NHS-SS-Biotin: stock solution 200mg/mL stored at -20°C
  working solution: 1mg/mL in PBS^{2+} (once diluted, it is quickly hydrolised!) = 1:200

Biotinylation:

- suck off media, treat cells with 0.5µM ***Okadaic Acid*** (OA) in KHB – or KHB only for the control
- 🕓 incubate for 30min @ room temperature
- suck off fluids, then ***wash*** 3x with ice cold PBS^{2+} and place cells on ice
- then prepare Biotin solution (1mg/mL; 750µL/well)
- ***2x*** add Biotin solution
- then place cell plate on rocker in cold room (velocity 35)
- 🕓 rock for 15min.
- then wash 3x with 1mL ice cold Quench solution
- ***2x*** then add 3mL to each well
- 🕓 incubate for 15min. on rocker in cold room (velocity 20)

Cell lysis:

- suck off Quench solution
- wash 3x with 2mL PBS^{2+}
- then add 300µL RIPA buffer with protease inhibitors to lyse the cells
- 🕓 rock for 30min.on rocker in cold room (velocity 35)
- scrape cells off and transfer contents of each well into a 1.5mL tube (label properly!)
- 🕓 centrifuge cell lysate at 4°C, 13 000rpm for 10 min.
- transfer supernatant into new 1.5mL tube, discard tube with pellet

sample storage:

- freeze centrifuged lysate or keep lysates on ice until protein conc. determination

Determination of protein concentration

Preparations:

- 7 BSA standards:

mg/mL BSA	0	0.1	0.25	0.5	1	1.25	1.75	
µL BSA	0	1	2.5	5	10	12.5	17.5	→ 2mg/mL stock!
µL MQ-H2O20	19	17.5	15	10	7.5	2.5		

- protein dilutes (in triplicates):

1 : 2 1 : 5 1 : 10 of the original protein lysate

+ 200µL BCA* in each tube (standards + dilutes)

**BCA: 2 solutions: A + B*

*50 : 1 → 5.5mL (7 * 200µL + 18 * 200µL + extra) = 5.39mL "A" + 110µL "B"*

↘ *number of samples*3*

🕓 incubate for 30min. at 37°C , then let samples cool down to room temperature

→ Hohenegger Lab: "Victor V3":

- turn on work station (from right to left): Victor-Annex, Victor V3, Computer (Dell): user = Victor, application = Wallac 1420 Workstation Victor V3
- Wallac 1420 Workstation Victor V3 (Victor must be on by now!):
  - tab "instrument control" → Protocol: "BCA Protein Determination"
  - click on symbol with traffic lights + wand () → a new window opens → choose "BCA Protein Determination" → press button "weiter" → select wells to be measured (by setting them as "measured" or "empty") → click button "weiter" → click button "weiter" → insert 96well-plate into Victor (without lid, without bubbles!) → click button "fertigstellen" → Victor measures
  - after finishing measuring, click on the protocol symbol () → a new window opens → save file: file/export... & print tab "table" (standard printing settings; printer is in the middle of the lab)
  - transfer saved Excel-file to your USB drive
  - *to view/print an old measurement: click tab Tools/Explorer → a new window opens → open file by double-clicking on it → a new window opens → file/export... & print "table" (just as above)*
  - turn off PC & workstation, discard plate

Excel calculations:

- open calculation template
- enter data: empty well, BSA standards, your sample data
- save file
- adapt equation in red labelled column: y = kx + d → =(H16-d)/k

Protein Pull-down

Preparations:

- ice-cold RIPA buffer + protease inhibitor cocktail
- Streptavidin-Agarose beads (30µL/well (6-well plate)) – stored @4°C (cold room)
- 1.5mL tubes for control cell lysate

Protein Pull-down:

- "measure" volume (µL) of lysate → ¼ of each lysate into new 1.5mL tube (=control cell lysate)
- add 300µL RIPA buffer into ¾ lysate tube
- add 30µL Streptavidin-Agarose beads into ¾ lysate tube
- put all tubes on the rotor in the cold room (4°C)
- 🕓 incubate/rotate over night

preparations for eluating:

- RIPA buffer (for washing)

- RIPA buffer + protease inhibitor cocktail (for washing)
- Western Blot 5x sample buffer + 100mM DTT (for eluating)

eluate:

- 🕓 centrifuge tubes with beads 14.000rpm @4°C for 5min
- then remove supernatant with syringe + 27G needle,
- ***2x*** add 1mL RIPA buffer, vortex very shortly,
- 🕓 centrifuge again 14.000rpm @4°C for 5min
- repeat previous step with RIPA buffer + protease inhibitor cocktail
- add 30µL 5x sample buffer with 100mM DTT
- add 20µL 5x sample buffer with 100mM DTT to control cell lysate tubes
- 🕓 then 30min @37°C with 800rpm on Thermomixer,
- then take supernatant (sample)
- freeze all samples @-20°C until Western Blot

Western Blot

Preparations:

- thaw samples
- 🕓 place on Thermomixer for 10min @800rpm and 37°C
- 🕓 centrifuge for 10min @13.000rpm
- save supernatant in new tube!

Prepare SDS-PAGE:

- assemble equipment...
- prepare gel (thickness: 1mm or 1.5mm; percentage): according to the available table (for 2 gels)
- load gel: use special tips! Load protein ladder left and/or right to all samples

- SDS-PAGE:

- 🕓 start at 80V for 10min,
- 🕓 then raise to 100V for about an hour (run gel until blue front entered the electrophoresis buffer)

- Blotting:

- use transfer apparatus,
- wet filters in TB_2 transfer buffer (stored at 4°C),
- place filters in the transfer apparatus,
- wet membrane (nitrocellulose or PVDF membrane) in the same buffer (PVDF: first in pure methanol, then in methanol-TB_2 buffer mix),
- place membrane on filter ,
- place gel carefully on membrane,
- add more wet filters on top,
- then get rid of air bubbles,
- 🕓 close the lid – run transfer for 30min at 20V.

- Antibody staining:
    - Block with 5% albumine in TBS (or 0.1% TBST) for 1h,
    - then directly transfer membrane into primary antibody solutions
    - incubate at 4°C over night – use a mild shaker
    - wash 3x with 0.1% TBST for 15min – use a mild shaker
    - incubate with second antibody solution for 1h at room temperature – use a mild shaker
    - wash 3x with 0.1% TBST for 15min – use a mild shaker
    - incubate with ECL solution for 3min in a plastic sheet to reduce required volume
- Signal detection:
    - go to dark room, turn on the developer,shut off the lights and lock the door
    - place the ECL solution-incubated membrane in a transparent plastic sheet, then into a radiocassette
    - remove any air bubbles
    - place hyperfilms on top of the membrane (different durations!)
    - develop hyperfilms (about 3min each)
    - label hyperfilms properly!!
- Result analysis:
    - scan hyperfilm as picture file
    - open ImageJ and determine background box and then “measure” optical density
    - note those arbitrary values in an excel sheet
    - (calculate averages of duplicates, set 100% to one of the test conditions and calculate other values)
    - plot data in a bar chart to compare the test conditions