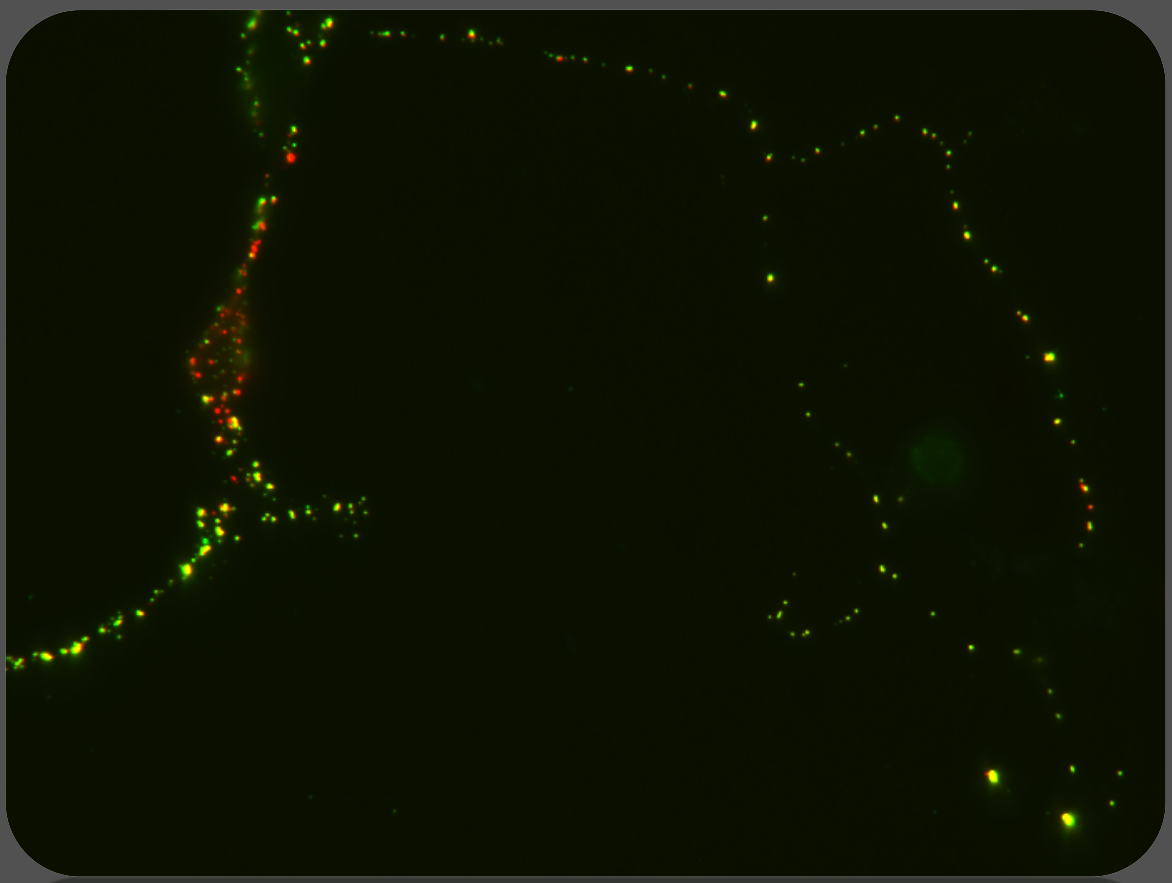
Molecular modulation of brain development and function: Lessons from “old” and “new” protein families

Molecular mechanisms of neurotrophin and Slitrk protein families mediating development and function of central nervous system

Cátia Cristina Moreira Proença



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
September, 2011

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Knowledge Creation

Aos meus pais e irmã

ao Pedro,

e à minha família.

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RESUMO

Compreender como famílias de proteínas interagem para coordernar o desenvolvimento e função do sistema nervoso central (SNC) é crítico para entender o funcionamento desta região em situações normais bem como em caso de doença. O tráfego de proteínas para compartimentos intracelulares alternativos pode levar a respostas biológicas completamente distintas. A família de neurotrofinas, constituída por três receptors (TrkA, TrkB e TrkC) e quatro ligandos (NGF, BDNF, NT3 e NT4) é um grupo de proteínas muito importante para o desenvolvimento e manutenção do sistema nervoso. O receptor TrkA e respectivo ligando, NGF, são expressos predominantemente no sistema nervoso periférico. Por outro lado, no SNC encontram-se níveis mais elevados do receptor TrkB e os dois ligandos BDNF e NT4. O receptor TrkC e o ligando NT3 são expressos em ambos os sistemas. O nosso conhecimento do funcionamento molecular das neurotrofinas é baseado principalmente em estudos feitos com neurónios do sistema periférico e com o receptor TrkA e NGF. Por outro lado, é ainda um mistério por que motivo o SNC expressa dois ligandos (BDNF e NT4) com a mesma afinidade para um só receptor (TrkB), os quais parecem modular funções diferentes. Levantámos então a hipótese de que estas diferentes funções, mediadas pelo mesmo receptor, são conseguidas por via de um controlo diferente do tráfego endocítico do TrkB iniciado por cada um dos ligandos. Os estudos que efectuámos demonstram que a activação inicial to receptor TrkB com BDNF ou NT4 é semelhante; porém, o BDNF induz uma ubiquitinação mais eficiente do receptor TrkB do que o NT4. Como resultado, verificámos que tratando os neurónios do córtex com BDNF, o tráfego para o lisosoma e degradação foi mais eficiente. Por outro lado, e considerando que tratando os neurónios com NT4 não houve tanta degradação de TrkB, observámos que este ligando leva a uma activação mais eficiente de proteínas sinal ajusante (nomeadamente Akt e MAPK). Assim sendo, estes resultados providenciam a

primeira explicação molecular para os efeitos distintos observados em estudos comparando as funções de BDNF e NT4.

Outra família de proteínas que foi identidicada recentemente como sendo crítica para o desenvolvimento do SNC é a família Slitrk. Os Slitrks têm algumas semelhanças estruturais com os Trks uma vez que também formam proteínas membranares tipo I, têm domínios repetidos de leucina na região extra-celular, contêm várias tirosinas com potencial para serem fosforiladas na região intra-celular, e também são expresses em níveis elevados no SNC. Apesar disso, ainda se sabe muito pouco sobre a função molecular destas proteínas. O Slitrk1, um dos membros dos Slitrks, está implicado em doenças psiquiátricas, nomeadamente síndrome de Tourrette e tricotilomania. O Slitrk6 é o membro cuja expressão é mais restricta, estando presente em níveis elevados no ouvido. A remoção do gene Slitrk6 no ratinho, levou a um desenvolvimento deficiente do ouvido, caracterizado por uma elevada morte neuronal neste orgão bem como um crescimento deficiente de axónios e dendrites. Para compreender melhor a função destas proteínas escolhemos o membro da família que é expresso em níveis mais elevados no cérebro: o Slitrk5. Nesse sentido, gerámos um ratinho do qual removemos o gene Slitrk5 (Slitrk5$^{-/-}$). Estudos feitos com este ratinho revelaram um comportamento que tem sido descrito na literatura como obsessivo compulsivo (OCD), que se reflecte em movimentos repetidos, bem como, ansiedade. Para melhor compreender este fenótipo, tratámos os ratinhos com um inibidor de transporte de serotonina selectivo, que é o fármaco mais utilizado para tratar humanos com esta doença, e verificámos que o tratamento levou a uma redução dos movimentos repetitivos. Por outro lado também verificámos que os ratinhos Slitrk5$^{-/-}$ possuem deficiências em zonas do cérebro que têm sido descritas como estando afectadas em pacientes com OCD. Nomeadamente, estudos de electrofisiologia revelaram que a ligação entre o córtex e estriado estava afectada. Também observámos que os neurónios do estriado estavam subdesenvolvidos. A nível celular, observámos que o Slitrk5 está

localizado em dendrites e sinapses e regula o tráfego dos receptores de glutamato AMPA, reduzindo os níveis membranares da subunidade GluA2. Estudos feitos com ratinhos Slitrk5$^{-/-}$ novos revelaram que a aprendizagem e memória dependente do hipocampo estava afectada. Por outro lado, também observámos que o Slitrk5 modula a degradação do receptor TrkB e de proteínas sinal ajusante. Estes resultados sugerem que o Slitrk5 é uma proteína que desempenha múltiplas funções no cérebro e consequentemente, a eliminação deste gene no ratinho leva a graves fenótipos neurológicos. Mais estudos serão necessários para a compreendermos completamente a relevância desta proteína em estados fisiológicos normais bem como em doença (incluíndo OCD). No entanto, os resultados aqui apresentados claramente favorecem uma papel activo do Slitrk5 para a função normal do SNC e que o nosso conhecimento sobre a função deste sistema é ainda limitado.

Em suma, com estes estudos providenciamos uma explicação molecular para as funções distintas mediadas por BDNF e NT4. Por outro lado, descobrimos um candidato novo que pode agora ser estudado em pacientes com OCD e criámos um modelo animal novo onde esta doença pode mais facimente ser estudada e manipulada.

ABSTRACT

Understanding how protein families interact to coordinate the development and function of the central nervous system (CNS) is critical for the understanding of this structure in normal conditions as well as in disease. Endocytic trafficking of membrane proteins to alternate intracellular targets can have significantly different biological outcomes. The neurotrophin family of proteins, composed of three receptors (TrkA, TrkB and TrkC) and four ligands (BDNF, NGF, NT3 and NT4), has a well-established role in the development and maintenance of the nervous system. TrkA receptor and its ligand NGF, are expressed mostly in the peripheral nervous system. In the CNS, TrkB and its two ligands, BDNF and NT4, are dominant, whereas TrkC and NT3 are expressed in both systems. Our current knowledge on the mechanisms of receptor activation, internalization and downstream targeting and signaling are mostly based on studies performed with sensory neurons relying on the TrkA/NGF system. On the other hand, it is still puzzling that in the CNS two ligands (BDNF and NT4) with similar affinity to the same receptor (TrkB) are expressed but seem to fulfill distinct biological responses. We hypothesized that these differences could be due to differential regulation of the endocytic trafficking pathway. In fact we observed that, even though NT4 and BDNF can equally activate TrkB receptor and its downstream signaling, BDNF promoted more efficient ubiquitination of the receptor than NT4. As a consequence, BDNF led to more efficient lysosomal degradation. On the other hand, even after long continuous treatment, NT4 did not induce efficient downregulation of TrkB. Consequently, NT4-induced activation of TrkB signaling was efficiently sustained, as seen by prolonged activation of the Akt and MAPK pathways. This data provides a new mechanism that can potentially explain the differential functions reported for these two ligands and adds to the current understanding of neurotrophin regulation and function in the CNS.

Another protein family that has emerged as being key for CNS development and function is the Slitrk family, which is composed of six members. The Slitrks share

some structural characteristics with Trks in that they form type one membrane proteins with extracellular leucine-rich repeat domains and intracellular phospho-tyrosines, and they are primarily expressed in the CNS. Despite this, little is known about Slitrks function in the brain. Slitrk1 has been associated with psychiatric disorders. Slitrk6, which is the member with the most restrictive expression, is highly expressed in the inner ear. Knocking out Slitrk6 in the mouse leads to reduced neuronal innervation and death in this organ. In order to better understand the function of this protein family we generated a mouse knockout for Slitrk5, which is the member with highest and broadest expression in the brain. We discovered that loss of this protein led to obsessive-compulsive-like behaviors in the mouse, which manifested in excessive self over-grooming as well as anxiety. To further delineate if this behavior represented OCD, we treated the mice with a selective serotonin reuptake inhibitor (fluoxetine), the most common drug used to treat humans with OCD. We observed that indeed fluoxetine ameliorated the over-grooming symptoms. Consistently with our knowledge of the pathophysiology of OCD in humans, we observed that the Slitrk5$^{-/-}$ mice have selective over-activation of the orbitofrontal cortex, impaired cortico-striatal transmission and morphological defects in the medium spiny neurons, namely reduced dendritic complexity. We also found that, at a cellular level, Slitrk5 localizes to synapses and modulates trafficking of AMPARs by reducing the surface expression of the subunit GluA2. Analysis of young Slitrk5$^{-/-}$ mice revealed impairment in hippocampal-dependent learning and memory tasks. Interestingly we also found that Slitrk5 delays BDNF-induced degradation of TrkB receptor and modulates its downstream signaling. These data suggests that Slitrk5 is a protein with multiple functions in the brain. In aggrement, lack of Slitrk5 in the mouse leads to severe neurological phenotypes. Further studies will be required for its full characterization, as well as to understand the relevance of this protein in normal human physiology and disease states (including OCD).

Nevertheless, our pioneer work clearly supports an active role of Slitrk5 for CNS function and that this system is still poorly understood.

Thus, with these studies we uncovered the mechanisms mediated by BDNF and NT4 modulation of TrkB endocytic trafficking and we also discovered a new candidate molecule for obsessive-compulsive disorder, and provide a new mouse model that can be used to study this condition.

Chapter 1 – General Introduction

GENERAL INTRODUCTION

The human brain is composed of approximately 100 billion neurons connected by up to 10 000 synaptic sites [1]. Understanding how neuronal numbers are controlled and how the connections between different brain structures are established are key questions in the field of neuroscience.

Secreted proteins play essential roles in the modulation of neuronal quantities, migration, morphology and function. The discovery of protein families that modulate these processes is critical for a basic understanding of nervous system function, as well as to identify molecular factors in disease. Several protein families have been described to play critical roles during brain development as well as adult nervous system maintenance. Among these, neurotrophins are one of the best characterized protein families known to regulate both these functions [2]. Initial studies in developmental neurobiology showed that targets of nerve innervation secrete limiting quantities of survival signals that ensure a balance between innervation and target tissue size. Nerve growth factor (NGF), a member of the neurotrophin family, was the first secreted polypeptide discovered to play this role [3]. It was later shown that NGF could be internalized and travels retrogradely long distances along the axon to reach the soma, where it regulates transcription of genes critical for neuronal development. In addition, NGF can also modulate neuron survival and differentiation. NGF remained an orphan ligand until the early 1990s (almost 30 years), when binding to two distinct classes of transmembrane receptors was identified: the p75 receptor and the tropomyosin related kinase (Trks) receptors, named after the protoncogene that resulted in its identification [4,5,6,7]. The discovery of a complete set of ligand-receptor signaling complex had a major impact in the field and provided the right tools to further delineate the downstream cascades involved. We now have a fairly good understanding of how neurotrophins function in the peripheral nervous system (PNS). However, neutrophin actions in the central nervous system (CNS) are less clear.

The continuous search for factors regulating central nervous system development led to the discovery of a new protein family named *Slitrk* [8]. *Slitrks* were discovered in a screen for molecules that were differentially expressed in mice with neural tube defects. Slitrks are tansmembrane proteins primarily expressed in the brain of both mice and humans. They can modulate neurite outgrowth and synapse formation. Interestingly, it was found that mutations in the Slitrk1 gene, a member of the Slitrk family, are associated with psychiatric disorders. Similarly to neurotrophins, it seems that this protein family can play important roles not only during CNS development but also for adult CNS maintenance [9]. We are still at early stages in terms of understanding the main functions of this protein family. It is plausible to speculate that, as opposed to neurotrophins, a set of receptors was identified before their ligands. Discovery of Slitrk's binding partners, such as a secreted ligand, other transmembrane protein, or even cytoplasmic partners, will provide invaluable tools to explore this family and extend our understanding of CNS development and function.

The goal of this thesis work was to contribute to a better understanding of the mechanisms of action of polypeptides critical for CNS function. On the one hand I was interested in consolidating our knowledge of neurotrophin's downstream signaling pathways in the CNS. On the other, we aimed to explore the newly identified Slitrk protein family, which is highly enriched in the brain.

1. NEUROTROHPHIN FAMILY

Neurotrophins are secreted polypeptides that were initially identified as survival factors for sensory and sympathetic neurons, and were later shown to be critical for nervous system development and function [10,11]. These peptides regulate processes such as cell survival, proliferation, differentiation and death. They also play critical roles in high order cognitive processes such as learning and memory

[12,13]. Altered neurotrophin levels have been implicated in the development of several neurodegenerative disorders including Alzheimer's and Hungtington's disease, as well as in psychiatric disorders, such as depression [14].

1.1 Neurotrophin family and receptors

The neurotrophin family is composed of four polypeptides: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4). Even though NGF was the first neurotrophin to be identified, very few neurons in the CNS require NGF for survival. The search for a functional analog to NGF in the CNS led to the discovery BDNF [11,15].

The p75 receptor belongs to the tumor necrosis factor (TNF) receptor superfamily and, neurotrophin binding to this receptor has been shown to lead to apoptosis [16]. P75 receptor expression is downregulated during postnatal development in the CNS but is rapidly induced after seizure or neuronal lesion. Although p75-mediated signaling has been shown to be important for nervous system development the discussion of the mechanisms involved in these pathways is outside the scope of this thesis. For detailed information about this subject, please refer to the following reviews [17,18,19].

Three *trk* genes have been identified in vertebrates coding for three protein receptors: TrkA, TrkB and TrkC that differ in their expression profile and neurotrophin affinity. NGF binds to TrkA; NT4 and BDNF bind to TrkB, and NT3 binds TrkC. In addition, NT3 can bind TrkA and TrkB in certain cellular contexts, even though with a 100x fold reduced affinity [2,20]. Expression of a specific Trk receptor confers responsiveness to the neurotrophin it binds; however the binding affinity can be regulated by p75 interaction with Trks. P75 modulates the binding affinity of NGF and BDNF, increasing their affinities to TrkA and TrkB respectively, as well as decreasing the affinities of NT3 and NT4 for the same receptors [4,21].

Different splice variants have been described for all Trk receptors that differ in neurotrophin affinity and function. A TrkB splice variant lacking exon9 in the extracellular domain that has reduced affinity for NT4 and NT3 but not for BDNF, was found in the developing nervous system of the chick [22]. A TrkB splice variant lacking exon9 in the extracellular domain that has reduced affinity for NT4 and NT3 but not for BDNF, was found in the developing nervous system of the chick [23]. Moreover, splice variants of TrkC, and especially TrkB, lacking the catalytic kinase domain, are highly expressed in the mature CNS [24]. This truncated TrkB variant is thought to act as a dominant negative since it can bind and internalize BDNF and possibly restrict its availability [25].

1.2 Neurotrophin expression and functions

Target-derived NGF plays critical roles for cell survival and differentiation of peripheral neurons. NGF expression in the CNS is more restricted and it promotes survival of cholinergic neurons in the basal forebrain. BDNF and NT4 are more widely expressed in the CNS with particular high levels in the cortex and hippocampus [2,26]. Their expression is developmentally regulated increasing during the first postnatal weeks [27,28,29]. NT3 is broadly expressed throughout the nervous system. In the periphery, it acts in coordination with NGF to support the survival of sympathetic neurons, being expressed in intermediate targets at high levels [30].

Trks are expressed in the nervous system in a temporal and selective manner. TrkA is more predominantly expressed in the PNS. It can be found in trigeminal ganglia, sympathetic neurons and in dorsal root ganglia. Neurotrophins are highly expressed in the target areas innervated by responsive PNS axons. On the other hand, TrkA expression in the CNS is less abundant, being restricted to the basal forebrain cholinergic neurons. TrkB is primarily expressed in the CNS and TrkC is broadly expressed in the nervous system, with high expression early in development [31].

The best-studied system showing neurotrophin-dependance for survival and development has been the PNS. All peripheral sensory neurons express one of the Trk receptors at some point in development and, presence or absence of corresponding neurotrophins at their innervated targets, determines survival or death. Ablation of individual Trk receptors leads to elimination of specific neuronal subpopulations. For example, NGF/TrkA ablation in mice leads to a complete loss of nociceptive neurons, whereas, null mutations of NT3/TrkC, leads to a lack of propioceptive neurons [31]. Moreover it was shown that mice null for both TrkB and TrkC have decreased synaptic density and lower numbers of pre-synaptic vesicles in the hippocampus [32].

An increasing body of data has suggested that unlike the PNS, neurotrophins are not necessary for survival of neurons in the CNS, but rather play critical roles in cell morphology and plasticity. Although initial studies using BDNF or TrkB null mice have reported brain neuronal loss, mainly using TUNEL staining, they were confounded by the early and severe PNS abnormalities [32,33]. Subsequent studies in BDNF- and TrkB-null mice have shown that despite developmental abnormalities, the neuronal numbers in the hippocampus and cortex are normal [26]. Accordingly, postnatal ablation of TrkB in the forebrain revealed a decrease in cortical size, likely as a result of reduced dendritic complexity, but normal neuronal numbers. These mice were, however, impaired in spatial reference memory tasks and had reduced long term potentiation (LTP) at CA1 hippocampal synapses [34]. The double BDNF/NT4 mutant shows no changes in spinal cord motor neuron numbers or in ganglion cells from the retina, but shows sensory neuronal loss [35,36]. Similarly to the TrkB null, the BDNF-deficient mice have decreased dendritic arborization and impairments in long term potentiation (LTP) at CA1 synapses [37,38]. Recently a new mouse line was generated in which the BDNF gene was conditionally ablated from post-mitotic neurons of the CNS. Surprisingly the overall morphology of the brain was normal with no major morphological defects and no change in neuronal

numbers. It did however have defects in dendritic arborization specifically in striatal neurons, suggesting that BDNF is negligible for mature hippocampal maintenance [27].

1.3 Neurotrophin-mediated signaling

Trks form single pass transmembrane proteins containing 3 leucine-rich repeats (LRR) flanked by cysteine-rich domains, and two immunoglobulin-like domains involved in ligand binding. Non-covalent homodimers of neurotrophins bind to Trks inducing receptor dimerization and activation. Dimeric Trks phosphorylate each other in the cytoplasmic auto-regulatory loop of the kinase domain and in other cytoplasmic tyrosines, further strengthening the catalytic activity of the kinase. Trks are highly homologous in their intracellular domain which is 73% conserved. Most research on Trk-mediated signaling has focused mainly on two phosphorylated tyrosines: Y496 in human TrkA (homologous to Y516 in TrkB) that is localized in the juxtamembrane domain within the NPxY motif and tyrosine Y791 in human TrkA (Y817 in TrkB), that is located at the carboxy-terminal (C-terminal). Phosphorylation of these tyrosines creates docking sites for adaptor proteins that couple to downstream signaling cascades including the Ras/mitogen activated protein kinase (a MAPK also known as extracellular signal regulated kinase - Erk) pathway, phosphatidyl inositol-3 kinase (PI3K)/Akt kinase pathway and the PLC-γ/cAMP response element binding protein (CREB) pathways [2,19,39]. For a summary of neurotrophin mediated signaling see figure 1.

Upon phosphorylation, the NPxY motif of Trks serves as a binding site for adaptor proteins containing the Src homology domain (SH2), or phosphotyrosine binding domain (PBD), such as the Src homologous and collagen-like adaptor protein (Shc), and the fibroblast growth factor receptor substrate 2 (Frs2) [40,41,42]. Most research has focused on signaling mediated by these two interactors. Phosphorylated Shc recruits adaptor molecules such as Grb2 and the guanine

nucleotide exchange factor Son of Sevenless (SOS) that converts Ras into its GTP bound form. This active form of Ras activates the PI3K, the p38, MAPK and c-Raf pathways [43]. One of the targets of MAPK is the ribosomal protein kinase 2 that phosphorylates CRE-binding proteins modulating transcription of critical genes.

Trk receptor mediated activation of Ras through Grb2 and Shc promotes MAPK signaling in a transient rather than sustained fashion [39]. Prolonged MAPK activation depends on the recruitment of Frs2 to Y490. Frs2 phosphorylation in turn provides binding sites for several other signaling players including the protein tyrosine phosphatase 2 (Shp2), Grb2 and the adaptor protein Crk. Association with Crk results in activation of the small GTPase Rap1 that that initiates the MAPK cascade. Recruitment of Grb2 provides an independent mechanism for Ras activation. Signaling through Y490 leads to survival and differentiation of cells [39,41,42,44,45,46]. Shp2 is a rare protein phosphatase implicated in signaling amplification. It acts as an adaptor protein recruiting Grb2/SOS complex, and it also mediates dephosphorylation of Sprouty, reversing its general receptor tyrosine kinase (RTK) inhibitory effects [47].

Phosphorylation of Y791 in human TrkA recruits and activates PLC-γ that triggers hydrolysis of phosphatidyl-inositol (4,5) 2 phostate (PIP_2) to generated inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from cytoplasmic stores, leading to the activation of Ca^{2+}-regulated protein kinases as well as Ca^{2+}-calmoduling regulated proteins. DAG activates protein kinase C (PKC) that is required for NGF induced neurite outgrowth [39]. The function of the signaling elicited by the PLC-γ pathway was studied in detail in mice with a homozygous mutation of Y816 in TrkB receptor. BDNF induced phosphorylation of Ca^{2+} -calmodulin protein kinase and CREB was blocked. Consistent with the role these proteins play in synaptic plasticity, hippocampal long-term potentiation (LTP) was impaired in these mice. BDNF-dependent activation of the MAPK pathway that occurs through the Shc site was intact [48].

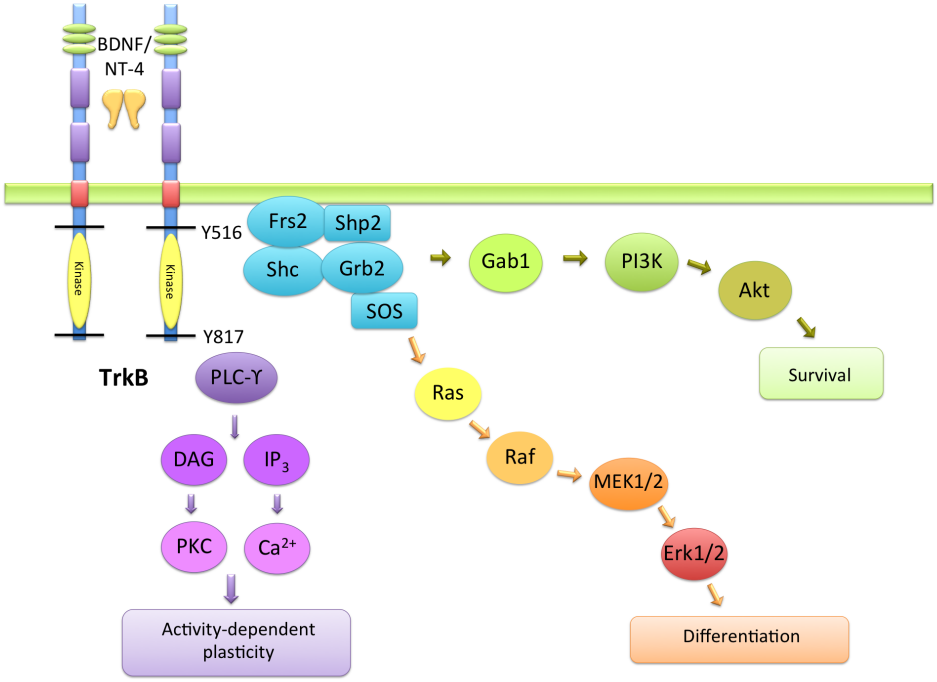


Figure 1: Summary of neurotrophin signaling. Neurotrophin binding to Trk receptors triggers dimmerization and activation of different signaling pathways. Depicted here is human TrkB receptor and recruitment of adaptor proteins to the two main phosphorylated tyrosines. Phosphorylation of the tyrosine residue located in the juxtamembrane region of Trk receptor recruits two complexes of adapter proteins the Shc/Grb2/SOS and the FRS2/Shp2/Grb2/SOS complexes. Activation of Ras triggers the MAPK/Erk signaling pathway, which stimulates neuronal differentiation including neurite outgrowth. Activation of PI3K through Ras/Gab1 promotes cell survival and growth. Phosphorylation of the c-terminal tyrosine recruits PLC-γ that results in increase in intracellular Ca^{2+} and modulation of synaptic plasticity.

1.4 Regulation of signal specificity

A question that arises from studies of RTK-mediated signaling is how specificity is achieved. The overall topology of RTKs, such as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR) or Insulin receptor, is

similar: all contain a ligand-binding domain in the extracellular region, a single transmembrane domain and a cytoplasmic domain that contains the tyrosine kinase plus regulatory sequences at the juxtamembrane region and the C-terminus. The signaling cascades previously described (RAS/MAPK and MAPK) are the canonical pathways that can be activated by virtually all RTKs [49], thus raising the question of how Trk-induced signaling is different from other RTKs and within the Trk family. Differential temporal and spatial distribution of signaling partners amongst different cell types provides one level of specificity. Specific neuronal populations express particular types of adaptors, and different Trks are expressed at different levels in the same cell type which can lead to diverse outcomes. A recent study reported that expression of TrkA or TrkC in mouse embryonic stem cells which were later differentiated into glutamatergic neurons, leads to neuronal death, whereas TrkB does not [50]. This suggests that even in similar contexts, different Trks are capable of engaging alternative signaling networks.

Differential sorting of adaptor proteins within intracellular membrane compartments also contribute to signal specificity and regulation. Recently, it was shown that transport of BDNF-activated TrkB to lipid rafts, through Fyn, was critical for efficient PLC-γ activation. In Fyn knockout mice, BDNF was unable to direct TrkB to intracellular lipid raft compartments and phopshorylation of PLC-γ was reduced. On the other hand, Akt and MAPK were excluded from lipid rafts and phosphorylation of these proteins occurred outside these domains [51].

Membrane trafficking controls both localization and accessibility of signaling players, providing some degree of specificity. RTK endocytosis is a critical step to regulate receptor activation of downstream cascades, signaling amplification as well as attenuation. Ligand binding to RTKs leads to receptor dimerization, activation of intrinsic kinase activity and tyrosine phosphorylation, triggering protein recruitment and activation of downstream signaling cascades. Ligand binding also triggers receptor internalization via clathrin-coated pits (CCP) that contain several adaptor

proteins such as AP2. CCP fuse with a specialized intracellular organelle the early endosome, also named sorting endosome. This process is regulated by Rab5 and early endosome antigen 1 (EEA1). The early endosome is characterized by a mildly acidic pH that leads to the uncoupling or receptor-ligand complex. Membrane proteins rapidly exit the early endosome and can follow two opposing routes that result in different signaling outcomes: recycling to the plasma membrane or degradation through the lysosome [52,53,54,55]. Phosphorylation, mono-ubiquitination and receptor-ligand uncoupling are key mechanisms modulating endocytic sorting. Transferrin receptor (TfnR) is the canonical constitutive recycled receptor. From the early endosome TfnR can either recycle directly to the membrane (rapid recycling) or it can be targeted to the recycling endosome and then to the membrane (slow recycling). EGFR is the best described RTK to undergo ligand-induced degradation through the lysosome. From the early endosome, EGFR is sorted to a late endosome, also known as multivesicular body, and subsequently, to the hydrolytic interior of the lysosome, where proteins are degraded [56,57,58,59,60,61].

Trks are multimonoubiquitinated upon ligand binding, via specific E3 ligases. The E3 ligase for TrkA has been identified as Nedd4-2. On the other hand, the E3 ligases for TrkB and TrkC are still unknown. Ligand-dependent ubiquitination of TrkA and TrkB is critical for lysosomal targeting of Trk receptors and this leads to signal attenuation; however, these receptors differ in their recycling and degradative rates [62]. Upon BDNF treatment Flag-tagged TrkB receptor is predominantly sorted to the degradative pathway, whereas, NGF leads to Flag-tagged TrkA recycling, both in PC12 cells as well as in cultured cortical neurons. The difference lies in a small stretch of amino acids in the juxtamembrane domain and transplantation of this domain into TrkB receptor reroutes TrkB to the recycling pathway. The contrasting endocytic sorting leads to different biological outcomes. Upon prolonged

neurotrophin treatment TrkA promotes sustained Akt phosphorylation as well as survival responses whereas TrkB does not [63].

In PC12 cells it has been shown that blocking NGF-dependent endocytosis of TrkA disrupts differentiation, even though survival was unaffected. Since Frs2 signaling is required for sustained MAPK activation and differentiation, this study suggests that efficient Frs2 activation requires endocytosis of the neurotrophin-receptor complex [64]. Endocytosis also seems to facilitate activation of the small G and attenuates Ras. On the other hand, PI3K activation occurs independently of endocytosis and this mechanism appears to attenuate this signaling pathway [65].

Accumulation of signaling partners at specialized structures contributes to more efficient signaling. In the PNS, binding of target-derived neurotrophins to pre-synaptic axons leads to Trk activation and endocytosis to early endosomes. A fraction of these endosomes evolves to specialized signaling endosomes that contain the ligand-receptor complex and signaling adaptors, which may trigger qualitatively different signals than those from receptors located at the plasma membrane. The signaling endosomes are retrogradely transported leading to signaling propagation throughout the axon and transcriptional regulation when they reach the soma. This transport is critical for cell survival, differentiation, innervation and synaptic plasticity [65,66,67]. Studies that used compartmentalized cultures of sensory neurons showed that neurotrophin stimulation directly in the cell body leads to activation of two pathways: the Erk1/Erk2 and Erk5, whereas neurotrophin treatment of distal axons leads to Erk5 activation in the soma but not Erk1/Erk2 [68]. These data suggest that activation of neurotrophin receptors within different cellular compartments leads to diverse functional outcomes. A new protein interacting with Trk receptors that localizes to signaling endosomes (named neurotrophic factor receptor associated protein - NTRAP) has been shown to be critical for retrograde activation of the transcription factor CREB in compartmentalized sensory neurons [69]. Another aspect to consider is that research studies usually employ saturating

amounts of ligands to study downstream signaling effects. However, this probably does not mimic endogenous contexts in which limiting amounts of ligands might induce specific signaling pathways.

2. SLITRK FAMILY OF PROTEINS AND FUNCTION

The advances in gene sequencing and cloning techniques of the past decades have contributed to the discovery of several protein families key for the development and function of the CNS. An example of this is the neurotrophin family that was covered in the previous section. However, our current knowledge of the molecular players involved in these processes is still limited and not sufficient to explain the complexity and diversity of this system. On the one hand, many open questions remain regarding the pathways and mechanisms of actions mediated by known protein families. On the other, the discovery and characterization of new molecular players is still necessary. This knowledge will allow us not only to understand the function of the normal nervous system, but also what fails in disease conditions.

Understanding the molecular mechanisms underlying pathological conditions are key question in neuroscience, being critical for the discovery of new molecular targets for pharmacological treatments. In this context, we were interested in studying new protein families that play critical roles for CNS maintenance and function. Slitrks are a recently discovered family of proteins primarily expressed in the brain that were discovered in a screen for proteins de-regulated in mice with neural tube defects [8]. So far, the few research studies addressing the function of these proteins in the CNS suggest that Slitrks mediate basic functions in neuronal development and also that they might be associated with psychiatric disorders ranging from obsessive-compulsive spectrum disorders to schizophrenia. Reminiscent of the neurotrophin family, it seems that the Slitrk family is key not only for the elaboration and development of the CNS but also in disease conditions.

Little is known about the main function that these proteins play in the brain. In order to contribute to a better understanding of this protein family, we recently

generated a Slitrk5 knockout mouse line and found that this protein plays critical roles for CNS function (see chapter 3).

2.1 Slitrk gene family

Slitrks are composed of six members, Slitrk1 through Slitrk6. They were initially identified in a screen for genes that were differentially expressed in mice with neural tube defects [8]. Slitrk5 had been previously discovered as a gene expressed in early hematopoietic progenitors but not in mature hematopoietic cells [70]. All Slitrks form single pass (type I) transmembrane proteins with an intracellular domain that varies in length [8] (Figure 2). At the extracellular domain, Slitrks contain two leucine-rich repeat (LRR) domains, which are each composed of 6 LRRs, flanked by cysteine rich capping domains [8]. Sequence analysis has revealed that the extracellular LRR domains of Slitrks resembled Slit proteins, and a conserved region in their intracellular carboxyl terminus (C-terminus) has a high degree of consensus with the last 16 amino acids of the neurotrophin receptor (Trk) [8]. Based on their similarity with Slits and Trks, these proteins were named Slitrks [8].

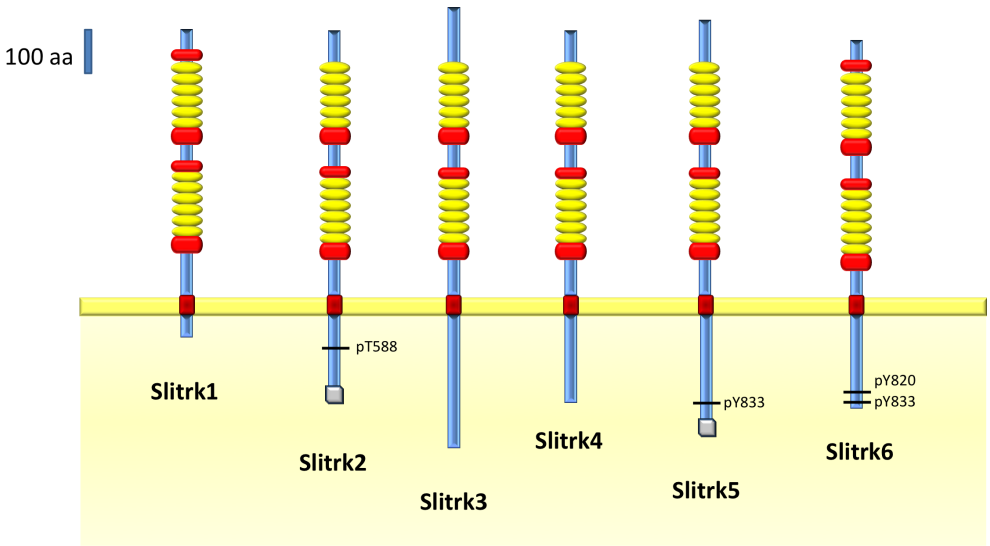


Figure 2: Schematic representation of the Slitrk protein family. Slitrks are composed of six members, Slitrk1 through Slitrk6. All contain extracellular LRR motifs that vary in numbers, flanked by cysteine-rich motifs, followed by a single transmembrane domain and an intracellular domain. All but Slitrk1 contain long intracellular domains that share some homology with Trk receptors at a C-terminus tyrosine. Phosphorylation of intracellular residues has been showed for Slitrk2, Slitrk5 and Slitrk6, however, the function is still unknown.

Slit is one of the most well-known LRR-containing proteins. These proteins have a tandem of four LRR domains at the N-terminus (D1-D4), each containing an array of five to seven LRRs [71]. Slit was originally discovered in *Drosophila* as a protein secreted by midline glia of the developing CNS [72,73]. Three Slit homologues (Slit1-3) have been discovered in mammals [74]. Later it was shown that Slit is a ligand for Robo receptors [75]. Slit-Robo signaling is involved in a variety of processes, key for proper nervous system development, such as repulsion of axons from the midline, axon guidance and repulsion, orchestrating tangential neuronal migration, regulating cytoskeletal dynamics and modifying cell adhesion properties [76,77].

LRR domains are composed of tandem repeats of LRR motifs and have curved solenoid structures that are suitable for mediating protein-protein interactions [78,79,80]. The LRR is a widespread structural motif of 20-30 amino acids with a defining sequence LxxLxLxxN/GxL (x being any amino acid). Given their extracellular LRR domains, Slitrks are considered part of the LRR superfamily. LRR-containing proteins are emerging as key regulators of the CNS functions, including neurite outgrowth, neuronal survival, myelin-based axon growth inhibition, synapse formation, dendritic morphogenesis, etc [81,82,83]. Given below is a brief summary of some LRR protein families and their main roles in CNS, including those in synapse formation.

2.2 LRR protein families and their function in the CNS

Several LRR proteins act as synaptic cell adhesion molecules (CAMs). These molecules play important roles in the establishment, maintenance and activity-dependent changes of synapses. For a protein to be classified as a synaptic CAM, it should be able to mediate cell adhesion through interacting with other proteins *in trans*; and it should interact *in cis* with scaffolding proteins that recruit synaptic proteins to the synapse. Many LRR-containing proteins meet these criteria and have been shown to regulate synapse formation and maturation.

The Amphoterin-induced gene and ORF (Amigo) family is composed of three members that are almost exclusively expressed in the CNS [81]. They contain six LRRs at the extracellular domain and an immunoglobulin-like domain located next to the transmembrane segment [84]. When the ectodomain of Amigo is expressed as an Fc fusion protein and substratum-attached, it promotes prominent neurite extension of cultured hippocampal neurons [84]. Moreover Amigo2 has also been implicated in activity-dependent survival of cerebellar neurons [85].

Synaptic adhesion-like molecules (SALMs) are a recently identified class of adhesion proteins primarily expressed in the brain [86,87]. The SALM family is composed of five members and all contain extracellular LRRs, an immunoglobulin C2-like (IgC2) domain, a fibronectin type III (FNIII) domain, a transmembrane domain, and a cytoplasmic C-terminal tail [86,87]. All SALMs have been shown to promote neurite outgrowth in cultured neurons, being the C-terminal PDZ binding domain crucial for this function [86,88]. SALMs interact with N-methyl D-aspartate receptors (NMDAR) and post-synaptic density protein 95 (PSD-95). Moreover, SALM2, 3 and 5 are also involved in synapse formation [87,89]. Overexpression of SALM2 increases the number of excitatory synapses and dendritic spines, while a knockdown has the opposite effect, in addition to decreasing the frequency of miniature excitatory postsynaptic currents (mESPCs) [87]. Overexpression of SALM3 and SALM5 in non-neuronal cells induces presynaptic differentiation of both

excitatory and inhibitory synapses in contacting axons [89]. The ligands for the SALM proteins are still unknown.

The netrin-G ligand (NGL) family form type one membrane proteins with nine LRRs at the extracellular domain, flanked by cysteine rich capping domains (LRRCT and LRRNT) and a C2 imunoglobulin domain [90]. NGLs are synaptic CAMs, primarily localized to the postsynaptic compartment of excitatory synapses. There are three known members: NGL-1, NGL-2 and NGL-3 and they interact with the pre-synaptic ligands, netrin-G1, netrin-G2 and LAR, respectively [81,90,91,92]. NGLs associate with main components of the synapse such as PSD-95 and NMDA receptors [86,93]. NGL-1 is highly expressed in the hippocampus, striatum and cerebral cortex and it promotes outgrowth of thalamocortical axons [94]. Both NGL-2 and NGL-3 induce pre-synaptic differentiation in a co-culture assay, increase the excitatory synapse density when over-expressed in cultured neurons and, when directly aggregated on dendrites, they recruit PSD-95 post-synaptically [93,95]. Disruption of NGL-2 or NGL-3 expression in cultured hippocampal neurons decreases the number and function of excitatory synapses [93,95].

The Leucine-rich repeat transmembrane (LRRTM) protein family, composed of four members, was initially described as a new family of transmembrane proteins expressed primarily and differentially in the central nervous system [96]. LRRTM1 and 2 are strong inducers of pre-synaptic differentiation in coculture assays and they localize to excitatory post-synaptic sites in neurons [83]. The LRRMT1 knockout mouse has been developed, and it was shown to have mild defects in excitatory synapse formation with just a modest increase in VGlut1 puncta size in the CA1, but not CA3, and no changes in puncta intensity or density. These data suggest that in this mouse model, possible compensatory mechanisms from LRRTM2 may be occurring [83]. LRRTM2 was recently identified as a post-synaptic ligand for Neurexin1 and this interaction was shown to be critical for synapse induction [97,98]. Knockdown of LRRTM2 decreases excitatory synapses *in vitro* and

attenuates the strength of evoked excitatory synaptic currents *in vivo.* LRRTM2 also interacts with PSD-95 and regulates surface expression of AMPA receptors [97,98].

In summary, LRR domains are involved in protein-protein interactions and LRR-containing proteins mediate a myriad of functions key for CNS development and function.

Table 1: Summary of LRR-containing proteins and their functions

Protein	Expression	Functions	Refs
AMIGO 1/2	Enriched in CNS Amigo 2 is ubiquitous	- TM protein that promotes prominent neurite extension in cultured hippocampal neurons - Exhibits homophilic and heterophilic binding activity - Modulates depolarization and NMDA-dependent survival of cerebellar granule neurons	[81,84]
FLRT3	Ubiquitous	- TM protein identified in a screen for genes upregulated after nerve injury - Promotes neurite outgrowth in cultured DRG neurons	[99]
Lingo-1	CNS	- TM protein that acts as co-receptor for Nogo receptor - Over-expression enhances responsiveness to myelin-associated inhibitors - Dominant negative form protects midbrain dopaminergic neurons against degeneration - Pathologically upregulated in SN of PD patients - Also interacts with EGFR and decreases its protein levels	[100,101,102,103]
Linx	DRG neurons	- TM protein that interacts with Trk and Ret receptors and augments neurotrophin and GDNF/Ret signaling - Knocking out Linx partially photocopies axonal projection defects in mice lacking NGF, TrkA, or Ret	[104]
Lrig1	Ubiquitous;	- TM protein that interacts with ErbB receptor family members (EGFR, ErbB2-4) and recruits cytoplasmic E3 ubiquitin ligases - Attenuates GDNF/Ret signaling in neuronal cells through its physical interaction with Ret	[105,106,107,108]
Lrig3	Ubiquitous	- TM protein that interacts with Xenopus FGFR through extracellular domain - Inhibits FGF-dependent MAPK phosphorylation - Negatively regulates FGF expression levels	[109]
LRRTM2	CNS, post-synaptic	- TM post-synaptic partner for α and β Neurexins - Promotes excitatory synapse formation - Interacts with PSD-95 and regulates surface expression of AMPARs	[83,96,97,98]
NGL-1	CNS: ST, CT, TCA, dendritic	- TM protein that binds netrin G-1 through LRRs - Stimulates outgrowth of embryonic thalamic axons	[94,110]
NGL-	CNS, HC, CT, post-synaptic	- TM protein that induces presynaptic differentiation and clustering of postsynaptic proteins - Increases the number of excitatory synapses	[93,95,110]
NGL-3	Brain	- TM protein that binds LAR through LRRs - Induces presynaptic differentiation - Promotes excitatory synapse formation	[95]
Omgp	CNS	- GPI-anchored protein that is expressed by neurons and oligodendrocytes - Mediates growth cone collapse and inhibition of neurite outgrowth	[111]
SALM 1-3	CNS	- TM proteins that form homo and heteromeric complexes - Promotes neurite outgrowth in young neurons (DIV 4-6) but not in older neurons (DIV 14-16) - SALM1-3 contain a PDZ-binding motif - SALM1 induces the dendritic clustering of NMDA receptors - SALM2 increases the number of excitatory synapses and dendritic spines - SALM3 & 5 induces excitatory and inhibitory pre-synaptic differentiation	[86,88,89,112]
Trks	CNS	- TM receptors that bind the neurotrophin family of growth factors - Influences the proliferation, differentiation, plasticity and survival of neurons	[2,19]

Abbreviations: Amigo, amphoterin-induced protein; FLRT3, fibronectin leucine rich transmembrane protein 3; Lingo-1, leucine rich repeat and Ig domain containing 1; Linx, immunoglobulin superfamily containing leucine-rich repeat 2; Lrig, leucine-rich repeats and immunoglobulin-like domains; NGL, Netrin G ligand; Omgp, oligodendrocyte myelin glycoprotein; SALM, Synaptic adhesion like molecule;

TCA, thalamocortical axons; ST, striatum; CT, Cortex; PD, Parkinson's disease; DA, dopamine; TM, transmembrane; DRG, dorsal root ganglia; GDNF, glial cell line-derived neurotrophic factor; SN, substantia nigra; TM, transmembrane; HC, hippocampus, AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; FGF, fibroblast growth factor; ErbB/EGFR, epidermal growth factor receptor; LAR, leukocyte antigen-related; GPI, glycosylphosphatidylinisotol; DIV, developmental day in vitro; PSD-95, synapse associated protein 95 kDa

2.3 Slitrks and their functions in the CNS

The Slitrk gene family has been implicated in psychiatric disorders, such as Tourrete Syndrome (TS), trichotillomania (TTM), obsessive-compulsive disorder (OCD) and schizophrenia [113,114,115,116]. Mechanistically, Slitrks can modulate neurite outgrowth, dendritic complexity, neuronal survival, and synapse formation [83,113,116,117]. Slitrks are highly expressed in the CNS, with its expression starting prenatally both in the mouse and humans [8,118,119].

Initial studies performed by over-expressing each of the Slitrk members in PC12 cells revealed that treatment of the transfected cells with NGF resulted in a decreased number of neurites per cell [8]. Neurite length was also decreased except for SLitrk1 and Slitrk4 [8]. Subsequently, the neurite outgrowth function of Slitrk1 has been analyzed in mouse cortical neurons [113,120]. Cortical neurons cultured from mouse embryos previously electroporated with human Slitrk1 cDNA showed increased dendritic length [113], suggesting that this Slitrk member plays an important role in promoting neurite outgrowth. More recently, it has been demonstrated that phosphorylation of Slitrk1 on Ser695 by casein kinase II is critical for the interaction of Slitrk1 with 14-3-3 proteins [120], which are ubiquitously expressed phosphorylation-binding proteins that regulate a number of important cellular processes including cell proliferation, neuronal migration and membrane excitability [120,121]. Mutation of this serine residue to an alanine residue abolished the interaction of Slitrk1 with 14-3-3 proteins as well as the induction of neurite outgrowth in cultured mouse cortical neurons [120].

Considering the more restricted distribution of Slitrk6 in the brain, with selectively high expression in the inner ear, a recent study performed a detailed

analysis of this organ in a mouse model that ubiquitously lacks Slitrk6 expression [117]. Results from this study demonstrated that Slitrk6 promotes the survival and neurite outgrowth of sensory neurons of the inner ear [117]. Histological examinations revealed that vestibular innervation was markedly decreased and sometimes misguided in the Slitrk6 null mice. The mutant mice also showed significant cell death in the spiral and vestibular ganglia. Furthermore, cochlear sensory epithelia taken from these mice and co-cultured with wild-type spiral ganglion neurons were less effective in promoting neurite outgrowth of the spiral ganglion neurons compared to co-cultures in which both types of cells were taken from wild-type mice [117]. Evidence for a trophic role of Slitrk6 was further strengthened by the observation that both BDNF and neurotrophin 3 (NT3) mRNA levels, as well as their corresponding receptor protein levels, TrkB and TrkC respectively, were down-regulated in the inner ear of Slitrk6 null mice. Interestingly, this was observed at an early developmental stage of the cochlea, when cell death had not occurred [117]. This study suggests that, at least in part, Slitrk6 may exert trophic actions by modulation of the neurotrophin system.Several LRR containing proteins have been shown to interact and modulate RTKs. For example Linx, a leucine-rich repeat and immunoglobulin (LIG) family protein, physically interacts with both Trk and Ret receptors, resulting in increased neurotrophin and GDNF signaling, respectively [104]. Besides Slitrk6, it would be interesting to investigate if other Slitrks could also modulate Trk receptor signaling.

Taken together, the results obtained with Slitrk1 overexpression in neurons [113,120] and the analyses the Slitrk6 null mouse [116,117], suggest that multiple Slitrk members play key roles in promoting neurite outgrowth. Even though the initial description of Slitrks indicated that they play a negative role on neurite outgrowth when over-expressed in PC12 cells [8], it remains unclear if this is of physiological relevance *in vivo.* It is likely that crucial binding partners involved in

neurite outgrowth that are endogenously expressed in neurons are lacking in PC12 cells.

Slitrks have also been implicated in synapse formation. A recent study performing an expression screen for synaptogenic proteins revealed the abundance of LRR containing proteins capable of inducing synapse formation [83]. Slitrk2 was one of the LRR proteins identified in this screen, and it was shown to induce excitatory pre-synaptic neuronal differentiation in a cellular co-culture system, suggesting that it could be a postsynaptic protein [28]. It is possible that Slitrks could interact with a pre-synaptic partner such as the Neurolgin-Neurexin system, via their extracellular LRR domains and could recruit intracellular post-synaptic proteins. Identifying the intracellular binding partners for Slitrks would contribute significantly to understanding the molecular basis of Slitrk functions in the nervous system.

2.4 Slitrk1 and OCD-spectrum disorders

TS is characterized by persistent involuntary vocal and motor tics and it is believed to belong to the OCD-spectrum [122,123]. Patients with OCD are characterized by having intrusive and persistent thoughts (obsessions) that cause anxiety and also by repetitive and ritualistic behaviors (compulsions) that they engage in order to reduce the anxiety caused by the obsessions [124,125,126]. Family studies have suggested that genetic factors are involved in the manifestation of OCD-spectrum disorders; however, finding susceptible genes for this disorder has been a daunting task [123,127]. OCD is present in 2-3% of the general population worldwide and epidemiological studies have shown that at least 50% of the people with OCD also suffer from other psychological disorders such as anxiety (including TS) or mood related disorders. Moreover, it is estimated that 90% of TS patients also suffer from OCD. It is this co-occurrence and the phenomenological similarities that motivated the hypothesis that these disorders might share common genetic and pathophysiological factors. Other so called OCD-spectrum disorders include

trichotillomania (TTM), that is characterized by a compulsive hair pulling behavior that leads to visible patches, and body dysmorphic disorder, characterized by obsession and extreme body image dissatisfaction and engaging in repetitive behaviors in order to change owns appearance, [125,128,129,130].

Studies performed on subjects that suffered from OCD as a result of brain lesions, as well as functional imaging analysis, have provided valuable insights into the pathophysiology of OCD. The cortico-striatal-thalamocortical circuit (CSTC) is known to play important roles in movement control and to facilitate adaptive motor actions and suppress others. These brain circuits, and mainly the basal ganglia, have been implicated in movement disorders including OCD, in which repetitive movements are core symptom [126,131]. The CSTC circuit is composed of two main parallel loops: the direct and indirect pathway, having opposing results in activating the orbitofrontal-cortex (Figure 3). In the direct pathway, neurons from orbitofrontal cortex project to medium spiny neurons in the striatum, expressing the D1 dopamine receptor. These neurons make connections with the globus pallidus interna-substantia nigra pars reticulata (GPi/SNr) complex, which then projects to the thalamus that in turn has reciprocal, excitatory projections to and from the cortical site of origin. The indirect pathway also originates in the frontal cortex and projects to medium spiny neurons expressing D2 dopamine receptor in the striatum, then projects to the globus pallidus externa (GPe), then to the subthalamic nucleus, then back to GPi/SNr, before returning to the thalamus and finally back to frontal cortex. The direct pathway contains two excitatory and two inhibitory connections, resulting in a net positive circuit back to the cortex. The indirect pathway has three inhibitory connections resulting in a net negative feedback loop to the cortex. Studies have suggested that OCD is associated with an over-activation of the direct pathway relative to the indirect pathway [124,129,132,133]. The origin of the over-activation observed is still being debated; however, evidence has mounting to implicate the glutamatergic system and the orbitofrontal cortex as the main players.

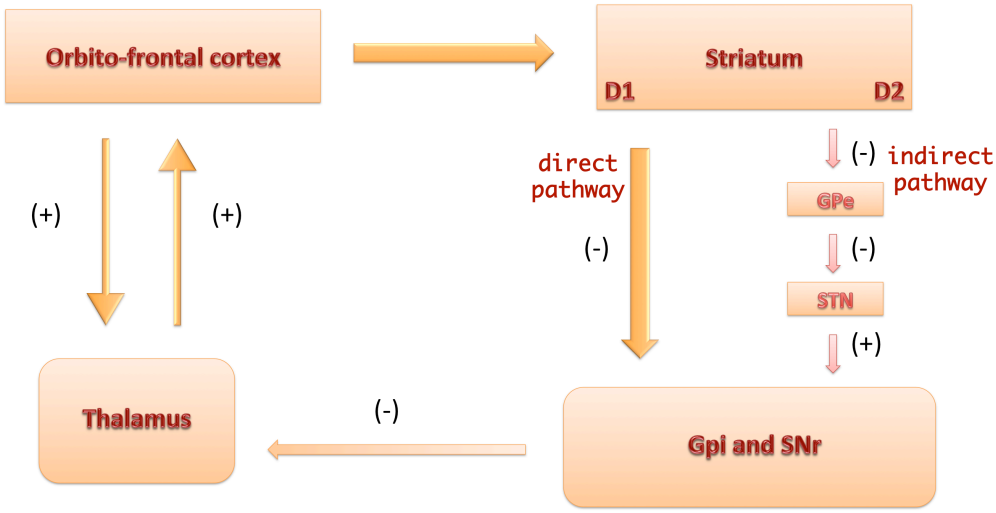


Figure 3. Schematic representation of the direct and indirect pathway, which are two opposing circuit loops in the basal ganglia. The direct pathway originates in the frontal cortex and projects to the striatum. Medium spiny neurons in the striatum, expressing D1 dopamine receptor, project to the globus pallidus interna/substantia nigra pars reticulata (GPi/SNr) complex, which projects to the thalamus that in turn has reciprocal, excitatory projections to and from the cortical site of origin. The indirect pathway also originates in the frontal cortex and projects to medium spiny neurons expressing D2 dopamine receptor in the striatum, then projects to the globus pallidus externa (GPe), then to the subthalamic nucleus, then back to GPi/SNr, before returning to the thalamus and finally back to frontal cortex. The direct pathway contains two excitatory and two inhibitory connections, resulting in a net positive circuit back to the cortex. The indirect pathway has three inhibitory connections resulting in a net negative feedback loop to the cortex.

The Slitrk1 gene was first implicated in TS in a rare variant study where two mutations were identified in three unrelated individuals with TS [113]. When looking for candidate genes that could be associated with TS, this group focused their attention in a particular subset of TS patients that had chromosomal abnormalities and no family history of TS. Fine mapping of the affected region led to the identification of several genes, including Slitrk1 that mapped 350Kb from the

breakpoint. Subsequent sequencing of Slitrk1 gene in a cohort of 174 individuals with TS, identified one proband (diagnosed with TS and attention-deficit hyperactive disorder) with a single base deletion, that led to a frame-shift and the expression of a truncated protein (named varCDf). The same mutation was also found in the patient's mother that suffered from TTM but not in unaffected relatives. Functional analysis showed that truncated Slitrk1 was ineffective in inducing dendritic outgrowth of cultured mouse cortical neurons as compared with wild type Slitrk1. An additional non-coding sequence variant (var321) in the 3'UTR was found in two unrelated individuals. This variant altered binding to the microRNA hsa-miR-189 and it negatively modulated Slitrk1 mRNA expression. These mutations were absent in over 3600 control samples, indicating that they are rare variants [113].

Subsequent studies were unable to find associations between these two reported variants in larger TS populations [134,135,136,137,138], arguing against a segregation of Slitrk1 and TS. Furthermore, it was reported that var321 was over-represented in the Ashkenazi Jew population, raising the possibility that population stratification might have led to a false positive result [136,137]. The largest screen for Slitrk1 and TS performed so far, which involved sequencing 1048 samples from the Tourette Syndrome Association International Consortium for Genetics, did not find association between var321 and TS [137]. Only two individuals were found who carried var321: one was diagnosed with TS, OCD and TTM, and the other with OCD but not TS. None transmitted the mutation to their affected offspring [137]. VarCDf was not analyzed. Additional studies provided more information regarding the ethnicity of the initially reported cases, and five additional subjects carrying var321 were reported [139]. Fine mapping of all seven carriers of the Slitrk1 region argued against population stratification confounding the original data [139].

In addition to these two variants, a recent study testing for association of common tag SNP's spanning Slitrk1 and TS identified an association to one specific tag SNP (rs959383) as well as two three-marker haplotypes, suggesting there may be

a common TS risk factor of low penetrance in linkage disequilibrium (LD) with the associated marker/haplotypes [140]. Furthermore, novel mutations in the Slitrk1 gene have been found to co-segregate with OCD-spectrum disorders [114,140,141]. Two non-synonymous mutations in the Slitrk1 extracellular region were discovered in two independent individuals of European descent, in a set of 44 families with TTM, and were absent in a group of almost 3000 healthy controls [114]. TTM is an OCD-spectrum disorder thought to be genetically associated with TS [130,142]. Moreover, a mutation in the 3'UTR of Slitrk1 gene was found in a group of 92 Austrian patients, that segregated in two additional family members with tic disorders, and which was absent in 192 control subjects [141].

Slitrk1 null mice have been generated and studied with the aim of better clarifying the potential role that Slitrk1 may play in neuropsychiatric disorders [143]. It has been demonstrated that these mutant mice display increased anxiety-like behavior (as measured by the elevated plus maze test) as well as depressive-like behaviors (as assessed in a forced swim task). Neurochemical analysis revealed that Slitrk1 null mice had increased levels of norepinephrine in the prefrontal cortex, striatum, and nucleus accumbens [143]. This is consistent with the pathophysiology of TS since patients with this disorder have been reported to have high concentrations of norepinephrine in their cerebrospinal fluid [144]. Although these mice did not recapitulate the hallmark motor characteristics of human TS, anxiety and depressive disorders are highly comorbid with TS [122,145], and administration of clonidine (an α2-adrenergic agonist commonly used to treat TS patients [146,147,148]) was able to rescue the anxiety-like behavior of Slitrk1 null mice [143]. These findings add support to a likely role of Slitrk1 in neuropsychiatric disorders. The generation of future mouse models for Slitrk1, especially those which conditionally knock-down the gene in a spatially and/or temporally-specific manner, will help to further delineate the exact roles that this protein plays, and will help to avoid potential developmental compensation from other Slitrk family members that

may occur with traditional knockout mouse models. Furthermore, it would be interesting to determine the consequences of the introduction of human Slitrk1 variants (eg. var321 or varCDfs) in future knock-in mouse models, and to assess these mice for their subsequent neuroanatomical and behavioral phenotypes. Such studies will help to directly evaluate the *in vivo* consequences of specific mutants in the Slitrk1 gene, and they would avoid possible compensatory mechanisms from other Slitrk members that could have occurred in the Slitrk1 mouse leading to a fairly modest phenotype and no tics.

2.5 Glutamate receptors implicated in psychiatric disorders

Glutamate is the major excitatory neurotransmitter in the CNS and as much as half of the entirety of brain synapses releases this agent. Glutamate neurotransmission is mainly mediated by two ionotropic reptors, the NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors [149]. It is then not surprising that modulation of the glutamatergic system is key for the regulation of a myriad of CNS properties ranging from high cognitive processes such as learning and memory, to disease [150,151]. Recent data has suggested a role for the glutamatergic system in OCD. Analysis of cerebral spinal fluid of patients with OCD, not undergoing pharmacological treatment, showed that glutamate levels were significantly elevated as compared to age-matched controls [152]. Correlation studies have suggested an association between the gene *GRIN2B* (coding for the NMDA receptor subunit 2B protein) and OCD [153]. Moreover, a gene encoding the neuronal glutamate transporter protein, *SLC1A1* gene, has also been associated with OCD [154]. Even though in small number and requiring larger sampling confirmation, these studies emphasize the importance of the glutamatergic system in OCD. Therefore, understanding the mechanisms of glutamate receptor expression and modulation is critical for the understanding of these disorders.

The synthesis, transport, stabilization and degradation of AMPA receptors are tightly controlled by a complex set of transmembrane and cytoplasmatic proteins [155,156]. Stargazin was the first transmembrane protein found to act as an auxiliary subunit to AMPA receptors [157]. Discovery of stargazin resulted from the characterization of a spontaneous mutation in inbred mice, distinguished by its striking behavior phenotype that includes absence epilepsy, cerebellar ataxia, and an abnormal motor syndrome [158]. Detailed analysis of the protein coded by stargazer mutation revealed that the protein stargazin belongs to a family of six members that are collectively called transmembrane AMPA receptor regulatory proteins (TARPs). TARPs are brain specific tetraspanning proteins that avidly and specifically interact with oligomeric AMPAR [159]. TARPs promote surface expression of AMPARs and also target AMPARs to the synapse through interactions with the synaptic scaffolding proteins PSD-95 and MAGUK. The stargazer mutant mouse exhibits a striking lack of surface AMPARs in cerebellar granule cells, confirming the critical role that these proteins play in correct targeting of AMPARs. TARPs are also key regulators of channel gating and pharmacology, and generally tend to enhance AMPAR mediated currents [160,161].

A recent study employing functional proteomic analysis of affinity purified native AMPARs from rat brain membrane fragments identified new binding partners for these receptors. The immunoprecipitation of the previously described TARPS validated this approach as an efficient screening method. In addition to TARPS, mass spectrometry analysis consistently identified homologs of a protein family named Cornichon, co-purifying with all AMPARs subunits analyzed (GluA1, 2 and 3) [162]. Cornichons form three-pass transmembrane proteins that promote forward trafficking of AMPARs from the ER, enhancing surface expression and modulating AMPARs channel properties by slowing deactivation and desensitization of the receptor. Interestingly this study revealed that the majority of AMPARs coassemble with cornichon homologs 2 and 3 (CNIH-2, CNIH-3) rather than with TARPS [162].

Subsequent studies raised questions regarding the mechanisms by which these proteins regulate AMPAR currents and location. Over-expression of CNIH2 in cerebellar granule neurons of the stargazer mouse failed to rescue synaptic currents, suggesting that CNIH2 is not associated with surface AMPARs. Moreover, in this study Flag-tagged CNIH2 was found associated with a Golgi marker and absent from the surface in cultured hippocampal neurons [163]. In contrast, parallel studies were able to find surface CNIH2 that co-localized with GluA1 [164]. The results from the first study suggest that cornichons may act as ER chaperones required to modulate AMPARs to acquire an appropriate conformational structure, therefore allowing efficient receptor currents. On the other hand, TARPS act as integral subunits of surface AMPARs critical to modulate receptor currents. An alternative possibility is that AMPARs may normally be associated with both CNIH-2 and stargazing (member γ-8).

Another recent proteomic approach identified CKAMP44 (cysteine-knot AMPAR modulating protein) as a type I transmembrane protein that interacts with TARP containing AMPARs. CKAMP44 is robustly and specifically expressed in the hippocampal dentate gyrus and, in contrast with TARPs and cornichons, it reduces glutamate-evoked currents [165].

Lastly, microarray analysis of mice with neuronal differentiation defects identified SynDIG1 as another type I transmembrane protein that interacts and modulates synaptic targeting of AMPARs. Knocking down SynDIG1 expression leads to a decrease in synaptic puncta size and AMPAR mEPSC frequency and amplitude [166].

Identification of glutamate receptor binding proteins and characterization of their mechanisms will contribute to a better understanding of this protein family and facilitate the discovery of potential molecular targets in a context of disease.

3. RESEARCH QUESTION AND GOAL OF THE THESIS

Understanding the molecular mechanism underlying CNS development and function is critical to comprehend the failure of these systems in disease. I was interested in further extend our knowledge of a known protein family, the neurotrophin, while at the same time exploring a new protein family that has just recently been described to be present in the brain.

Our understanding of the molecular mechanisms of neurotrophin dependent signaling is mainly based on studies performed in the PNS, whereas their characterization in the CNS is more limited. Amongst all Trk receptors, TrkB has some distinct characteristics, as it was found to be expressed primarily in the CNS and serve as a high affinity receptor to more than one neurotrophin. In fact, TrkB binds with equal affinity to BDNF and NT4, which are similarly distributed throughout the brain. It is an interesting question why nature evolved to express two ligands for the same receptor that apparently fulfill a similar role. NT4 was the latest neurotrophin to appear in evolution and it is not present in fish, suggesting that in the CNS an extra level of variability was necessary to achieve more complex functions. Despite binding the same receptor, studies have shown, however, that knocking down each of these neurotrophins can lead to different outcomes. I was interested in understanding how BDNF and NT4 differentially modulate the TrkB receptor in order to achieve distinct functions. Considering the prominent role of endocytic protein trafficking in regulating RTK signaling, I decided to approach this question by investigating the downstream targeting of TrkB receptor upon activation by BDNF or NT4.

Comprehensive analyses of the molecular players involved in CNS development and function have shown us that our knowledge is still limited to explain the complexity and diversity of this system. In addition to expansion of existing protein families, such as the appearance of NT4 in the neurotrophic family, evolution has constantly brought us new molecules to allow the viability of more

complex organisms. I became interested in a newly identify protein family, Slitrk, given their structural similarities with Trk receptors, their high expression in the brain and the little information we had on their molecular functions. Proteins containing LLR domains are emerging as major determinants of brain development and wiring. The few studies performed to date on the Slitrk family have highlighted their potential association with psychiatric disorders, raising the significance of delineating the function of this family. Thus, we were interested in understanding the molecular mechanisms regulated by these proteins. We also would like to identify new pathways altered in psychiatric disorders and eventually contribute to the discovery of new therapeutic targets for intervention in patients affected by these conditions. I accomplished this by studying the function of one of the highly expressed members of the family, Slitrk5, through analyses of Slitrk5 knockout mice.

Chapter 2: Endocytic trafficking of TrkB receptor mediated by BDNF and NT4

AUTHOR'S CONTRIBUTION FOR CHAPTER 2

For the following chapter all of the experiments were performed by the author Cátia Proença, except the analysis of the samples for mass spectrometry (Table 1), which were performed in collaboration with Moses Chao Laboratory at Skirball Institute, New York, USA.

INTRODUCTION

Neurotrophins are secreted polypeptides known to play critical roles for nervous system development and function. There are two types of neurotrophin receptors: the p75 and Trk receptors. Trks are a family of three members TrkA, TrkB and TrkC. TrkB being the most highly expressed in the brain. TrkB receptor is mainly activated by BDNF and NT4, although it can also bind NT3 to a lesser extent [39]. Comparative analysis of BDNF and NT4 expression has shown that these neurotrophins are widely expressed in the brain; however, NT4 is expressed at much lower levels that escape histological detection. Studies using sensitive RNAse protection assays with total rat brain showed that NT4 expression is maximal at embryonic day 13 (E13) followed by a decline at around birth, after which it increases again [29]. The levels of BDNF also increase during postnatal rodent brain development peaking at P20. This developmental regulation of BDNF and NT4 expression was observed in the cortex, hippocampus and cerebellum [28,29].

Recent structural analysis revealed that the neurotrophin–binding domain in Trk receptors is the second immunoglobulin like domain (Ig_2) [167,168]. Neurotrophin homodimers form a bridge between two Ig_2 homodimer molecules stabilizing this complex [167]. The binding affinity of BDNF and NT4 to purified Ig_2 domains has been determined using surface plasmon resonance. NT4 shows a slightly higher affinity for TrkB Ig_2 (K_D of 260 pM) as compared BDNF (K_D of 790 pM), while NGF did not bind, as expected [168]. The association constant for BDNF with Ig_2 was consistent with the previously reported affinity to the full length receptor (of 990 pM) [168]. Interestingly, after acidic ligand washing, NT4 appeared to dissociate faster than BDNF from TrkB receptor [168]. Consistent with a similar binding mechanism, it has been shown that BDNF and NT4 lead to comparable activation of TrkB receptor and its downstream signaling molecules (such as PLC-γ, Shc and MAPK) in heterologous cells [169].

Despite the similarities in receptor binding and activation, BDNF and NT4 can mediate different cellular mechanisms. BDNF has been established as a major regulator of synaptic transmission and plasticity. It can modulate processes such as ion channel activity, neurotransmitter release, LTP, transcriptional activation during LTP and it is also involved in higher order cognitive tasks, ranging from learning and memory to psychiatric disorders [13,14,170,171,172,173]. The role of NT4 for synaptic transmission is less pronounced, however, a recent study highlighted the ability of NT4 to rescue deficits in LTP in the Schaffer collateral-CA1 area, induced by amyloid β peptide treatment, in a CaMKII (Ca^{2+} Calmodulin dependent kinase II)-dependent manner [174]. Studies comparing the effects of exogenous application of BDNF and NT4 in the development of retinal ganglion cells have shown that BDNF promotes cell polarization and extension of a single neurite, whereas NT4 induces the formation of highly branched symmetrical arbors with short neurites [175]. The BDNF and NT4 knockout mice ($BDNF^{-/-}$ or $NT4^{-/-}$) also show strikingly different phenotypes. Complete $BDNF^{-/-}$ mice have severe cardiac and breathing defects leading to early post-natal lethality [176,177,178]. Therefore, approaches such as utilization of $BDNF^{+/-}$ (heterozygous) mice, generation of region specific conditional deletions and local delivery of gene knockdown agents have been employed. These studies have strengthened the impact of BDNF for proper CNS development and function [27,179,180]. In contrast to BDNF-deficient mice, the $NT4^{-/-}$ mouse has been studied to a much lesser extent. $NT4^{-/-}$ mice are viable and do not exhibit major developmental defects, allowing for behavioral and physiological studies to be easily performed [181]. These mice have defects in long-term memory in a cue and contextual fear conditioning task, but short term memory is intact. Hippocampal slices show normal basal synaptic transmission, in contrast to the $BDNF^{-/-}$; however, they have a selective defect in long lasting LTP in the Schaffer collateral – CA1 synapses, that correlated with a selective defect in long versus short term memory [181]. In the periphery, mice lacking NT4 exhibit a selective loss of nodose-petrosal

and geniculate ganglia neurons [36]. This neuronal population was also shown to be dependent on BDNF, however, whereas two BDNF alleles are necessary to support the survival of these neurons, a single NT4 allele is sufficient [176].

The differences observed in the modulation of synaptic plasticity mediated by these two neurotrophins might be explained by the fact that BDNF is released in an activity-dependent manner, whereas NT4 is processed through the constitutive secretory pathway [182]. Regarding the phenotype of the null mice, it is plausible to speculate that endogenous expression of high levels of BDNF are sufficient to rescue major defects in the $NT4^{-/-}$ mice. In contrast, the endogenous expression of NT4 might not be enough to rescue the lack of BDNF. In this line of thought, a very interesting study in which the *BDNF* gene was replaced by *NT4*, maintaining the *BDNF* promoter intact, showed that not only *NT4* was able to rescue the $BDNF^{-/-}$ phenotype (these mice are viable), it was also more potent at inducing synaptic maturation and sensory neuron survival. NT4 levels in the *knock-in* mouse were comparable to BDNF in wild type mice [183]. In fact, some studies that compared the biological actions of BDNF and NT4 in parallel have suggested that NT4 is a more potent neurotrophin in certain contexts. One study that analyzed the ability of different neurotrophins to counteract monocular deprivation in rats showed that infusion of NT4 in the visual cortex was more potent than BDNF in counteracting monocular deprivation. However, as shown in other studies, it did not enhance basal nor evoked neuronal activity [184]. NT4 is more effective than BDNF at inducing axonal outgrowth of peripheral neurons of the mouse nodose ganglia in organotypic cultures [185]. Neuropeptide Y (NPY) is synthesized by neurons of CNS and peripheral nervous system (PNS) and is implicated in regulating appetite and energy homeostasis. Explants of thalamocortical cocultures that were transfected with either BDNF or NT4 showed that transfection with NT4 evoked substantially larger numbers of NPY-positive neurons and mRNA per neuron; however, in contrast with BDNF this was independent of calcium influx. Both effects could be abolished by a

TrkB inhibitor [186]. Finally, analysis of neurotrophin induced dendritic growth, arborization and spine formation of the ferret visual cortex showed that NT4 is more potent than BDNF at inducing dendritic density and spines in the layer 5 and layer 6, of both apical and basal dendrites. In basal dendrites of the layer 4, BDNF had a more prominent role [187].

The above studies suggest that even though BDNF and NT4 signal through the same receptor they can mediate distinct cellular outcomes. Interestingly, TrkB is the only Trk receptor similarly activated by two neurotrophins and the most expressed in the brain. NT4 was the last neurotrophin to be discovered owing to its divergent sequence, however it has a similar structure as BDNF and binds TrkB in the same regions [188,189,190]. It remains an open question why would nature evolve to generate two different ligands that activate the same receptor, apparently to a similar extend, and have a similar spatial distribution [29].

The duration, specificity and intensity of signaling through RTKs can be modulated by ligand-mediated endocytosis and post-endocytic sorting of activated receptors. Internalization of activated RTKs and consequent targeting to the lysosome results in downregulation of the activated signaling receptor [53]. On the other hand, post-endocytic recycling of RTK contributes to sustained signaling [54]. We asked the question if the distinct biological outcomes achieved with BDNF and NT4 could be due to differential sorting of TrkB receptor to distinct endocytic trafficking routes. We found that NT4 leads to efficient TrkB activation, endocytosis and downstream signaling at early time points. Surprisingly, we found that BDNF leads to more rapid TrkB ubiquitination than NT4. After prolonged neurotrophin treatment, BDNF leads to faster degradation of the TrkB receptor than NT4. As a result, NT4 was cable of maintaining longer sustained downstream signaling activation. Thus, these results suggest that the differences on the TrkB-mediated outcomes induced by these two neurotrophins are not associated with altered TrkB activation, but rather lay on the duration of this effect.

MATERIALS AND METHODS

Reagents and antibodies

Human recombinant BDNF and NT4 were obtained from PeproTech (Rocky Hill, NJ). Immunoprecipitations were performed with the following antibodies: rabbit anti TrkB antibody from Upstate (catalog 07-225; 1:100) or goat from R&D (AF1494 1:100); rabbit SHP2 from Santa Cruz (SC-280); Flag antibodies were purchased from Sigma (M2 and F2555 both used at 1:100). For western blot the following antibodies were used: mouse TrkB antibody 1:1000 (610101), mouse SHP2 1:1000 (610621), and mouse Grb2 were obtained from BD Biosciences 1:1000 (610112); phosphotyrosine PY99 1:4000 (SC-7020), actin-HRP 1:4000 (sc-1616), Erk1 (sc-93), Erk2 (sc-154) both used 1:4000 were from Santa Cruz. Tubulin 1:4000 (T6074) was from Sigma. PhosphoErk 1:4000 (#9101S), phosphoAkt 1:1000 (#4060S), Akt 1:1000 (9272) were from Cell Signaling. For immunocytochemistry, the rabbit and goat TrkB antibody and both Flag antibodies were used; EEA1 was from BD Biosciences (610457). Alexa conjugated fluorescent secondary antibodies were from Molecular Probes. All other compounds were from Sigma-Aldrich.

Cell culture

All reagents used to prepare primary neuronal cultures were purchased from Invitrogen, except glucose that was from Sigma. The cortex and/or hippocampus, were dissected from E18 Sprague Dawley rat in Hanks Balanced solution (HBSS) supplemented with 0.37% glucose. Digestion was performed in the same medium supplemented with 0.05% trypsin for 10-15 minutes at 37°C. Neurons were mechanically dissociated with fire-polished Pasteur pipettes and plated in plating medium (PM) (MEM containing 10% FBS, 1 mM pyruvate, 0.37% glucose, and 0.1 mg/ml of Primocin from Invogen) for 24 hours. After one day in culture PM was replaced by Neurobasal supplemented with B-27, 0.5mM glutamine, 0.1 mg/ml

Primocin and 2µM Ara-C (Cytosine β-D-arabinofuranoside hydrochloride). Cells were grown on poly-D lysine-coated surfaces: on glass coverslips a density of $15x10^3$ cells/cm^2 was used for immunocytochemistry; on polystyrene dishes, a density of $76x10^3$ cells/cm^2 was used for biochemistry. Neurons were kept in a humidified incubator at 37°C and 5% CO2. 293 cells stably expressing TrkB were a kind gift of Moses Chao lab at NYU Medical center and were maintained in regular 293 media, DMEM (Invitrogen) containing 10% heat inactivated fetal bovine serum (GemCell), with 100 U/ml penicillin, 100 U/ml streptomycin (Pen/Strep) (Invitrogen), 2 mM glutamine (GlutaMAX from Invitrogen), supplemented with 250 µg/ml of Geneticin (Invitrogen).

Degradation assay

Cell surface biotinylation was used to specifically detect receptors present in the plasma membrane and to measure their proteolysis. Neurons were washed twice with ice-cold PBS supplemented with Ca^{2+} and Mg^{2+} (PBS^{++}) and incubated with 300 µg/ml of sulfo-NHS-S-biotin (Pierce Chemical, Rockford, IL) for 20 min on ice with gentle agitation. Unreacted biotin was quenched and removed with 50mM Glycine in PBS^{++}. Biotinylated cells were then transferred to pre-warmed medium containing ligand for the indicated times, and then cells were immediately chilled on ice and lysed in Ripa buffer. Biotinylated proteins were isolated from cell extracts by immobilization on high capacity streptavidin-conjugated Sepharose beads (Pierce Chemical, Rockford, IL). Washed beads were eluted with SDS sample buffer, and eluted proteins were resolved by SDS-PAGE.

Biochemical internalization assay

Cells were prepared as for degradation assay however; a cleavable form of biotion was used (Sulfo-NHS-SS-Biotin from Pierce, 21331). After neurotrophin treatment and incubation, remaining surface-bound biotin was cleaved with

glutathione solution containing: 50mM glutathione in 75mM NaCl, 10mM EDTA, 1%BSA, 0.075 NaOH; 2 times for 15 min each on ice and with gentle agitation. After glutathione wash, cells were quenched with TBS buffer followed by lsysis in RIPA buffer and avidin pull down.

Western blotting and immunoprecipitation

Protein lysates for western blot and immunoprecipitation were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0; 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) containing protease and phosphatase inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Extracts were rotated at 4°C for 15 minutes and cleared by centrifugation (12,000∗g for 15 min). For immunoprecipitation, antibody was added at 1:100 concentration a rotated for 2 hours at 4°C, followed by pull down with either protein A beads (Sigma) or protein G (Roche), overnight at 4°C. Lysates were washed 3-6 times in lysis buffer. Western blotting was performed using 10% BIS-TRIS pre-cast gels from Invitrogen. Protein samples were boiled with 4X LDS Nupage sample buffer from Invitrogen (NP0007) for 2 minutes before loading. Electrophoresis was done at 80mA using MES-SDS running buffer from Invitrogen. Transfer was done at 4°C, 100V for 90 minutes. PVDF membranes were blocked in 5% milk-TBS-T or 3% BSA-TBS-T if PY99 was being used in (0.1% Tween). Antibodies were probed over-night at 4°C.

Immunocytochemistry

DIV5-7 rat cortical neurons were serum-starved over-night in Neurobasal supplemented with 2% glucose. TrkB antibody was added to the covers-slips at 1:1000 dilution on ice for 15 min. Cells were fixed in 3.7% formaldehyde solution (from EMS) for 15 min at RT. Unreacted formaldehyde was quenched with glycine. Permeabilization was done with 0.2% Triton-X with gentle agitation at RT for 6

minutes. Primary antibodies were incubated for 60 min at RT, followed by washes in PBS with gentle agitation, followed by alexa-conjugated secondary antibody incubation for 20 min at RT. Antibodies were prepared in blocking solution 3%BSA, 10% donkey serum in PBS.

Endocytosis assay

DIV5-7 rat cortical neurons were grown on glass coverslips pre-coated with PDL. Medium was changed 12h before the experiment to Neurobasal containing 2% glucose. Endogenous surface pool of TrkB receptor was live fed by adding the antibody directly to the media on ice (rabbit Upstate antibody at 1:1000 concentration). After surface labeling, neurons were incubated at 37°C for an initial 10 min temperature-adaptation period and a further 15 min with BDNF or NT4, 50ng/ml. After internalization, cells were fixed and TrkB receptor remaining on the surface was labeled with saturating concentrations of secondary antibody conjugated with alexa488 (1:300) in blocking solution, followed by permeabilization, after which the internalized pool was labeled with secondary antibody conjugated with alexa568, 1:300 in blocking solution. Control neurons were incubated without neurotrophins. Quantification was performed blind to treatment. Internalization index = ((Inter / (Intern + Surface))*100. For endocytosis measured as co-localization with EEA1, after antibody-fed TrkB and neurotrophin treatment (15 min), neurons were fixed, permeabilized and stained for EEA1. Quantification of TrkB co-localizing with EEA1 was done by manually counting total TrkB puncta and TrkB puncta overlapping EEA1, using the manually count objects tool in Metamorph software.

Fluorescence Microscopy

Images were acquired on an inverted microscope, Nikon Eclipse TE2000-U, light source was PhotoFluor from Chroma, using Metamorph Software. Objective

used was PlanApo 60xA/1.40 oil Nikon. Image quantification was performed using ImageJ and Metamorph software.

SILAC (stable isotope labeling by amino acids in cell culture)

Preparation of cortical and hippocampal neurons was done as previously described in this chapter. Neurons were cultured in regular complete Neurobasal or an identical medium formulated without L-arginine and L-lysine (Specialty Media, Philipsburg, NJ) supplemented with heavy ^{13}C isotope-containing amino acids (Cambridge Isotope Laboratories, Andover, MA) or isotopically normal amino acids (Sigma-Aldrich). Culture media were refreshed every 3 days by removing half of the volume present on each plate and replacing it with fresh medium. After culturing neurons for 10 days in vitro, BDNF or NT4 (PeproTech, Rocky Hill, NJ) was added to neuronal cultures at a concentration of 25 ng/ml for 15 min, and cells were washed with ice-cold PBS and immediately placed on ice in ice-cold lysis buffer containing appropriate inhibitors. Lysates from cells cultured in differently labeled media were mixed in a 1:1:1 stimulated:control total protein ratio (based on Bradford assay) and combined with agarose conjugated anti-phosphotyrosine antibody pY99 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing 20 µl of beads/ml of lysate for overnight incubation at 4 °C. Beads were washed four times with lysis buffer. Immunoprecipitated proteins were eluted by boiling in Laemmli SDS-PAGE reducing buffer (Bio-Rad) for 5 min. All samples were separated by SDS-PAGE using 10% Tris-HCl gels (Bio-Rad). Gels were stained with Coomassie Brilliant Blue (Bio-Rad), and gel lanes were cut horizontally into 23 sections. Excised gel bands were cut into small pieces and destained in 25 mM ammonium bicarbonate, 50% acetonitrile; dehydrated with acetonitrile; and dried. The gel pieces were rehydrated with 10 ng/µl trypsin solution in 25 mM ammonium bicarbonate and incubated overnight at 37 °C. Peptides were extracted twice with 5% formic acid, 50% acetonitrile followed by a final extraction with acetonitrile. Extracts were pooled, dried by vacuum

centrifugation, and reconstituted in 5 µl of 0.1% formic acid, 2% acetonitrile for HPLC sample injection. The peptide mixtures resulting from tryptic in-gel digestions were analyzed using nanoflow LC-MS/MS. Proteome changes, experiments were performed using a nanoACQUITY Ultra Performance Liquid Chromatography system (Waters) coupled directly to a Q-TOF Premier mass spectrometer (Micromass). Raw mass spectrometry data were processed using ProteinLynxGlobalServer 2.2 software (Waters). Proteins were identified using Mascot software (version 2.1, Matrix Science, London, UK) and parsed using ProteinCenter (Proxeon, Odense, Denmark) MS spectra of labeled and non-labeled peptide pairs were tracked in the raw LC-MS/MS files. Experimental Tyr(P) IP SILAC ratio quantification was carried out using the open source software MSQuant (kindly provided by Peter Mortensen and Matthias Mann (Source- Forge, Inc.). As an additional measure to achieve proper quantification, weighted mean ratios were calculated manually for proteins observed in more than one band and in replicate experiments. Ratios were calculated using MSQuant for proteins that had peptide signals with observable unlabeled components. Ratios of less than 10:1 (the dynamic range limit of MSQuant for our data) were corrected for labeling efficiency.

RESULTS

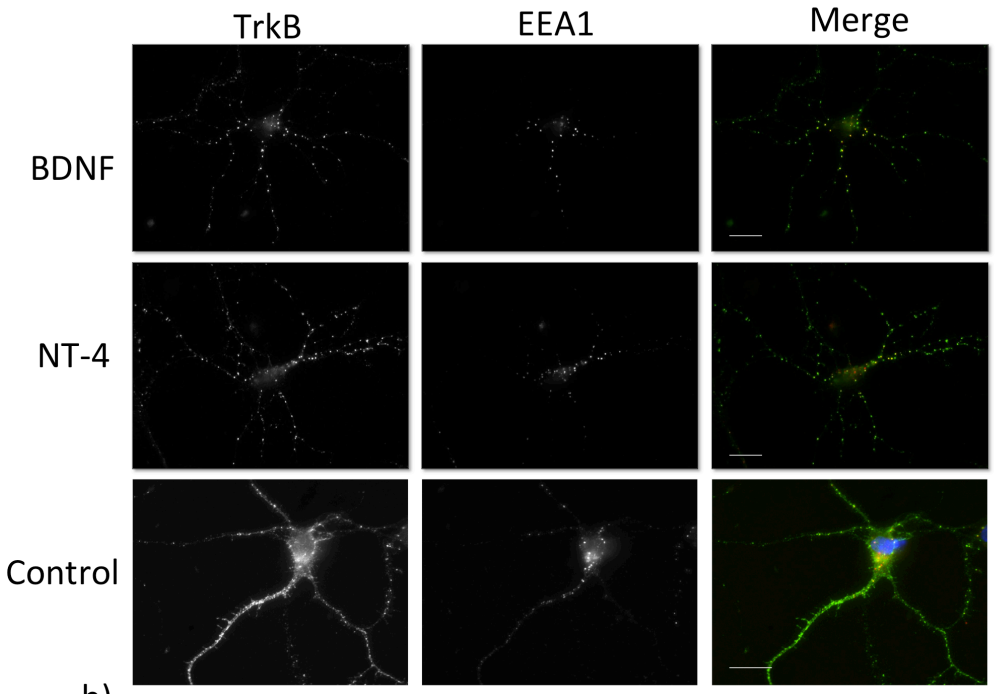
NT4 leads to efficient endocytosis of TrkB receptor and targets TrkB to the early endosome compartment

TrkB is the neurotrophic receptor with highest expression in the brain and the only member that is activated with similar affinity by two neurotrophins [168]. However they seem to mediate distinct biological outocomes. In order to understand the mechanisms mediating these differences we sought to investigate how BDNF and NT4 might differentially modulate the endocytic trafficking of TrkB receptor.

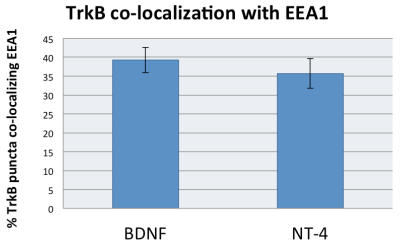
We started by analyzing the initial endocytic steps of TrkB upon BDNF or NT4 stimulation. It is known that BDNF treatment induces endocytosis of TrkB in a clathrin-dependent manner to an early endosome compartment. However, the mechanisms of NT4 mediated TrkB trafficking are unknown [191]. To measure TrkB endocytosis endogenous TrkB receptor was labeled with a live-fed antibody. We first aimed to understand if BDNF and NT4 target TrkB to the same intracellular compartment. To explore this we quantified the co-localization of TrkB with an early endocytic marker, EEA1. As shown in figure 1 a, both BDNF and NT4 lead to TrkB internalization that can be observed by the puncta-like staining corresponding to early endosomes. Non-treated neurons show mostly surface TrkB staining; consistent with the fact that antibody-induced TrkB activation was minimal. Quantification of TrkB co-localization with EEA1 revealed a comparable endocytosis of the receptor at a 15 min time point, mediated by BDNF or NT4, suggesting that both ligands induce endocytosis through a similar mechanism. The fraction of TrkB that localizes to the early endosome containing EEA1 is similar in cells treated with BDNF or NT4 (BDNF 39.2% ± 3.3%; NT4 35.7% ± 3.9%; p value 0.5) (Figure 1 a, b). Although at this time point there is a relatively small fraction of TrkB co-localizing with EEA1, this could be potentially explained by the fact that this marker is exclusively associated with endosomes of the somatodendritic compartment and not

with axonal endosomes [192]. To overcome a possible underestimation with these studies, we also measured total internalized TrkB divided by the surface fraction. In this experiment, total surface TrkB receptor was live labeled with a fed antibody. Cells were then incubated with BDNF, NT4 or left untreated for 15 min at 37°C. Quantification of the ratio of internalized receptor versus the surface pool revealed that BNDF and NT4 induce similar endocytosis of TrkB receptor (BDNF 72% ± 1.5%; NT4 74.3% ± 1.8%; p value 0.4) (Figure 1 d, e).

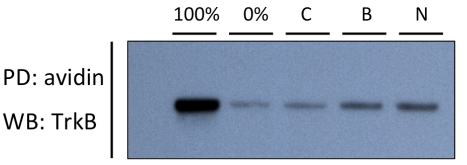
To complement the immunocytochemical data, we also measured TrkB internalization using a cleavable biotin assay. In this assay, membrane proteins are surface biotinylated and after internalization had occurred, remaining surface biotin is removed such that only internalized proteins are pull down. For technical reasons this experiment was performed with HEK293 cells stably expressing low levels of TrkB protein, such that receptor auto-activation does not occur. As a positive control, we used biotinylated cells without cleavage and, as a negative control biotin cleavage was done without the 37°C incubation step. In agreement with our previous observations, BDNF and NT4 also elicit similar TrkB endocytosis in this experimental setting (Figure 1 c).


TrkB
EEA1
Merge
BDNF
NT-4
Control

b)


TrkB co-localization with EEA1
% TrkB puncta co-localizing EEA1
45
40
35
30
25
20
15
10
5
0
BDNF
NT-4

c)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PD: avidin
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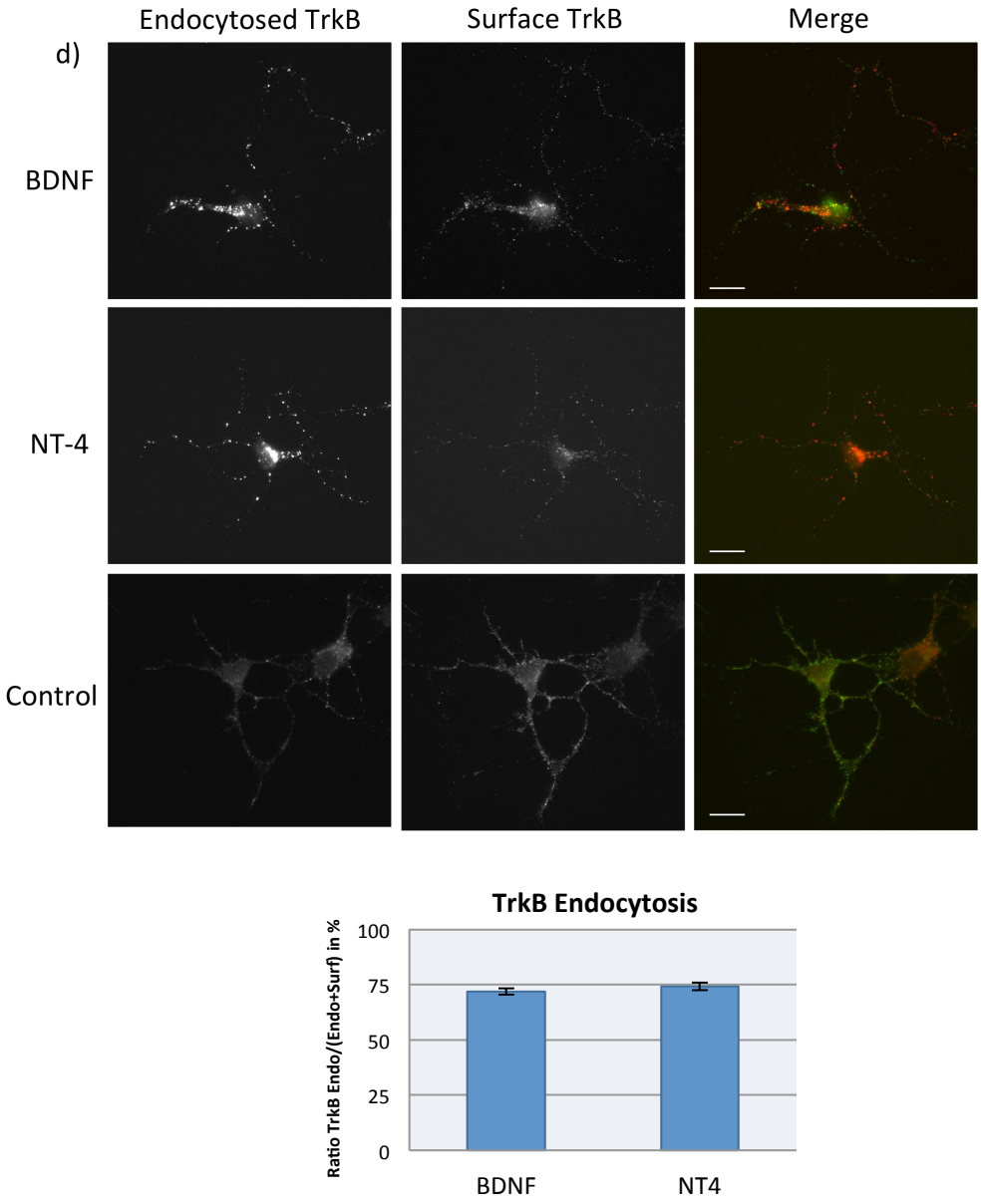


Figure 1: Endocytosis of TrkB receptor mediated by BDNF and NT4. a) Rat cortical neurons were live fed with TrkB antibody on ice, and subsequently treated with 50ng/ml of BDNF or NT4 for 15 min at 37 °C. Co-immuno localization with the early endocytic marker EEA1 was analyzed; **b)** Quantification of percent TrkB co-localizing with EEA1 upon BDNF or

NT4 treatment (BDNF 39.2% ± 3.3%; NT4 35.7% ± 3.9%; p value 0.5); **c)** Biochemical internalization assay with. HEK293 cells stably expressing TrkB receptor were surface biotinylated with cleavable sulfo-NHS-S-biotin that is cell impermeable. After a 50ng/ml BDNF/NT4 treatment and endocytosis had occurred (15 min at 37°C), remaining surface biotin was cleaved with a reducing agent, and avidin was used to pull down internalized proteins. Precipitates were run on Western blot and probed with TrkB antibody depicting the internalized pool only. As a positive control, biotin was not cleaved and as a negative control, biotin was cleaved without the incubation step. **d)** Endocytosis assay of TrkB receptor in rat cortical neurons in which the endogenous surface pool of TrkB was labeled using a live fed antibody on ice. After surface labeling, neurons were incubated at 37°C for an initial 10 min for temperature-adaptation and a further 15 min with BDNF or NT4, 50ng/ml. After internalization, cells were fixed and TrkB receptor remaining on the surface was labeled with saturating concentrations of secondary antibody conjugated with alexa488, followed by permeabilization, after which the internalized pool was labeled with secondary antibody conjugated with alexa568. Control neurons were incubated without neurotrophins. **e)** Quantification of internalized TrkB (BDNF 72% ± 1.5%; NT4 74.3% ± 1.8%; p value 0.4). Image quantification was done using ImageJ software.

BDNF and NT4 activate TrkB receptor with similar kinetics

Ligand binding to Trk receptors leads to receptor activation and cells use phosphorylation as a mechanism to modulate trafficking of RTKs. Previous studies have highlighted the importance of phosphorylation in modulation of receptor sorting. An EGFR mutated in the kinase domain, is endocytosed upon ligand binding with similar kinetics to the wild type receptor, however, rather than being sorted to the degradative pathway, a significant portion recycles back to the cell surface [193]. We asked the question if BDNF and NT4 differentially induce TrkB phosphorylation while at the early endosome compartment. Previous studies comparing TrkB activation with BDNF and NT4 used cell lines transfected with the receptor [169]. However studying ectopic expressed RTKs poses some limitations since

overexpression itself can promote dimerization and activation in a ligand independent-manner. Moreover, endogenous molecular players that could be differentially recruited by one of the ligands in neurons may not be expressed in these cell lines. Therefore, we decided to analyze phosphorylation of endogenous TrkB in rat cortical cultures. Neurons were treated for 15 min with the respective neutrotophin. An initial immunoprecipitation step with a TrkB-specific antibody was performed and phosphorylation was assessed by phosphotyrosine western blot. At this time point, when roughly 75% of TrkB receptor has been internalized (figure 1d), BDNF and NT4 led to similar activation of TrkB receptor (ratio of BDNF induced phosphorylation 1.4 ± 0.09; NT4 ratio 1.3 ± 0.01; p value = 0.24, figure 2 a and b). Even though this data strongly suggests that phosphorylation of TrkB by BDNF and NT4 is not different, it did not exclude the possibility that these two neuretrophins activate TrkB with different kinetics. Thus, we performed a kinetic study of TrkB phosphorylation at different time points following neurotrophin stimulation (1, 2.5, 5, 7 and 10 min). As shown in figure 2 c, BDNF and NT4 efficiently activate TrkB receptor with similar kinetics, further supporting the hypothesis that activation of TrkB by these two neurotrophins is similar (including endocytosis, phosphorylation and kinetics).

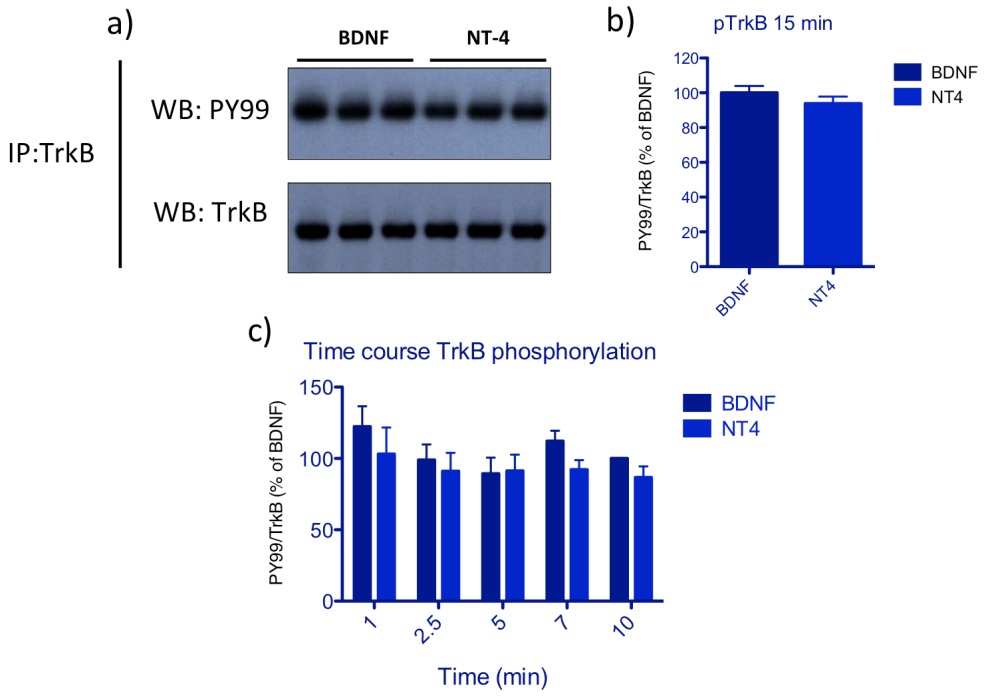


Figure 2: BDNF and NT4 induce similar phosphorylation of TrkB receptor. DIV5 rat cortical neurons, were treated for 15 min with 50ng/ml of BDNF or NT4. Neurons were subsequently lysed and TrkB was immunoprecipitated with a specific antibody. A phosphotyrosine antibody (PY99) was used to measure TrkB phosphorylation. **a)** Representative western blot with PY99 antibody of immunoprecipiated TrkB. Phosphorylation levels were normalized to total TrkB levels and relative quantification to BDNF is shown in **b)** (BDNF 94.6 ± 3.9%; NT4 ratio 90.3 ± 3.9%; p value = 0.42). **c)** Time course phosphorylation of TrkB receptor (2 way ANOVA 0.27). Quantification was done using ImageJ software and it represents an average of three independent experiments.

To gain insight into the signaling cascades activated by NT4 we decided to employ a functional proteomic approach for proteins that become tyrosine phosphorylated upon NT4 or BDNF treatment. We used stable isotope labeling with amino acids (SILAC) coupled to mass spectrometry. This technique has been shown to be a highly effective means for characterization of protein-protein interactions

and cellular signaling [194]. To achieve maximum isotope incorporation, rat cortical and hippocampal neurons were grown for 10 days in neurotrophin of interest, lysed, and a phosphotyrosine antibody was used to culture. At this time point neurons were treated for 15 minute with the precipitate protein complexes that become tyrosine phosphorylated upon neurotrophin treatment. We compared proteins recruited upon NT4 or BDNF treatment or untreated control.

Signaling proteins known to interact with BDNF-activated TrkB receptor, such as Shc and PLC-γ, were pulled down with significantly higher ratios in cells treated with BDNF than in the untreated control, validating this approach. Interestingly the intracellular signaling events initiated by BDNF and NT4 were very similar. We observed that proteins such as Shp2, PLC-γ, Grb2 and Shc, known to mediate downstream signaling cascades of Trks, were immunoprecipiated with a similar ratio in the BDNF and NT4 condition, showing that NT4 activates and elicits downstream signaling as efficiently as BDNF (Table 1).

Table 1: Summary of proteins recruited by BDNF and NT4

Protein	NT4	SD	BDNF	SD
	Weighted mean ratio		Weighted mean ratio	
N-Shc	9.90	7.07	11.85	6.76
TrkB	7.99	3.27	10.46	3.79
Hrs	4.08	1.33	6.43	1.39
PLCγ	4.53	2.51	5.31	1.84
STAM1	2.63	0.63	5.50	1.62
Shp2	4.17	2.99	5.11	3.50
MAPK 1/2	4.75	1.26	5.00	1.05
p44MAPK	4.57	1.29	4.98	1.18
ARMS	3.03	0.00	2.89	0.00
Grb2	2.25	0.33	2.75	0.28

Phosphotyrosin immunoprecipiation of cortical neurons stimulated for 15 min with BDNF or NT4. Only proteins with mass spectrometry ratios higher than the control are shown.

To further confirm recruitment of specific targets, some proteins were selected for western blot analysis. Neurons treated for 15 min with BDNF or NT4 were lysed and TrkB receptor was immunoprecipiated with a TrkB-specific antibody. In agreement with the results obtained in the mass spectrometry experiments, we observed that BDNF and NT4 efficiently recruit the adaptor protein Grb2, known to mediate Ras/MAPK as well as PIP_2 signaling (Figure 3 a and b). The other selected target was Shp2. Shp2 is a SH2 containing tyrosine phosphatase that promotes the activation of the Ras/MAPK signaling pathway. Shp2 becomes phosphorylated and is recruited to Trk receptor upon neurotrophin treatment. SHP2 also acts as a scaffolding protein and recruits Grb2 and PI3K [47]. We examined Shp2 activation by measuring its phosphorylation and also the recruitment of Grb2 elicited by BDNF or NT4. Both neurotrophins induced activation of Shp2 (BDNF ratio 0.8 ± 0.2; NT4 ratio

0.76 ± 0.25; p value = 0.75) and Grb2 recruitment (BDNF ratio 1.17 ± 0.40; NT4 ratio 1.64 ± 0.54; p value = 0.21). (Figure 3 c, d and e) with similar efficiency. These data further support the idea that the difference on biological activity between BDNF and NT4 is not associated with different signaling elicited upon neurotrophin binding to TrkB.

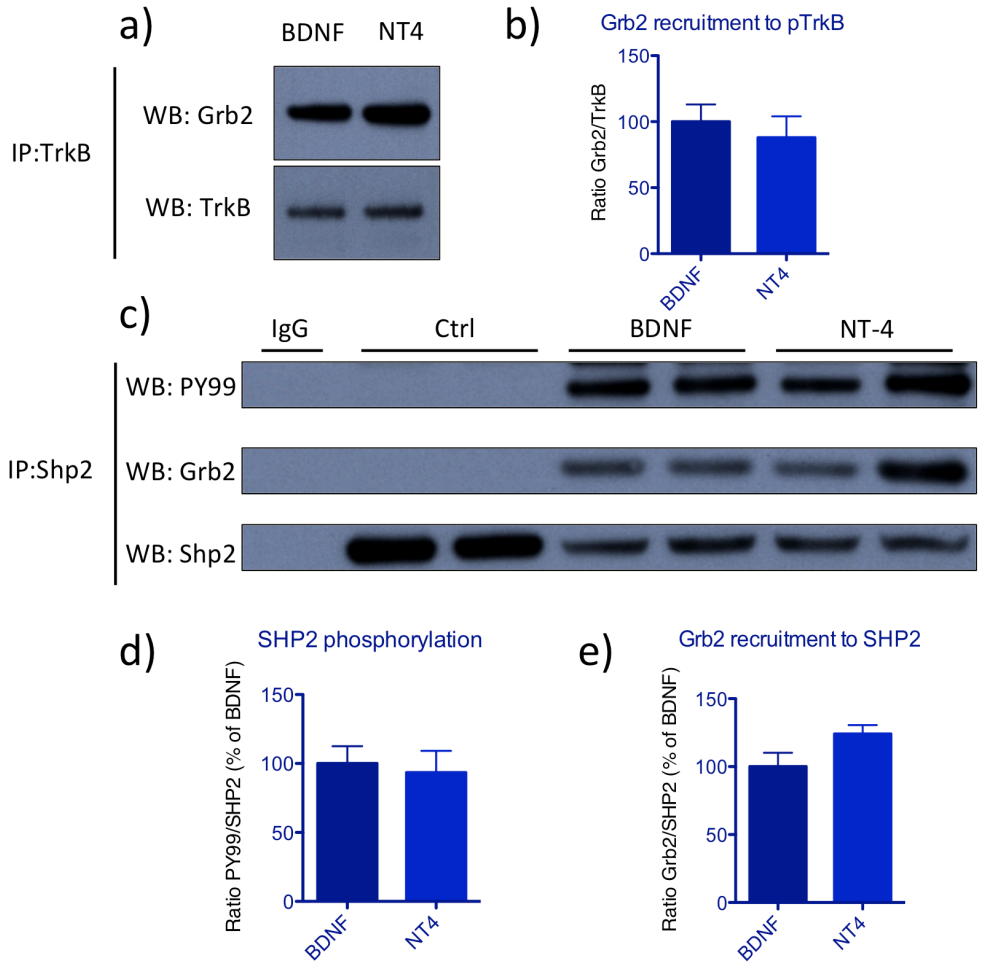


Figure 3: Short-term signaling mediated by BDNF and NT4 is similar. a) Grb2 recruitment to activated TrkB receptor. Cortical neurons were treated for 15 min with 50ng/ml with BDNF or NT4. TrkB receptor was immunoprecipitated and Grb2 recruitment was analyzed by western blot. b)

Quantification of an average of 3 experiments show no statistical difference between BDNF and NT4 (BDNF 100 ± 12.7% NT4 87.94 ± 14.6%, p = 0.63). c) Activation of Shp2 and recruitment of Grb2 to Shp2. At the same time point, Shp2 was immunoprecipitated and phosphorylation was analyzed by PY99 western blot, recruitment of Grb2 with Grb2 antibody. d) and e) represents quantification of an average of 3 independent experiments (phoshoShp2 BDNF 100 ± 10.0%, NT4 93.4 ± 14.6%, p = 0.79; Grb2 recruitment BDNF 100 ± 9.0%, NT4 124.1 ± 5.0%, p = 0.29).

BDNF leads to more efficient ubiquitination of TrkB receptor

Some RTKs require phosphorylation to recruit specific E3 ubiquitin ligases that bind to phosphorylated tyrosines in the receptor. Phosphorylation of EGFR recruits and transphosphorylates the E3 ligase Cbl, that in turn leads to receptor ubiquitination [195]. Ubiquitinated receptors are recruited by a complex of proteins (the ESCRT complex) to the late endosome and degraded in the lysosome [196]. In the case of TrkA, the E3 ligase Nedd4-2 binds specifically to the unphosphorylated form of the receptor, leading to its ubiquitination and targeting to the degradative pathway. Considering that TrkB is internalized and activated by BDNF and NT4 in a similar fashion we expected that ubiquitination would follow a similar pattern. To investigate the kinetics of ligand induced TrkB ubiquitination, cultured cortical neurons were treated for different times with neurotrophin, TrkB was immunoprecipiated using a TrkB-specific antibody and ubiquitination was analyzed by western blot with an ubiquitin antibody (P4D1). We observed that ubiquitination of the TrkB receptor increases with time after neurotrophins treatment. Interestingly, BDNF induces more efficient ubiquitination of TrkB receptor than NT4, with a faster kinetics (figure 4 a and b). As early as one minute after neurotrophin treatment, TrkB ubiquitination induced by BDNF is significantly higher than that induced by NT4. This effect is maintained for up to 10 minutes; however, at a 15 min time point, this difference is no longer significant, suggesting that NT4-induced receptor ubiquitination occurs at a slower pace than that induced by BDNF, reaching both a peak at 15 min. Considering the role of ubiquitination in sorting cargo to the

degradative pathway, we questioned if neurotrophin-mediated degradation of TrkB was differently triggered by BDNF or NT4.

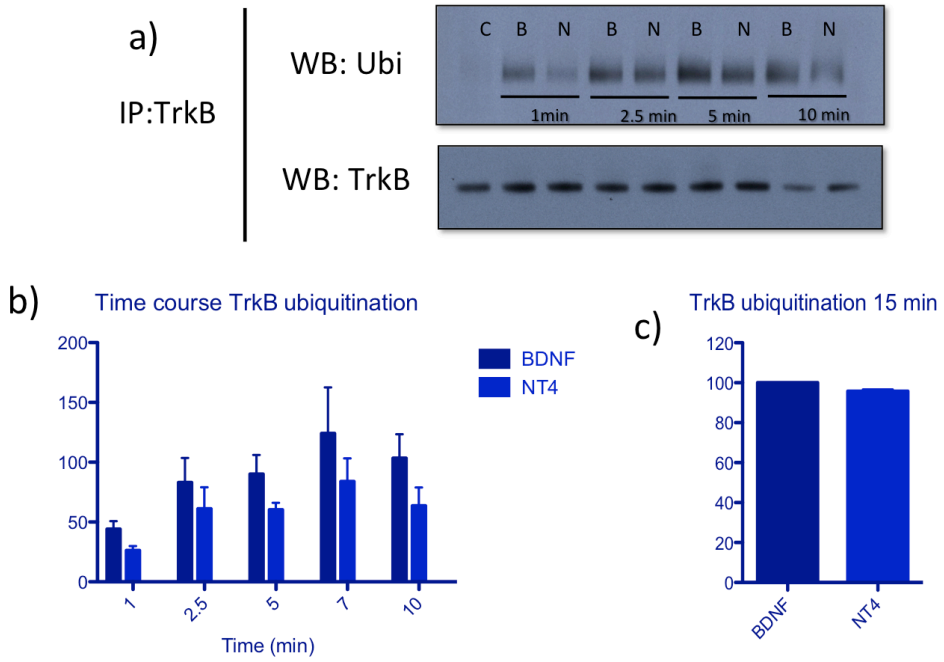


Figure 4. Time course ubiquitination of TrkB receptor mediated by BDNF and NT4. a) Cortical neurons were treated for different times with 50ng/ml of BDNF, NT4 or left untreated. TrkB receptor was immunoprecipitated and ubiquitination was analyzed by western blot with an ubiquitin antibody (P4D1). b) Quantification of four independent experiments. A 2 way ANOVA statistical analysis revealed a significant difference across time and with treatment ($p= 0.004$). c) Quantification of TrkB ubiquitination at 15 minute time point ($p = 0.7$).

BDNF targets TrkB receptor efficiently to the degradative pathway whereas NT4 leads to sustained TrkB activation and decreased degradation

It was previously shown that BDNF leads to effective downregulation of TrkB receptor in cultured cerebellar granule neurons; however, it is still unknown if NT4 can lead to effective TrkB degradation [197]. Considering that TrkB receptor is ubiquitinated with a faster kinetics in neurons treated with BDNF than those treated with NT4, we hypothesized that sorting to the degradative pathway would be more efficient after BDNF treatment. To test this, the time course of BDNF and NT4-induced TrkB downregulation was analyzed using a surface biotinylation degradation assay. In this assay, cultured cortical neurons are surface biotinylated with membrane impermeable sulfo-NHS-S-biotin that labels only membrane proteins. Following biotinylation, neurons were returned to 37°C and were treated for different times with BDNF or NT4, or with vehicle. At the end of treatment, cells were lysed and the initially biotinylated receptors are pulled down with avidin sepharose. Finally, western blot analysis with TrkB antibody allowed quantification of remaining non-degraded receptors, since degradation obviously leads to loss of the epitope (figure 5). Exposure of neurons to 50 ng/ml of BDNF or NT4 for one hour reduced the TrkB protein levels to 80%. After 4 hours of BDNF treatment, TrkB protein levels were reduced to 30% of controls. At this time point, cells treated with NT4 only had a reduction of TrkB levels to 60% of those seen in controls. This difference was more evident at a 5-hour treatment when BDNF treatment led to a reduction to less than 20% of controls, whereas in cells treated with NT4 almost half of receptors were still available (reduction to 40%).

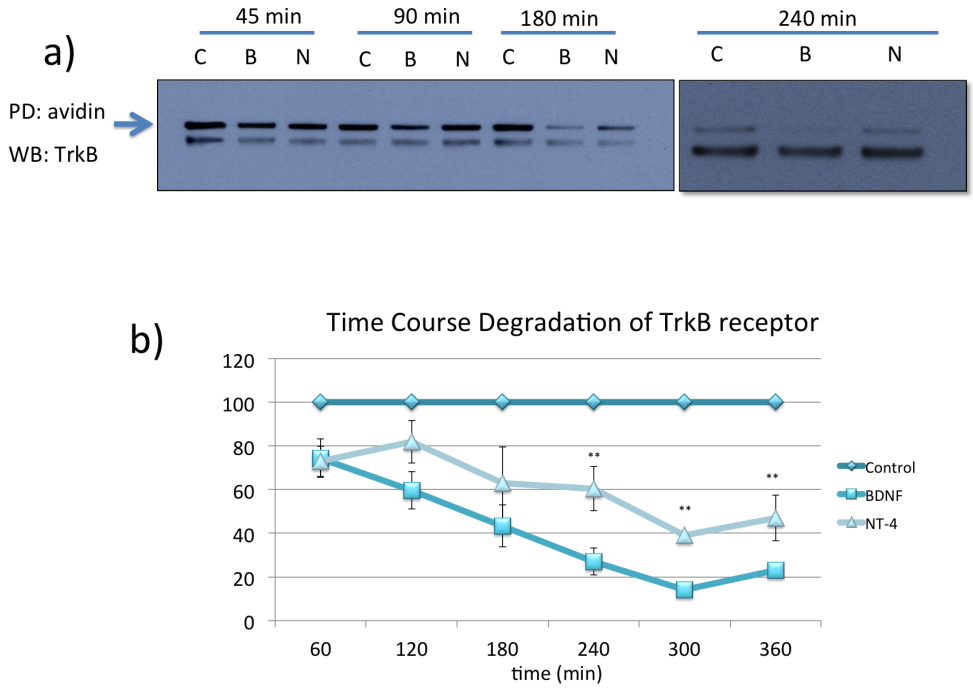


Figure 5. Time course degradation of TrkB receptor mediated by BDNF and NT4. Cortical neurons were surface biotinylated with a cell impermeable sulfo-biotin. Subsequently neurons were incubated for the indicated times with 50 ng/ml of BDNF or NT4. A) Avidin pulled down precipitates were analyzed by western blot with a TrkB antibody. The upper band at 140KDa represents full length TrkB, whereas the lower band likely represents truncated TrkB (TrkB.T1) b) Quantification of TrkB degradation as percentage of the untreated control; represents an average of 5 independent experiments. Asterisks represent a p value ≤ 0.04.

Our data suggests that NT4-induced degradation of TrkB is different than that induced by BDNF. Thus, it is possible that NT4 sorts activated TrkB receptors to a different trafficking destination that BDNF-activated TrkB. Therefore, we sought to investigate the degradation pathway mediated by these two ligands. Previous

studies have suggested that BDNF induced TrkB degradation is proteasomal-dependent [197]. Subsequent studies with TrkA showed that both the proteasome system and the lysosome play a sequence coordinated role in the degradation of this receptor [198]. Extensive studies analyzing RTK downregulation have highlighted the revelance of the lysosome for this pathway [53]. To test if neurotrophin mediated downregulation is lysosomal-dependent we performed the same surface biotinylation degradation assay in the presence of a lysosome inhibitor: leupeptin (100 µg/ml) (figure 6). Given the unstable nature of this inhibitor in aqueous solutions we tested degradation at a 3-4 hour time point. Treatment with 50ng/ml with BDNF alone led to a degradation index of an average of 27.0 ± 3.2% that was significantly different than cortical neurons treated with the same concentration of BDNF but in the presence of leupeptin 68.5 ± 6.4 % (p value = 0.004). In the case of NT4, treatment with 50ng/ml led to a degradation index of 68.9 ± 5.8 %, whereas in the presence of NT4 and leupeptin it was 90.3 ± 1.7% (p value = 0.025). Thus, in both cases leupeptin significantly inhibited neurotrophin-induced degradation, however, in the case of BDNF this effect was much more pronounced. In fact, at such time point, even in the absence of neurotrophins and comparing the untreated group with and without leupeptin, we observed that surface biotinylation alone led to a degradation of 20% of TrkB, as can see by the quantification showing that after 3 hour incubation period, the untreated control had a degradation index of 80.8 ± 4.8%. This most likely represents endogenous protein turnover. Considering that leupeptin only blocked 20% of NT4 mediated degradation whereas it blocked 40% of BDNF, we asked the question if degradation triggered by NT4 is mediated by the proteasome rather than the lysosome. On the other hand, the rather short incubation period analyzed might have occluded an effect on NT4 considering the slow degradation kinetics mediated by this ligand. To clarify this matter we decided to test both proteasomal and additional lysosomal inhibitors at a longer time point (5h). A 5h treatment with BDNF or NT-4 in the presence of leupeptin did not lead to

significant blockage of TrkB degradation, possibly due to the labile nature of this drug in aqueous solutions (Figure 7 a). Thus, we tested additional lysosome inhibitors: ConcanamycinA and Bafilomycin (at the same time point), which impair the acidification of the lysosome by inhibiting the vacuolar proton pump [198,199]. Bafilomycin significantly blocked neurotrophin-induced degradation (BDNF 60% p= 0.01 and NT4 85% of control, p = 0.03). Concanamycin also reduced neurotrophin-mediated degradation however it was non-significant. (Figure 7b)

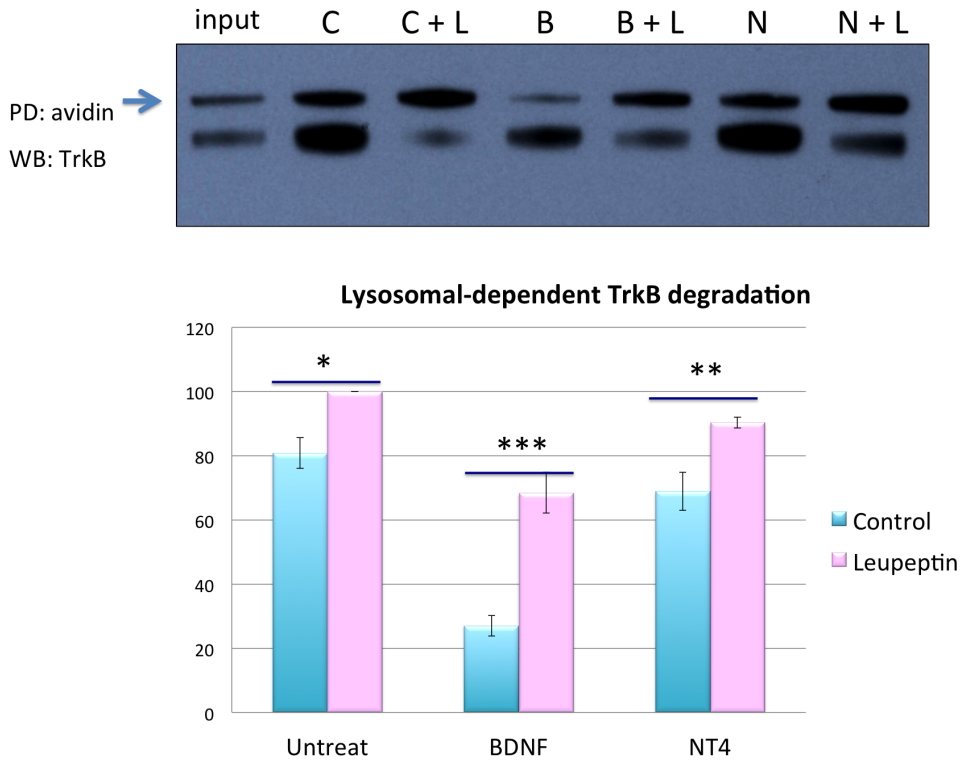
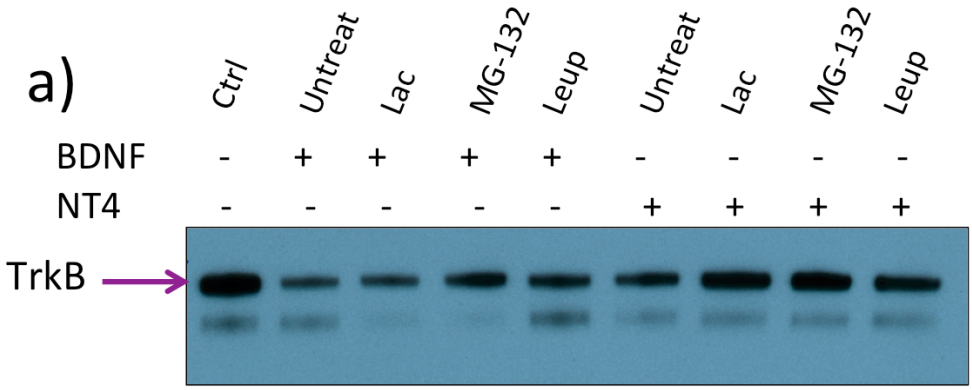


Figure 6. Lysosomal-dependent degradation of TrkB mediated by BDNF or NT4. A surface biotinylation degradation assay was performed with cortical neurons treated for 3-4 hours with 50ng/ml BDNF, NT4 or untreated in the presence (C + L, B + L N + L) or absence of 100 µg/ml leupeptin (C, B, N). a) Representative western blot with TrkB antibody. The upper band has an approximate size of 140KDa represents full length TrkB, whereas the lower band likely represents truncated TrkB (TrkB.T1) b) Quantification of an average of three

independent experiments. 50ng/ml with BDNF alone (B) led to a degradation index of 27.0 ± 3.2%M; BDNF and leupeptin (B + L) 68.5 ± 6.4 % (p value = 0.004). 50ng/ml of NT4 (N) led to a degradation index of 68.9 ± 5.8 %, NT4 and leupeptin (N + L) 90.3 ± 1.7% (p value = 0.025).

In order to test if the proteasome is involved in neurotrophin-mediated degradation of TrkB we tested the previously reported inhibitor lactacystin, which is an irreversible proteasome inhibitor that binds to the catalytic domain of 20S subunit [200]. Lactacystin has been shown to block BDNF-mediated TrkB degradation in cerebellar neurons at a 3h time point, as well as NGF-induced TrkA degradation in PC12 cells at a 2h time point [197,198]. In our studies, lactacystin (8 µM) did not significantly impair BDNF or NT4-induced degradation of TrkB receptor in cortical neurons (Figure 7).

We decided to test a second proteasome inhibitor MG-132 (10 µM) that is a reversible, and cell-permeable proteasome inhibitor, reducing the degradation of ubiquitin-conjugated by the 26S proteasome. Using this inhibitor both BDNF as well as NT4 elicited degradation was significantly blocked (Figure 7a and b). Thus suggesting that these neurotrophins mediate degradation through the same mechanisms and that both the proteasome and lysosome might be implicated in TrkB degradation. However it is still puzzling how does NT4 induce slower TrkB downregulation.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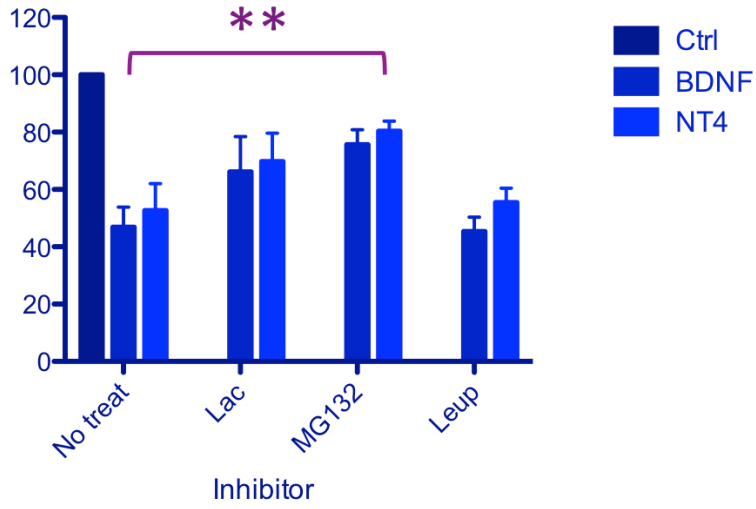
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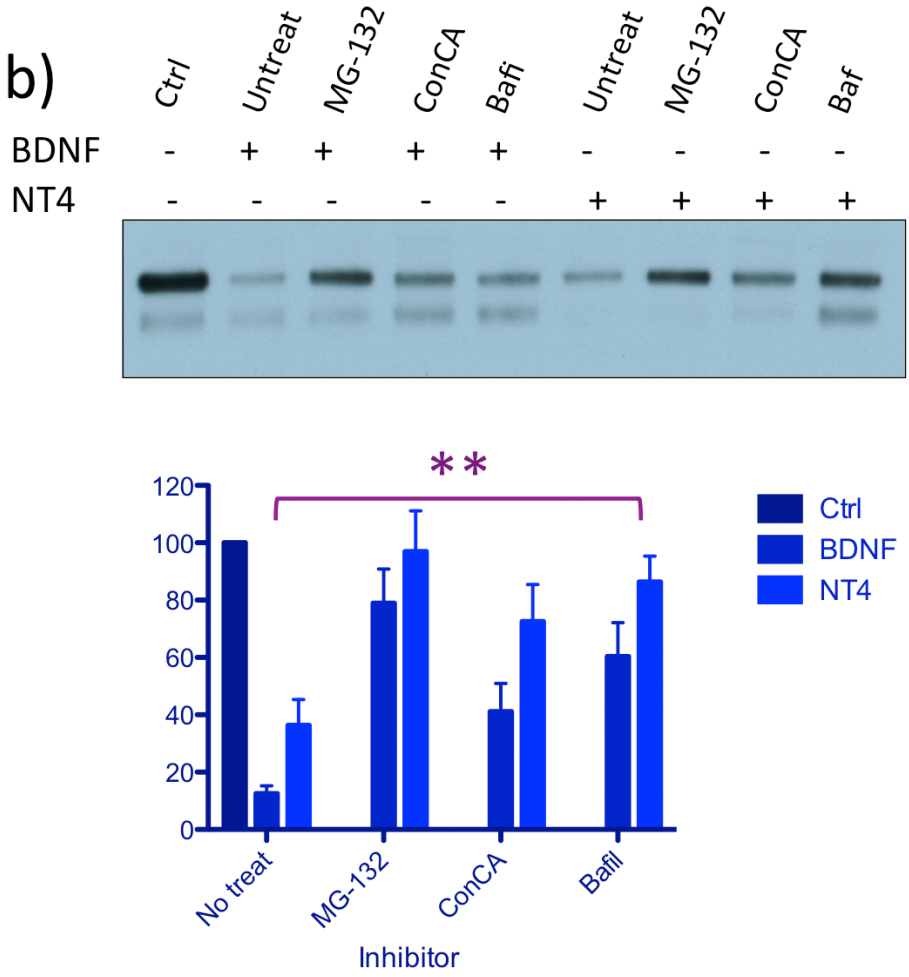


Figure 7: Lysosomal and proteasomal degradation of TrkB receptor triggered by BDNF and NT4. Cultured cortical neurons were surface biotinylated and treated for 5 hours with 50ng/ml of neurotrophin in the presence of absence of the indicated inhibitor. 7 a) Lactacysin (8 µM); MG-132 (10 µM); leupeptin was refreshed every hour (100 µg/µl). 7 b) MG-132 (10 µM); ConcanamycinA (20nM) and Bafilomycin (200nM). Quantification of four independent experiments show a significant treatment of MG-132 (5h BDNF ± MG-132 $p = 0.005$; 5h NT4 ± MG-132 $p = 0.02$) and Bafilomycin (5h BDNF ± Bafilomycin $p = 0.014$; 5h NT4 ± Bafilomycin $p = 0.03$)

Studies on EGFR have shown that ligands (such as TGF-α) that lead to recycling rather than degradation, interact less efficiently with the receptor within the acidic environment of the early endosome, whereas the EGF-EGFR complex remains tightly bound even at lower pHs [201]. Recent studies on TrkA receptor have also shown that NT3 induces less efficient activation of the receptor than NGF at more acidic pHs [202]. Thus we decided to test the efficiency of NT4 mediated TrkB activation and ubiquitination at lower pHs that mimic environment of the early endosome (pH 5.5 and 6.5), at a time point where there is no difference at pH=7 (30 min) (Figure 8). Indeed we observed that BDNF induces more efficient ubiquitination of TrkB receptor at lower pHs, as compared with NT4 that explain the more efficient downregulation of TrkB with BDNF stimulation. Interestingly, phosphorylation was similar at all pHs tested, suggesting that these neurotrophins differ specifically in the mechanisms of TrkB ubiquitination.

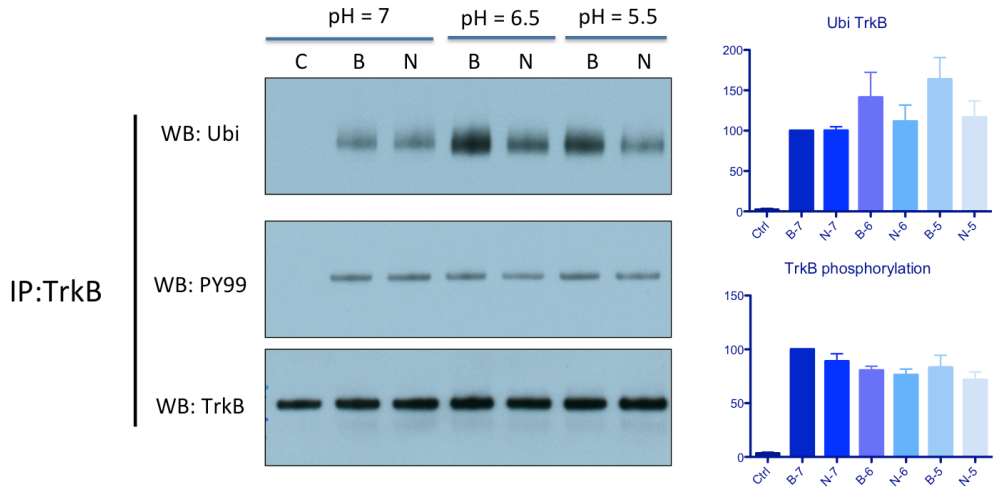
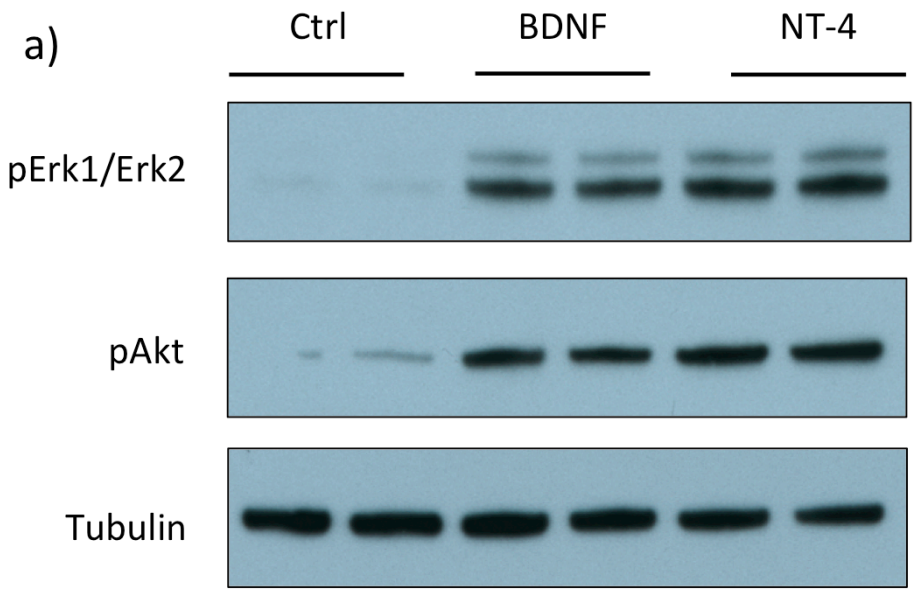


Figure 8. BDNF and NT4 induced TrkB activation at acidic pHs. Cortical neurons were treated with BDNF or NT4 (50ng/ml) for 30 min with medium adjusted at different pHs. TrkB was immunoprecipitated and ubiquitination (P4D1) and phosphorylation (PY99) were

analyzed by western blot. Quantification shows an average of three independent experiments.

Endocytic sorting of RTKs away from the degradative pathway can have a large impact on biological outcomes. Degradation of the receptor leads to signaling attenuation. Studies done with the EGFR have shown that TGFα, which promotes recycling of the receptor rather than degradation, is a more potent mitogen than EGF, which efficiently sorts EGFR to the lysosomal-degradation pathway [57]. Considering that NT4 maintains a stable pool of TrkB receptor even after long sustained treatments, we expected that this ligand would also maintain active downstream signaling cascades during the same time period. To test this, we treated cortical neurons with BDNF or NT4 for five hours and then analyzed the activation of the two major signaling pathways MAPK, through Erk1/Erk2 phosphorylation and Akt (figure 9). We observed that after 5-hour treatment, NT4 led to significantly higher phoshphorylation of Akt and Erk1/Erk2 as compared with BDNF, most likely due to the amount of TrkB receptor that is still available at this time point.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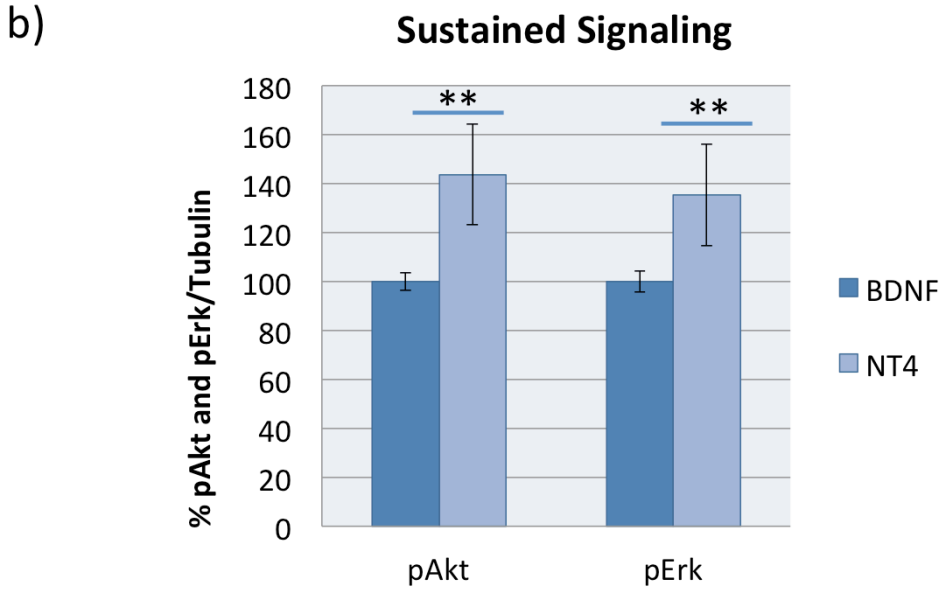
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Figure 9. Sustained signaling mediated by BDNF or NT4. a) Cortical neurons were treated for five hours with 50ng/ml with BDNF, NT4 or left untreated. Lysates were immunobloted for phoshpo Akt (pAkt) and phospho Erk (pErk). b) Quantification represents phosphorylation of Akt and Erk normalized to tubulin (pAkt BDNF = 100 ± 3.6%, NT4 = 143.5 ± 20.5%, p = 0.043; pErk BDNF = 100 ± 4.3%, NT4 = 135.2 ± 20.7%. p = 0.031). Average of three independent experiments.

DISCUSSION

The results presented here reveal for the first time that differential sorting of endocytosed TrkB receptor, in cortical neurons, can be achieved by treatment with two different neurotrophins: BDNF and NT4. BDNF and NT4 induce similar TrkB endocytosis measured biochemically in 293 cells stably expressing TrkB receptor. In cortical neurons TrkB endocytosis was measured by immunocytochemistry using co-localization with an early endosome marker (EEA1) and also by assessing the ratio of internalized-surface receptor. Moreover, we observed that initial phosphorylation of TrkB and recruitment of adaptor signaling proteins, such as Grb2 and Shp2, which mediate TrkB downstream signaling, was similarly triggered by these two ligands.

Differences between BDNF and NT4-mediated TrkB signaling have previously been shown for Shc mediated MAPK activation [203]. In this report, a mouse line was created with a point mutation in the NPQY site of TrkB in which Y515, which is in the consensus site for Shc recruitment, was replaced by a phenylalanine [2]. Analysis of viability of several sensory neuron populations revealed a selective death of NT4-dependent neurons, whereas BDNF supported populations were mostly viable. This correlated with a reduced ability of NT4 to activate MAPK signaling in cortical neurons derived form the mutant mouse [203]. Our own mass spectrometry data in which we stimulated cortical neurons with BDNF or NT4 and immunoprecipitated proteins with a phosphotyrosine antibody revealed similar activation and/or recruitment of the protein Shc (Table 1 of). Our studies do not distinguish between Shc recruitment to TrkB or Shc tyrosine-phosphorylation so it is possible that NT4 leads to more efficient Shc interaction with TrkB receptor, however, BDNF is equally capable of activating this protein.

Interestingly, and despite the similarities in the initial endocytic steps, we observed that BDNF induces faster and more efficient ubiquitination of TrkB. Considering the central role ubiquitination plays in sorting membrane receptors to the degradative pathway we hypothesized that more efficient ubiquitination would

be coupled to more efficient ligand-induced receptor degradation. The time course of BDNF and NT4-induced TrkB downregulation was analyzed. Exposure of cortical neurons to BDNF for 2-3 hours reduced the surface pool of TrkB to half, whereas a longer treatment (4-5 hours) with NT4 was necessary to achieve a similar downregulation. Previous studies analyzing BDNF induced TrkB degradation in cerebellar neurons showed a $T_{1/2}$ of 1-2 hours [197]. The differences in the degradation kinetics can be due to the cell types used (cortical vs cerebellar neurons), and also to the use of slightly different technical methods. In all, prolonged BDNF treatment leads to fast downregulation of TrkB. However, as shown here, even after prolonged activation with NT4, TrkB receptor was still stable.

We then asked the question if the mechanisms mediating TrkB degradation were similar with BDNF or NT4 activation. To test this we performed the same degradation assay, in the presence or absence of a lysosomal inhibitor, leupeptin. Interestingly leupeptin was much more efficient in blocking BDNF induced degradation than NT4 induced degradation. One possible explanation for this difference might rely on the time point analyzed, at which BDNF induces significantly higher degradation than NT4 (27% vs 68%), therefore, the degree of leupeptin-induced inhibition might be masked in the case of NT4. On the other hand, it is possible that NT4 might sort TrkB to different degradation pathways such as the proteasome. Indeed, previous studies in cerebellar granular neurons have suggested that TrkB follows a proteasomal dependent degradation pathway [197]. Subsequent studies with TrkA showed that both the proteasome system and the lysosome play a sequence coordinated role in the degradation of this receptor [198]. In order to better understand the mechanisms of neurotrophin-mediated TrkB degradation, we analyzed additional lysosomal as well as proteasomal inhibitors. Contrary to previous work on TrkB, we did not observe a significant blockage of neurotrophin-induced degradation with lactacystin, however, MG-132 had the strongest effect in reverting TrkB degradation [197]. We also observed that the lsysosomal inhibitor Bafilomycin

significantly blocked neurotrophin-mediated TrkB degradation. It is interesting that lacatcystin, a strong and irreversible inhibitor that binds the β-catalytic domain of the 20S subunit of the proteasome, failed to block TrkB degradation. In addition, this inhibitor has not been shown to block other proteases [197]. On the other hand, considering that peptide aldehydes, such as MG-132, might also inhibit certain lysosomal cysteine proteases and calpains, its possible that this inhibitor may be blocking the ubiquitin-dependent lysosomal degradation of the receptor [204]. All together, these results suggest that BDNF and NT4 sort TrkB receptor to the same degradation pathway however with different kinetics.

Even though NT4 is widely expressed in the brain, little work has been done on NT4 mediated TrkB activation and signaling. In large this is mainly due to the mild phenotype of the NT4 null mouse in comparison with the BDNF null, that has severe developmental defects and is lethal [176]. However, it is interesting that the complete TrkB null mouse shows neuronal death in the hippocampus whereas the BDNF null does not, suggesting the need for a second ligand, perhaps NT4 [33,205]. The lack of major developmental abnormalities in the CNS of NT4 null mice is most likely due to compensation by BDNF, given the overlapping expression of these two neurotrophins and the levels at which they are expressed. Research groups employing an RNA protection assay to quantify NT4 in the adult brain, estimated that in the rat there is approximately 3 ng of NT4 RNA per g of tissue [29]. Studies comparing the levels of both neurotrophins in the rat brain with quantitative PCR suggest that the levels of NT4 are half of BDNF in the adult [28]. NT4 protein levels have not been measured in detail, on the other hand, ELISA analysis of BDNF in the mouse brain, suggests that this neurotrophin is present at 200 ng per g of tissue [27]. In addition BDNF is released in an activity-dependent manner whereas NT4 is sorted to the constitutive secreted pathway, suggesting that the levels of BDNF can be unregulated in the absence of NT4 [182]. Perhaps due to the scarceness of NT4, the system evolved in a way that it became more sensitive to this neurotrophin, enabling

long lasting effects on its receptor. There are several examples in the literature suggesting that NT4 is a more potent trophic factor than BDNF. Replacement of BDNF with NT4 in the BDNF locus not only rescued the major abnormalities of the BDNF null mouse but also supported the survival of more vestibular neurons than in wt mice. In addition, both synaptic maturation and function were enhanced [183]. [183]. A similar effect was observed on the survival of nodose-petrosal neurons, whereas a single NT4 allele is sufficient to support survival of these cells, while two functional BDNF alleles were necessary to achieve the same effect [176]. Our data showing that NT4 activation of TrkB does not lead to massive down-regulation of the receptor thus ensuring sustained signaling, presents the first molecular evidence that could explain those findings. Moreover, we show that prolonged NT4 treatment leads to significantly higher sustained phosphorylation of Akt and MAPK pathway than BDNF treatment, supporting the idea that NT4 is a more potent neurotrophin.

Activation of TrkA by NGF and NT3 is the other example in the neurotrophin family of two ligands activating the same receptor and mediate ligand-dependent neuronal survival. Therefore it is plausible to speculate that activation of TrkB by BDNF or NT4 functions in a similar way. NGF and NT3 act synergistically to support the survival of sympathetic neurons in a TrkA dependent manner. However, they do so in a mechanistically distinct fashion [65]. NT3 is expressed at higher levels in intermediate targets whereas NGF is an established final target derived survival factor [30]. Studies employing compartmentalized sensory neuronal cultures showed that treatment of distal axons with NGF leads to robust phosphorylation of TrkA, and its effectors Akt and Erk. Moreover, a fraction of endosomes containing activated TrkA is retrograde transported resulting in accumulation of the same signaling proteins in the soma [30,206]. In contrast, NT3 treatment of distal axons did not elicit somatic accumulation of phosphorylated proteins and rather showed a selective local effect [30,202]. One could speculate that NT4 activates TrkB in a similar way as NT3 activates TrkA. However, there are striking differences that

distinguish these two systems. The most obvious one is their distinct spatial expression with TrkB, BDNF and NT4 being strongly and widely expressed in the CNS, whereas TrkA and NGF are mostly confined to the periphery [26]. It is thought that neurotrophins are not necessary for cell survival in the CNS and in fact, long-range retrograde movements of activated TrkB receptor has not been observed in these cellular systems. Initial studies showing cell death in the CNS in TrkB null mice, are confounded by massive neuronal death in sensory system [26]. Therefore, it is reasonable to speculate that both BDNF and NT4 lead to confined TrkB mediated signaling, which is consistent with the compact structure of the CNS. Secondly, contrary to BDNF and NT4 that bind TrkB with the similar affinity, NT3 has a much lower affinity for TrkA, and studies comparing these two ligands usually employ amounts 10 fold higher for NT3 than for NGF [168,207]. Lastly, it has been shown that NGF activated TrkA is predominantly trafficked to recycling endosomes whereas BDNF activated TrkB is efficiently sorted to the degradative pathway [63,206]. It is interesting to hypothesize that the NT4-TrkB signaling complex is trafficked in a similar manner as NGF-TrkA whereas, instead of promoting degradation, it would sort the receptor to a signaling endosome that could be retrogradely trafficked and sustain long term signaling. Moreover, experiments showing short-range movements of activated TrkB receptor in cortical neurons (Deinhardt K, Chao M, personal communication) were only done in the presence of BDNF and not NT4. Therefore, it is possible that NT4 induces long-range retrograde movements of TrkB. In order to ultimately answer this question, the use of compartmentalized cultures where the axonal and somatodendritic compartment are separated, would have to be employed. Unfortunately, for CNS neurons these are particularly challenging from a technical standpoint.

Our studies show that minor differences in the initial activation and trafficking steps of TrkB receptor are critical for long-term signaling. We were still puzzled about how NT4, that binds TrkB with similar affinity as BDNF, might sort this

receptor do alternate trafficking destinations that are less efficiently coupled to degradation. Our data shows that BDNF induces more efficient ubiquitination of TrkB receptor however; we did not observe differences in endocytosis rates, suggesting that upon internalization the receptor is initially sorted to a similar compartment (as suggested by co-localization with EEA1). Ligand-mediated receptor ubiquitination and sorting to the degradative pathway is a common mechanism cells use to regulate RTK signaling. The capacity of BDNF to induce faster ubiquitination of TrkB might be due to its ability to bind tightly to the receptor, even at the mildly acidic pH of the early endosome, whereas NT4 binding may become unstable in this compartment. This was suggested by the surface plasmogen studies where the binding affinity of both BDNF and NT4 was measured. In this report, the acidic wash of the receptor showed that TrkB uncoupled from NT4 much more efficiently than it did from BDNF [168]. Efficient ligand/receptor uncoupling has been associated with increased EGFR recycling and decreased degradation [57]. EGF, TGFα and E4T are all ligands for EGFR capable of inducing similar phosphorylation and internalization of the receptor, however only EGF induces efficient recruitment of the E3 ligase Cbl, ubiquitination and degradation of EGFR. In contrast, TGFα and E4T triggered recycling of EGFR and inefficient ubiquitination [57,201]. The EGF-EGFR complex is resistant to the mildly acidic pH of early and late endosomes and remains intact along the endocytic route and this was critical to ensure degradation of EGFR, whereas both TGFα and E4T binding to EGFR are unstable at the early endosome [57]. Interestingly studies have shown that TGFα is a more potent mitogen than EGF, stressing how critical modulation of endocytic trafficking is to achieve specific biological outcomes [57,201]. Considering the efficient uncoupling of NT4 from TrkB upon exposure to an acidic solution suggests that NT4 might behave as TGFα, and would uncouple from TrkB faster at the early endocytic compartment leading to more efficient recycling and consequently less degradation and sustained signaling.

Based on these previous studies, we postulated that the NT4-TrkB interaction is more labile within the acidic environment of the endosome, whereas BDNF remains bound to TrkB even at lower pHs. Indeed we observed that at acidic pHs BDNF induced more efficient ubiquitination of TrkB receptor than NT4 but phosphorylation was similar. The differential ubiquitination might promote a configuration that sorts the receptor either to an alternate compartment or a different microenvironment within the early endosome that might facilitate the recruitment of an E3 ligase (still unidentified) and is more efficiently coupled with the degradation pathway.

In all these studies reinforce the importance of addressing both neurotrophins that activate TrkB and how mild differences in initial trafficking events are reflected in opposing outcomes. Mutations in the NT4 gene have recently been associated with development of glaucoma in humans [208]. TrkB phosphorylation induced by NT4 carrying these mutations was reduced, as compared to wild type NT4, drawing attention to the relevance of precisely regulating receptor activation and signaling and understanding the mechanisms of TrkB activation mediated specifically NT4 and not only BDNF [208].

Chapter 3: Slitrk5 as a new key player for CNS function

AUTHOR'S CONTRIBUTION

For Chapter 3, the results published in the following article were used.

Shmelkov SV*, Hormigo A*, Jing D*, **Proenca CC***, Bath KG, Milde T, Shmelkov E, Kushner JS, Baljevic M, Dincheva I, Murphy AJ, Valenzuela DM, Gale NW, Yancopoulos GD, Ninan I, Lee FS, Rafii S. *Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive-like behaviors in mice*. Nat Med. 2010 May;16(5):598-602.

[*] These authors contributed equally to this work.

Different researchers have contributed for this manuscript. Specifically all the electrophysiological experiments were done in collaboration with Ninan Ipe; the initial behavioral analysis (grooming, rotarod and elevated plus maze) were performed in collaboration with Kevin Bath, Iva Dincheva, Adilia Hormiongo and Sergey Shmelkov; the Golgi staining was performed by Denqian Jing and the transgenic mouse was generated by Regeneron. All the other experiments were performed by the author Cátia Proença.

INTRODUCTION

The Slitrk gene family was discovered in a screen for proteins that had altered expression in mice with neural tube defects [8]. This family comprises six members that were named Slitrk1 through Slitrk6 and they are primarily expressed in the brain. Given their expression at early developmental stages as well as high and broad expression in the CNS, efforts have been made in trying to understand the main functions of these proteins.

Several human and mouse genetic studies have associated Slitrk genes with psychiatric disorders [9]. The Slitrk1 gene was the first member in the family to be associated with a disorder, when mutations in this gene were found in patients with Tourrete's Syndrome (TS) [113]. Later studies found mutations in Slitrk1 in individuals with Trichotillomania (TTM) [114]. Given their common behavioral characteristics these disorders fall into the broad spectrum of obsessive-compulsive disorder (OCD) [130,142,209]. The Slitrk2 gene was the second member in the family to be found associated with a psychiatric disorder. A study performing systematic re-sequencing of X-chromosome genes found two novel missense variants in Slitrk2 in patients with schizophrenia and in their affected siblings [115].

The Slitrk1 null mouse was developed in order to provide a suitable model to study TS [143]. This mouse developed anxiety and depressive-like behaviors as well as increased norepinephrine levels in the brain, that is consistent with the pathophysiology of TS [144]. However, they did not develop behavioral characteristics that resemble the hallmarks of TS, such as tics or repetitive movements [143].

Evidences are mounting to indicate an involvement of the Slitrk gene family with neuropsychiatric disorders; however, the underlying molecular mechanisms remain are unknown. Some studies suggested that Slitrks can modulate neurite outgrowth and one report has showed that Slitrk2 has synaptogenic activity [9]. The goal of this thesis was to contribute to a better understanding of the functions these

proteins play in the CNS. We focused on the Slitrk5 member given its wide distribution and strong expression in the brain and we hypothesize that this protein would be involved in OCD-spectrum disorders. In order to investigate the function of this protein and to delineate the expression pattern of the Slitrk5 gene in mouse tissues, we generated a knockout/knockin mouse by replacing the Slitrk5 gene with a reporter gene.

METHODS

Reagents

The following antibodies were used: FLAG rabbit (F255); mouse M1 and M2, mouse Tubulin, HA (rabbit and mouse H3663, H6908) were all from Sigma. Actin (sc-1616) from Santa Cruz; GluA1 mouse (MAB2263) and rabbit (AB1504); GluA2 mouse (MAB397) and rabbit (AB1768), VGlut1 guinea pig (AB5905) were from Millipore. PhosphoErk (#9101S), phosphoAkt (#4060S), Akt (9272) were from Cell Signaling. GluA2/3 rabbit (ab53086), MAP2 chicken (AB5392), MAP2 rabbit (AB32454), PSD95 rabbit (ab18258) were from Abcam. Tau mouse (MAB3420), Synapsin rabbit (AB1543) from Chemicon. C-Myc (mouse sc-40), Erk1 (sc-93), Erk2 (sc-154) was from Santa Cruz; GFP from Invitrogen, and finally Transferrin receptor mouse (13-6800) from Zymed.

Animals.

Male C57BL6/J mice were used for all behavioral experiments. Slitrk5 heterozygous mice were crossed and litters were weaned at P21. After genotype male littermates heterozygous, knockout and wild type were used for behavioral testing. Mice were housed a maximum of five per cage in a temperature- and humidity-controlled vivarium maintained on a 12-h light/dark cycle. Mice had ad libitum access to food and water. All procedures regarding animal care and

treatment were in compliance with guidelines established by Weill Cornell Medical College's Institutional Animal Care and Use Committee and the National Institutes of Health.

Generation of the *Slitrk5* knockout *lacZ* knock-in mice and determination of *Slitrk5* expression pattern

The *Slitrk5* encoding region was replaced with *TM-lacZ* inserted at amino acid 47 of *Slitrk5* after the initiator methionine (amino acid 7 after the signal sequence cleavage site). Velocigene Allele Identification Number: VG737. Mice were generated on C57BL/6 and SV129 mixed background and subsequently backcrossed to C57BL/6 background for five generations. Genotyping: forward primer – 5'-GACCCCCTTCCGTCTACAC-3', reverse primer – 5'- TGGACAAAGTTCCTGCTTGGATAC-3' and the probe – 5'-CTCGTCCAAATCCC-3' for wild type; forward primer – 5'-GGGCGCCCGGTTCTT-3', reverse primer – 5'- CCTCGTCCTGCAGTTCATTCA-3' and the probe – 5'-ACCTGTCCGGTGCCC-3' for the knockout. Expression pattern of *Slitrk5* was determined by the detection of β-galactosidase activity in mouse tissues. Fresh-frozen tissues were sectioned and subsequently incubated for 4- 16 hours with X-gal (1 mg/ml, Calbiochem), then counterstained with Nuclear Fast Red (VectorLabs).

Fluoxetine treatment

Fluoxetine was dissolved in tap water and delivered ad libitum in the drinking water (tap) in glass bottles, and daily water intake was measured for the first 7 days. An intake of 2.0-2.5 ml per day was determined, similar to standard tap water intake controls. Fluoxetine mixtures were changed every 48h to insure delivery of fresh drug. A dose of 18 mg/kg per day fluoxetine was given for 21 days, which corresponded to 160 mg/L and was based on prior fluoxetine dosing regimens in C57BL/6 mice.

Cell culture

All reagents used to prepare primary neuronal cultures were purchased from Invitrogen, except glucose that was from Sigma. The striatum, cortex or hippocampus, were dissected from E18 Sprague Dawley rat or E16 C57BL6 (WT, Het or KO) mouse embryos in Hanks Balanced solution (HBSS) supplemented with 0.37% glucose. Digestion was performed in the same medium supplemented with 0.05% trypsin for 10-15 minutes at 37°C. Neurons were mechanically dissociated with fire-polished Pasteur pipettes and plated in plating medium (PM) (MEM containing 10% FBS, 1 mM pyruvate, 0.37% glucose, and 0.1 mg/ml of Primocin from Invivogen) for 24 hours. After one day in culture, PM was replaced by Neurobasal supplemented with B-27, 0.5mM glutamine, 0.1 mg/ml Primocin and 2µM Ara-C (Cytosine β-D-arabinofuranoside hydrochloride). Cells were grown on 1mg/ml poly-D lysine-coated surfaces: for immunocytochemistry a density of $15x10^3$ cells/cm^2 was; for biochemistry, polystyrene dishes were used at a density of $76x10^3$ cells/cm^2. Neurons were kept in a humidified incubator at 37°C and 5% CO2. COS7 and HEK293 cells were maintained in DMEM (Invitrogen) containing 10% heat inactivated fetal bovine serum (FBS) (GemCell), supplemented with 100 U/ml penicillin, 100 U/ml streptomycin (Pen/Strep) (Invitrogen), and 2 mM glutamine (GlutaMAX from Invitrogen).

Lentiviral vector production and constructs

The third generation lentiviral contructs used in this work were a kind gift of Stefano Rivella lab at Weill Cornell Medical College. Lentiviral particles were produced by transfecting low passage HEK293. Cells were plated on 10 cm dishes at 4-5 x 10^6 density the day before transfection on regular growth media. Medium was changed 2 hour before transfection to Iscoves DMEM (CellGro), 10% FBS (Hyclone), PenStrep (Invitrogen) and 2 mM glutamine. For transfection the Ca^{2+} phosphate method was used. The plasmid DNA mix was prepared by adding 3 µg ENV plasmid

(VSV-G), 5 µg Packaging plasmid (pMDLg/p RRE), 2.5 µg of pRSV-REV and 10 µg of Gene Transfer plasmid together (human FlagSlitrk5 and GFP was constructed into pCCL vector). The plasmid solution was made up to a final volume of 450 µl with 0.1xTE/dH_2O (2:1). Finally 50 µl of 2.5M $CaCl_2$ was added, mixed and incubated for 5 minute at RT. Precipitates were formed by drop wise addition of 500 µl of 2xHBS solution (281mM NaCl, 100mM HEPES, 1.5 mM Na_2HPO_4 pH= 7.08 – 7.16 – several solutions of HBS with different pH were tested for efficiency before transfection of viral particle constructs) to the DNA-TE-$CaCl_2$ mixture while vortexing. The precipitate was added immediately after the addition of HBS (a maximum of 3 dishes were transfected with the same solution). Cells were incubated and media was changed 12-16 hour after transfection. The supernatant was collected at 24h and 48h after changing the media and it was used to transduce the neurons.

Second generation lentivirus were used to transduce neurons with shRNA against mouse Slitrk5. pGIPZ vectors containing a target sequence for mouse Slitrk5 were obtained from Open Biosystem (5'-catcatcagtctctctgaa-3' and 5'-cagctgctattcttgaata-3'). Packaging vectors were obtained from Addgene. The protocol for HEK293 cell transfection and collection of viral particles was the same as for third generation lentivirus, only differing in the vector composition. Vector solution was as following: 7.5 µg of PsPAX2 packaging plasmid, 2.5 µg pMD2.G envelope plasmid, and 10 µg of pGIPZ vector.

Rat full lenght HA-Pick1, rat MycPick1 (Δ121), rat FlagPick1 (Δ135), rat MycGluA2 were a kind of Edward Ziff lab, at NYU medical Center. Human FlagSlitrk5 was sub-cloned into pcDNA3.1+; human Slitrk5GFP was sub-cloned into pCCL vector; mouse full length Slitrk5 was initially amplified from cDNA library produced in the lab and constructed into pcDNA3.1+ vector.

Western blotting

Brain tissue or cultured cells was lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0; 5 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) containing protease and phosphatase inhibitors (2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). After mechanical trituration of tissue or cells, lysates were rotated at 4°C for 20 min for maximum protein extraction, followed by a 10 min centrifugation at 14000 rpm. The pellet was discarded. Protein quantification was performed using Biorad reagent. After bringing the samples to the same concentration, proteins were separated on a 10% Nupage Bis-Tris Gel (Invitrogen) and transferred to PVDF membranes (Biorad). These were then blocked for 1 h in TBS with 0.1% Tween 20 (TBS-T) and 5% low-fat milk or 3% BSA if phosphotyrosine antibody was used for immunoblot. The incubation with the primary antibodies was performed overnight at 4°C in TBS-T with 3% BSA, followed by washes in TBS-T and incubation with HRP secondary antibodies at room temperature for 1 h at 1:4000 dilution. Immunoreactive proteins were visualized by ECL detection and film autoradiography. Striping was done by washing the membranes in 0.1M Glycine pH 2.5 for 15 min, followed by another wash in 1% SDS for 15 min.

Synaptosomal fractionation

The procedure of obtaining the PSD enriched fractions was adopted from elsewhere [210]. The striatum of three 6 and 14 months old Slitrk5$^{-/-}$ mice or their wild type littermates were pooled together and subjected to dounce homogenization in 1 ml of solution A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, with protease and phosphatase inhibitors) and centrifuged at 1,400 g for 10 min. The supernatant was subjected to a second centrifugation at 14,000 g for 30 min to obtain a crude P2 fraction. The pellet was resuspendend in 1ml of solution B (0.32 M sucrose, 1 mM $NaHCO_3$). This homogenate was layered on top of the

sucrose gradient (1 M sucrose and 1.2 M sucrose) and centrifuged at 82,500 g for 2 hrs. Purified synaptosomes were collected from the 1 M and 1.2 M sucrose interface. The synaptosomes were resuspended in Solution B and subjected to centrifugation at 82,500 g for 45 min. The pellet was resuspended in 2% SDS and 25 mM Tris. Protein lysates were subjected to western blot analysis. The normalization was done with the actin.

Open field test

The open field apparatus consisted of a (40 cm x 40 cm x 49 cm) white Plexiglas arena. The arena was set up in a dim room (2.0 Lux) under a digital camera connected to a video recorder and a computer under the control of the EthoVision tracking system (Noldus Information Technology). The arena was digitally divided into twelve equally sized quadrants. A single mouse (3 months old) was placed into the center of open-field arena and their positions in the field were recorded over a 10 min session. Anxiety level was assessed by the quantification of two indices: (i) the percentage of time spent in the center quadrants and (ii) percentage of entries into the center quadrants. An entry into a given quadrant was only registered if the center mass of the mouse traveled inside of the quadrant.

Elevated plus maze

The elevated plus-maze was constructed of white Plexiglas, and raised 70 cm above the floor, and consisted of two opposite enclosed arms with 14 cm high opaque walls and two opposite open arms of the same size (30 cm x 5 cm). The elevated plus-maze was set up under an infrared sensitive digital camera connected to a video recorder. A single testing session lasting 10 min was carried out in a dark room. To begin a trial, the test animal was placed in the center of the plus-maze facing an open arm, and their behavior was recorded for 10 min. The maze was cleaned with a 50% ethanol solution and dried after each trial to eliminate possible

odor cues left by previous subjects. The number of entries into both the open and enclosed arms was recorded (an entry is when the animal puts all four paws into one arm); the time spent in those two areas; and the frequency of center crosses was also recorded. Anxiety levels were measured by the relative amount of exploration devoted to the open arms relative to that to the enclosed arms This was quantified by two indices: (i) percentage of time spent in the open arms and (ii) percentage of entries into in the open arms.

Marble burying test

Mice (3 months old) were placed individually in a standard shoebox cage (internal dimensions 265 x 160 x 140 mm, L x W x H; floor area 424 cm^2) without a lid but with a filter top so that mice could not cling to the cage lid. The cage contained 20 opaque (black or blue) glass marbles evenly spaced on a 5 cm thick layer of sawdust. The mice were left in the cage with marbles for a 30-min period after which the test was terminated by removing the mice from the cage. An overhead photograph was taken of the cage, converted to grayscale in Photoshop and imported into imageJ. The dimension of an uncovered marble were measured using the particle analyze function. Using the uncovered marble as a baseline, the image of the interior of the cage was thresholded and the particle analyze function was used to count the number of marbles in which greater than 33% of the area was covered. This method insured an unbiased counting strategy. Each mouse was used only once in the experiment.

Rotarod test

Mice (3 months old, 10 *Slitrk5*$^{-/-}$ and 9 wild type) were tested for three trials in the same daily schedule, with intervals of 20 min between trials, for three consecutive days (nine trials total). Mice were tested rotating the rotarod (Economex, Columbus Instruments) at the following speeds: 6 rpm without

acceleration (low speed), 12 rpm without acceleration (high speed), and 4 rpm (baseline) with the acceleration of 0.2 rpm/s over 2 minutes. The total time on rotarod before falling was determined in seconds and statistical analysis was performed using a two-tailed Student's t-test for the constant speed and two-way Anova with repeated measures for rotation with acceleration.

Cylinder test

Mice (3 months old, 10 *Slitrk5*$^{-/-}$ and 9 wild type) were individually placed in a clear cylinder (diameter 14 inches and height 19 inches) and videotaped with a reflective mirror for 5 minutes. Vertical rearings (the number of instances the mouse lifted both of its front limbs off the surface, supporting its weight on the hind limbs) were counted. The total time that each mouse spent rearing on its hind limbs was scored. The time the mouse spent on its hind limbs is presented as percent of total time in the cylinder.

Golgi impregnation and tracing

We impregnated fresh brains in Golgi-cox using the FD Rapid GolgiStain Kit (Neurodigitech) solution for 14 d at 25 °C in the dark and then transferred them to 30% sucrose at 4 °C for 72 h. We prepared 150-µm coronal serial sections with a vibrotome; slides were soaked in 50% sucrose and air-dried for 72 h in the dark. Quantitative microscopy was performed on a Microbrightfield imaging system. Two hundred striatal neurons were chosen by systemic random sampling, and 40 'traceable' neurons for each genotype were reconstructed three dimensionally with the Neurolucida system. The morphological traits of cells were analyzed with Neuroexplorer.

Electrophysiology

We killed 4 month-old mice by pentobarbital anesthesia to obtain corticostriatal slices for electrophysiological recordings. Coronal brain slices (400 µm) were made on a vibrotome (Campden Instruments) and submerged in artificial cerebrospinal fluid in a brain-slice keeper (Scientific Systems Design) for 90 min at 25 °C and gassed with 95% O_2, 5% CO_2 before transfer to the recording chamber. The artificial cerebrospinal fluid contained 118 mM NaCl, 2.5 mM KCl, 10 mM glucose, 1 mM NaH_2PO_4, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 25 mM $NaHCO_3$. 100 µM picrotoxin was included in the recording solution. Recording electrodes were filled with 2 M NaCl solution, and population spikes were recorded from the striatum with the IE-210 amplifier (Warner Instruments) using Digidata 1440A and pClamp 10 software (Molecular Devices) at 32 °C. For synaptic stimulation, we placed bipolar electrodes in the white matter between the cortex and the striatum to activate corticostriatal fibers. Population spike amplitude was calculated by the mean of the amplitude from the first peak positivity to the peak negativity of the population spike and the amplitude of the peak negativity of the population spike to the second peak positivity. The presynaptic fiber volley amplitude was measured as a difference between the initial positive and the following negative peak. Three consecutive responses were averaged for measuring the spike and fiber volley amplitude. Paired-pulse responses were evoked at interstimulus intervals of 20, 40, 80, 120 and 200 ms using a stimulation intensity of 0.9 mA. Paired-pulse ratio is defined as the ratio of second population spike amplitude to the first population spike amplitude. Population spike amplitudes were analyzed by Clampfit software (Molecular Devices).

Endocytosis assay

To measure AMPAR endocytosis endogenous GluA1 or GluA2 receptors were live labeled using mouse antibodies at a dilution of 1:100 for 10-15 min at 37°C. After a brief wash in pre-warmed DMEM, neurons were either returned to conditioned

media or stimulated for 3 minutes with 100 µM AMPA and 50 µM APV or 50 µM NMDA, washed in DMEM, returned to conditioned medium and incubated for the different periods of time. After endocytosis had occurred, neurons were fixed in 3.7% formaldehyde for 15 minutes at room temperature (RT). Remaining surface receptors were labeled with Alexa647 anti mouse at a dilution of 1:300 for 20 min at RT. After 20 min with gentle agitation and PBS, cells were permeabilized with 0.2% Triton-X for 6 min with gentle agitation. Endocytosed receptors were labeled with Alexa568 at a dilution of 1:300 for 20 min at RT. Images of a given fluorophore were obtained with the same exposure times. Quantification of endocytosis was performed with ImageJ, GFP was used as a mask to measure both internalization and surface signaling within the exact same area. The experiments done with neurons from WT and KO, co-immunolabeling with Tuj antibody (β-Tubulin) was performed and this was used as a mask to measure intensity of internalized and surface signal within the exact same area. Quantification was performed blind. Internalization index = ((Inter / (Intern + Surface))*100.

Surface biotinylation and degradation assay

Surface biotinylation was performed to specifically detect receptors present in the plasma membrane. Cultured neurons were placed on ice and rinsed two times with PBS containing 1mM $MgCl_2$ and 2.5 mM $CaCl_2$ (PBS^{++}) followed by incubation with 1mg/ml of sulfo-NHS-S-biotin (Pierce Chemical, Rockford, IL) on ice with gentle agitation. Biotin solution was initially prepared 100mg/ml in DMSO and further diluted to 1mg/ml in PBS^{++}. Unbound biotin was quenched with 50mM Glycine solution in PBS^{++} two times for 5 minutes. Cells were then lyzed and biotinylated proteins were pulled down using streptavidin-conjugated sepharose beads (from Pierce) over-night at 4°C with rotation and analyzed by immunoblot as described previously. For degradation assay, after surface biotinylation cells were re-incubated at 37°C for a given amount of time before lysis.

Coculture assay

The co-culture assay was adapted from elsewhere [211]. Primary hippocampal neuronal cultures from E16 mice were used for synapse formation assays. Transfected COS7 cells were added to hippocampal neurons at 6-7 days *in vitro*. After 1-2 days of co-culture, cells were fixed in 3.7% formaldehyde for 15 min at RT. Synapse formation was determined by quantifying co-immunostaining between the presynaptic marker synapsin and transfected COS7 cells, that were not in contact with neuronal dendrites that were visualized with dendritic marker MAP2. Images were acquired using a Nikon TE2000 inverted fluorescente microscope and quantification was done using ImageJ. For quantification, first the COS7 area was selected, and then either a tau image, or an over-exposed synapsin picture was used to select the area to quantify synapsin staning. A MAP2 image was used to remove puncta associated with neuronal dendrites. Afterwards, a threshold image highlighted the synapsin puncta only within the target area (overlapping tau, contacting COS7 and not contacting MAP2). This image was used to measure puncta intensity (ImageJ mean intensity) as well number (manual particle picker).

Object placement test of spatial memory

Before starting object placement task, mice were acclimated to the open field apparatus, for 2 days before testing. The first day of acclimation consisted of a 10-min open field trial; the second day consisted of a 5-min open field trial. Testing consisted of two trials, the sample trial (T1) and recognition trial (T2) separated by a 15 minute inter trial interval (ITI). During the T1 trial, the mouse explored two identical objects (objects 1 and 2) at one end of an open field that consisted of two Lego trees for 5 min. During the T2 trial, the mouse explored the same two objects, with object 1 in the original location and object 2 in a new location. Object start locations and new object locations were counterbalanced across mice and trials.

Trials were recorded and analyzed using the Noldus Ethovision XT software (Noldus Information Technology, Leesburg, VA, USA). An area of 0.5 cm surrounding the objects was delineated using the software, and object exploration was defined as when the nose of the mouse was within this object surround area.

Morris Water Maze

Spatial reference memory was assessed in a 78 cm diameter watermaze filled with opaque white water and with visual external cues. Before experimental acquisition, one habituation session was performed. This consisted of allowing the mice to swim for 60 seconds until they found a hidden platform that was submerged 1cm bellow the water surface in the center of one of the four quadrants of the pool. Once they found the platform, they were allowed to sit on it for 10 seconds. If an animal failed to find the platform in the allotted time it was placed on the platform by the experimenter for 10 seconds (both during habituation and acquisition). During acquisition mice received 4 training trials per day during 4 days with an inter-trial interval of approximately 15-20 minutes. Latency to find the hidden platform was recorded. After 4 days of training a 24 hour probe trial was conducted to test if the mice had learned the spatial location of the platform. In this trial the platform was removed and mice were allowed to swim for 60 seconds. The time spent in each of the four quadrants was measured and the percent time the mice spent on the target quadrant was used as an indication of memory. Statistical analysis was done using SPSS software and ANOVA test with repeated measures with post-Hoc Bonferroni test was performed.

RESULTS

Bioinformatic analysis of the Slitrk protein family

There are six Slitrk genes in humans that are conserved in euteleostomi, which comprises most vertebrates, commonly known as bony vertebrates (Figure 2 Chapter 1). An alignment analysis of the human Slitrk protein sequences (using the ClustalW2 website [212]), reveals a modest 40% similarity within the members (Table 1, accessions: Q96PX8, Q9H156, O94933, Q8IW52, O94991, Q9H5Y7). Interestingly, Slitrk2 and Slitrk5 are the most similar with a total protein homology of 49%. Slitrk2 was the only member shown to have synaptogenic activity in a coculture assay and my own findings suggest that Slitrk5 has the same capability (see following sections) [83]. LRR-containing synaptogenic proteins (such as LRRTM or NGLs) interact via their extracellular LRR domains, with pre-synaptic proteins and contribute to synaptic formation and stability [90,97,98]. It is not surprising then that the highest homology between Slitrk2 and Slitrk5 is amongst the extracellular domain with 53% identity, also the highest observed in the family (Table 2). In fact, it is at the intracellular domain that Slitrks diverge the most, with an average homology of only 21% (and 39% for Slitrk2 and Slitrk5), suggesting that Slitrks might interact with similar extracellular ligands but mediate different intracellular signaling cascades (Table 3). The homology is higher on the C-terminal tail of Slitrk2-6 where it goes up to 58%. This region contains a conserved tyrosine that resembles the last 15 amino acids of Trk (Y791 in human TrkA) and spans the Trk PLC-γ site [8]. PLC-γ is recruited and activated by phosphorylated Trks and leads to the release of Ca^{2+} from internal stores and PKC activation, that ultimately modulates synaptic plasticity, channel activity and activates downstream transcription factors [2,39,213]. If Slitrks can recruit PLC-γ is still an open question. In addition, Slitrk2, 3 and 5 share more similarities with Trk receptors in that they contain an NPxY motif near their intracellular juxtamembrane region. Phosphorylation of the NPxY motif serves as a binding site for adaptor proteins such as Shc (that initiates Ras and phosphoinositide

3 kinase downstream signaling) [41,42,44] and it can also signal for endocytosis [214]. The stretch of amino acids that spans the NPxY motif in human Slitrk5 shares 25% amino acid identity with the TrkA NPxY region, and higher than 60% with Slitrk2, and 3 suggesting they might also recruit Shc and other adaptor proteins that could initiate intracellular signaling cascades. The only Slitrk5 residue reported to undergo phosphorylation is the Y833, in a proteomic screen performed with HeLa cells. However, the function and conservation of this site still needs to be clarified [215].

Table 1: Alignment of human Slitrk proteins

Name	Length	Name	Length	Alignment score
Slitrk1	696	Slitrk2	845	41
Slitrk1	696	Slitrk3	977	37
Slitrk1	696	Slitrk4	837	39
Slitrk1	696	Slitrk5	958	38
Slitrk1	696	Slitrk6	840	36
Slitrk2	845	Slitrk3	977	44
Slitrk2	845	Slitrk4	837	43
Slitrk2	845	Slitrk5	958	49
Slitrk2	845	Slitrk6	840	38
Slitrk3	977	Slitrk4	837	40
Slitrk3	977	Slitrk5	958	39
Slitrk3	977	Slitrk6	840	38
Slitrk4	837	Slitrk5	958	44
Slitrk4	837	Slitrk6	840	40
Slitrk5	958	Slitrk6	840	39
			Average	**40**

Table2: Alignment of extracellular domain of human Slitrk proteins

Name	Length	Name	Length	Alignment score
Slitrk1	605	Slitrk2	600	44
Slitrk1	605	Slitrk3	626	39
Slitrk1	605	Slitrk4	600	42
Slitrk1	605	Slitrk5	624	40
Slitrk1	605	Slitrk6	582	41
Slitrk2	600	Slitrk3	626	48
Slitrk2	600	Slitrk4	600	48
Slitrk2	600	Slitrk5	624	53
Slitrk2	600	Slitrk6	582	43
Slitrk3	626	Slitrk4	600	46
Slitrk3	626	Slitrk5	624	46
Slitrk3	626	Slitrk6	582	41
Slitrk4	600	Slitrk5	624	50
Slitrk4	600	Slitrk6	582	45
Slitrk5	624	Slitrk6	582	43
			Average	**45**

Table 3: Alignment of intracellular domain of human Slitrk proteins

Name	Length	Name	Length	Alignment score
Slitrk1	53	Slitrk2	203	5
Slitrk1	53	Slitrk3	302	22
Slitrk1	53	Slitrk4	198	5
Slitrk1	53	Slitrk5	273	18
Slitrk1	53	Slitrk6	212	5
Slitrk2	203	Slitrk3	302	31
Slitrk2	203	Slitrk4	198	26
Slitrk2	203	Slitrk5	273	39
Slitrk2	203	Slitrk6	212	25
Slitrk3	302	Slitrk4	198	22
Slitrk3	302	Slitrk5	273	22
Slitrk3	302	Slitrk6	212	23
Slitrk4	198	Slitrk5	273	25
Slitrk4	198	Slitrk6	212	26
Slitrk5	273	Slitrk6	212	27
			Average	**21**

Generation of Slitrk5$^{-/-}$ mouse and Slitrk5 expression

In order to understand the function of Slitrk genes we decided to focus our attention in the member of the family that is most strongly and widely expressed in the brain, the Slitrk5 [118]. We decided to generate a Slitrk5 knockout mouse (Slitrk5$^{-/-}$) and analyze its behavior in order to obtain insights about Slitrk5's function. Analysis of the genomic structure of *slitrk5* gene revealed that the coding region is localized to a single exon. Using Velocigene technology [216], we replaced the entire encoding exon with *lacZ* gene (Figure 1a). The expression analysis of *lacZ* demonstrated that *Slitrk5* is widely expressed throughout the central nervous system, including cortex and striatum (Figure 1b). Co-staining with a neuronal marker NeuN showed that *Slitrk5* expression is restricted to neurons and that the majority of neurons express *Slitrk5* (Figure 1c). We developed a custom made antibody for the intracellular domain of Slitrk5 and performed western blot analysis to further assess the protein distribution across the brain. We can observe how Slitrk5 is expressed at similar levels in the cortex, striatum and hippocampus in the adult murine brain (4 month old) (Figure 2).

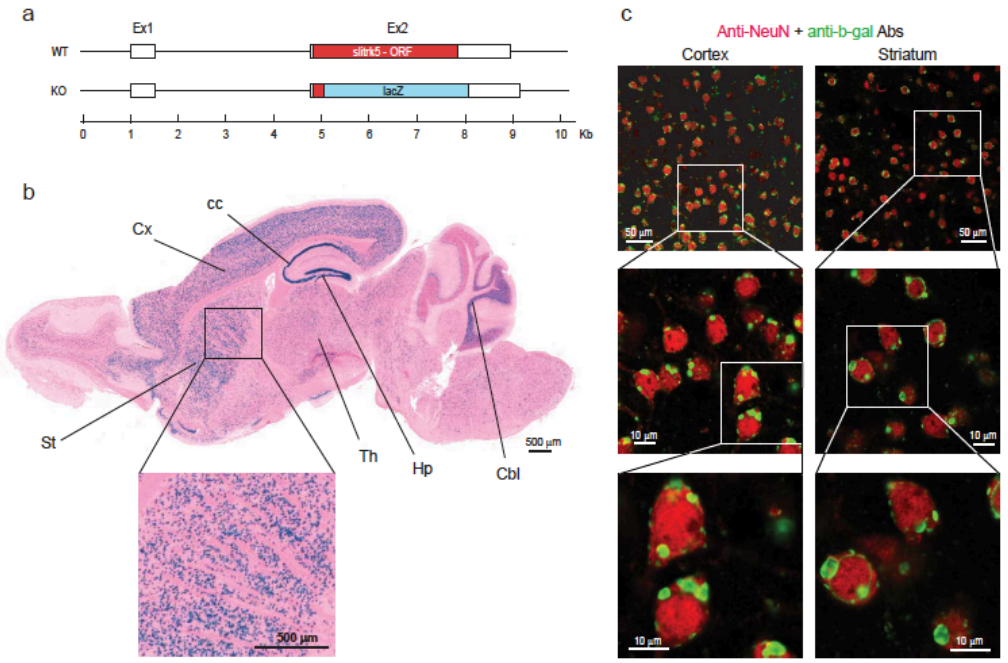


Figure 1: Targeted inactivation of Slitrk5 in mice and its expression pattern in the mouse brain. a) Genomic structure and the design of the *Slitrk5*-knockout, *lacZ*–knock-in mouse. The entire open reading frame (ORF) is localized to exon 2 (Ex2); exon 1 (Ex1) is noncoding. The *Slitrk5*-encoding region was replaced with *lacZ* downstream of the signal sequence cleavage site. WT, wild-type; KO, knockout. **b**) X-gal staining of mouse brain tissue, showing ubiquitous expression of lacZ in the gray matter of the various parts of the brain, including cortex and striatum. Cx, cortex; St, striatum; Hp, hippocampus; cc, corpus callosum; Th, thalamus; Cbl, cerebellum. The higher magnification image shows the distribution of *lacZ*-expressing cells in the striatum of the *Slitrk5*-knockout, *lacZ*–knock-in mouse. **c**) Immunostaining of cortex and striatum with antibodies to β-galactosidase (anti–β-gal) and NeuN (anti-NeuN), indicating that the majority of neurons express *Slitrk5*.

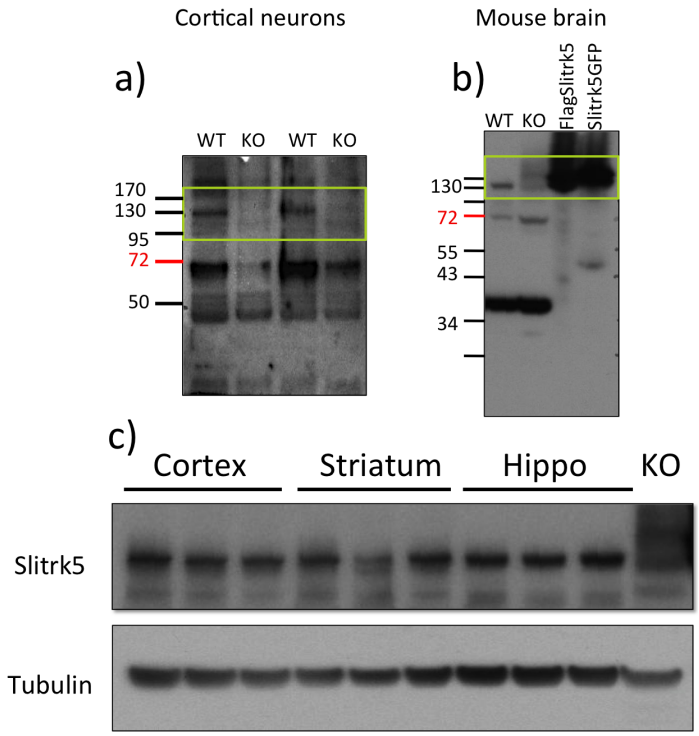


Figure 2. Slitrk5 antibody characterization and expression across the brain. a) mouse cortical neuronal cultures and **b)** brain lysates showing Slitrk5 expression from Slitrk5$^{-/-}$ (KO) and wild-type (WT) animals. As a positive control lysates from 293 cells transfected with FlagSlitrk5 and SlitrkGFP were used. As expected, Slitrk5GFP is shifted upwards, considering the additional GFP tag. **c)** Western blot of Slitrk5 expression across the brain from adult (4 month old) mice (Cortex, Striatum and Hippo (hippocampus)). Tubulin was used as a control. Slitrk5 is similarly expressed in these brain regions.

Slitrk5$^{-/-}$ mice were born in accordance with mendelian distribution. Gross anatomical and thorough histological examination of young *Slitrk5*$^{-/-}$ mice did not show any abnormalities. However, the analysis of older *Slitrk5*$^{-/-}$ mice revealed a behavioral phenotype. Starting at 4-6 months of age, *Slitrk5*$^{-/-}$ mice develop facial hair loss and severe skin lesions. Over time these lesions produced ulceration with hemorrhage (Figure 3a). The penetrance of this phenotype increases with age and

most of the knockout as well as the heterozygous mice are affected. The lesions in heterozygous mice are similar to those in homozygous animals but their emergence is delayed by 7-9 months. We hypothesized that this phenotype could be the result of excessive grooming. The lesions were not found in the wild type littermates even if they were housed in the same cage with *Slitrk5*$^{-/-}$ knockout mice, indicating that this phenotype can be attributed to self-grooming. This type of behavior is similar to that previously observed in mice deficient for the *Sapap3* gene [217]. Targeted deletion of this gene, which encodes a postsynaptic scaffold protein, leads to compulsive overgrooming behavior, increased anxiety, and response to selective serotonin reuptake inhibitors [217].

The grooming behavior of *Slitrk5*$^{-/-}$ mice was assessed by counting the number and the length of grooming events in the knockout and wild type littermates before any lesions or hair loss developed, to exclude the overgrooming as a result of irritation in a wound area. Our data demonstrated a significant increase in the length of grooming events in *Slitrk5*$^{-/-}$ mice compare to their wild type littermates (Figure 3b).

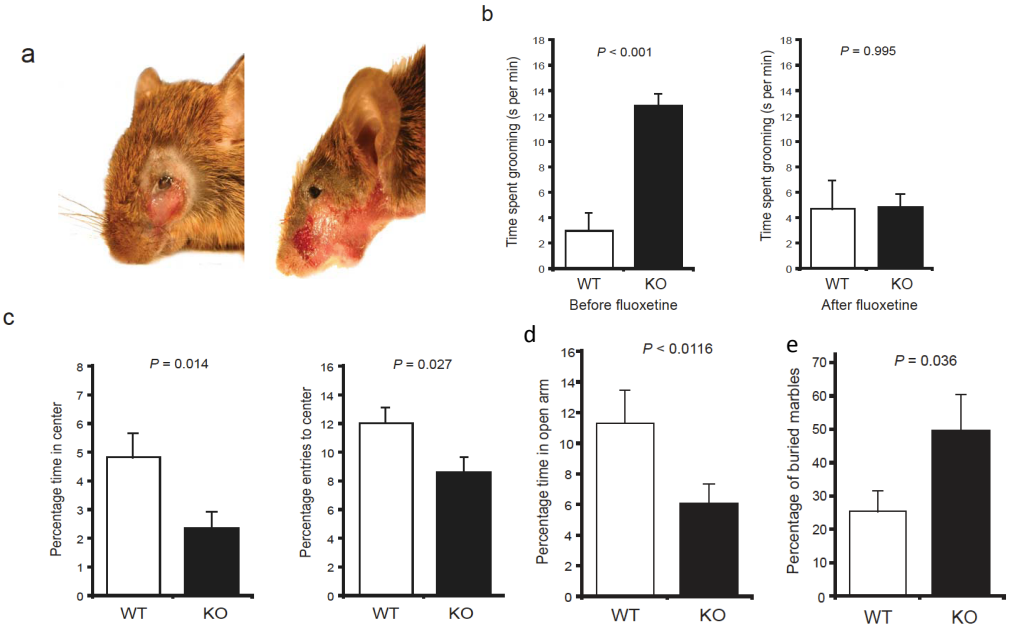


Figure 3. Facial lesions, OCD-like behavior and its alleviation with fluoxetine treatment in *Slitrk5*-knockout mice. **a**) Phenotypic characteristic of *Slitrk5*$^{-/-}$ mice: excessive grooming leads to severe facial lesions. **b)** Time spent grooming in *Slitrk5*$^{-/-}$ mice (*n* = 9) compared to their wild-type littermates (*n* = 8) before and after treatment with fluoxetine. Error bars depict the s.e.m. **c)** Anxiety-related behavior of *Slitrk5*$^{-/-}$ and WT mice in the open-field test. Percentage of time spent in the center and entries into the center of the open field are shown. **d),** Anxiety-related behavior of *Slitrk5* mice in the elevated plus test. Percentage of time spent in open arms is shown. Total traveled distance is not different between knockout and wild type littermates. All open-field results are presented as means ± s.e.m. determined from analysis of 20 mice per genotype **e)** OCD-like behavior of *Slitrk5* mice in the marble burying test. Percentage of marbles in which greater than 33% of the area was covered are shown. All results are presented as means ± SEM determined from analysis of eight mice per genotype.

As OCD is linked to a deficit in serotonin production, and since selective serotonin reuptake inhibitors (SSRI) are the major therapeutic agents for this disorder, we sought to test the effect of chronic fluoxetine on overgrooming behavior in *Slitrk5*$^{-/-}$ mice. Indeed, treatment of *Slitrk5*$^{-/-}$ mice with fluoxetine led to a significant reduction in the length of grooming compared to pre-treated animals. The length of grooming in *Slitrk5*$^{-/-}$ mice after fluoxetine treatment was the same as in wild type littermates. The length of grooming events in wild type mice was not affected by fluoxetine (Figure 3b). Thus, treatment of *Slitrk5*$^{-/-}$ mice with an SSRI rescues the compulsive behavior in these animals.

Slitrk5$^{-/-}$ mice display anxiety-like behaviors

In order to determine whether *Slitrk5*$^{-/-}$ mice exhibited additional behavioral phenotypes that also occur in OCD related conditions, we assessed anxiety-like behaviors in these mice. We performed the elevated plus maze and the open field

tests, standard measures of anxiety-like behavior that places subjects in conflict situations. In comparison with littermate wild type mice, *Slitrk5*$^{-/-}$ mice displayed decreased exploratory behavior as demonstrated by a reduction in the percentage of time spent in the center compartment and the number of entries into the center compartment in the open field test (Figure 3c), and by reduction of time spent in open arms in the elevated plus maze test (Figure 3d). This reduction in exploration could not be explained by changes in locomotor activity, as there were no significant differences in total distance traveled. To further assess the behavioral consequences of Slitrk5 inactivation, we also tested *Slitrk5*$^{-/-}$ mice in a marble burying paradigm, a behavioral task that assesses both OCD-like and anxiety-related behaviors. We found that *Slitrk5*$^{-/-}$ mice displayed an increase in marble burying behavior, which is consistent with the findings that this mouse models core symptoms in OCD spectrum disorders (Figure 3e). We also assessed motor function in *Slitrk5*$^{-/-}$ mice by using the cylinder test (data not shown) and by measuring the latency to fall from the rotarod and found no difference in gross motor skills and no impairment in motor learning compared to the wild type mice, indicating that these functions are not affected in *Slitrk5*$^{-/-}$ mice (Figure 4).

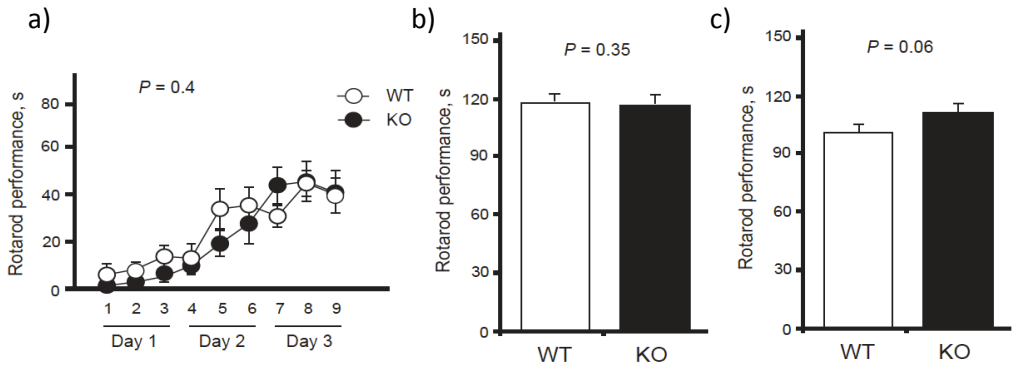


Figure 4. **Motor coordination in *Slitrk5* mice in the rotarod test. a)** Rotarod test with acceleration. Mice were tested for 3 consecutive days, 3 trials per day. Baseline speed is

4 rpm, acceleration is 0.2 rpm/s for 2 min. Statistical analysis was performed using two-way Anova with repeated measures. Both *Slitrk5* mice and wild type controls have the capacity of motor learning ($P < 0.001$). There is no difference in motor learning ability between the two groups for the duration of the test ($P = 0.4$) and for each individual day ($P = 0.5$). **b)** Low speed (6 rpm) rotarod test without acceleration. Statistical analysis was performed using a two-tailed Student's t-test. **c)** High speed (12 rpm) rotarod test without acceleration. Statistical analysis was performed using a two-tailed Student's t-test. All results are presented as means ± SEM determined from the analysis of nine wild type and ten *Slitrk5* mice.

Slitrk5$^{-/-}$ mice have impaired striatal function

Since cortico-striatal circuitry has been previously implicated in the pathogenesis of OCD, we performed detailed anatomical, histological and functional analyses of cortex and striatum in *Slitrk5*$^{-/-}$ mice. Initially, we evaluated the difference in baseline activity of selected brain regions between wild type and *Slitrk5*$^{-/-}$ mice by assessing FosB expression, an established marker for neural activity [218]. FosB was found to be upregulated exclusively in the orbitofrontal cortex of *Slitrk5*$^{-/-}$ mice (Figure 5 a,b). Other brain regions such as the caudate putamen, and thalamus do not show upregulation of FosB expression. These findings are of particular interest as it has been consistently shown in functional imaging studies that there is an increase in activity of orbitofrontal cortex in patients with OCD [126,219,220,221]. Conversely, alterations in neural activity in the thalamus have been less consistent [126,220,222].

As a next step, we measured the volume of the striatum relative to whole brain volume using Cavalieri estimation. Our data showed that the volume of striatum in *Slitrk5*$^{-/-}$ mice was significantly reduced. In both young and old animals the ratio of striatal volume to the total brain volume was decreased, while volume ratios of other brain structures, such as the dorsal hippocampus and the septum, to the total brain volume were not changed, indicating that the anatomy of striatum is

specifically affected by Slitrk5 deficiency (Figure 3 c, g and h). This is an important finding since it was earlier reported that specifically the volume of the caudate is decreased in some patients with OCD [223,224,225]**,** though this finding has not been consistent across all OCD patient studies in which increased or no change in striatal volumes have also been reported [126,220,222].

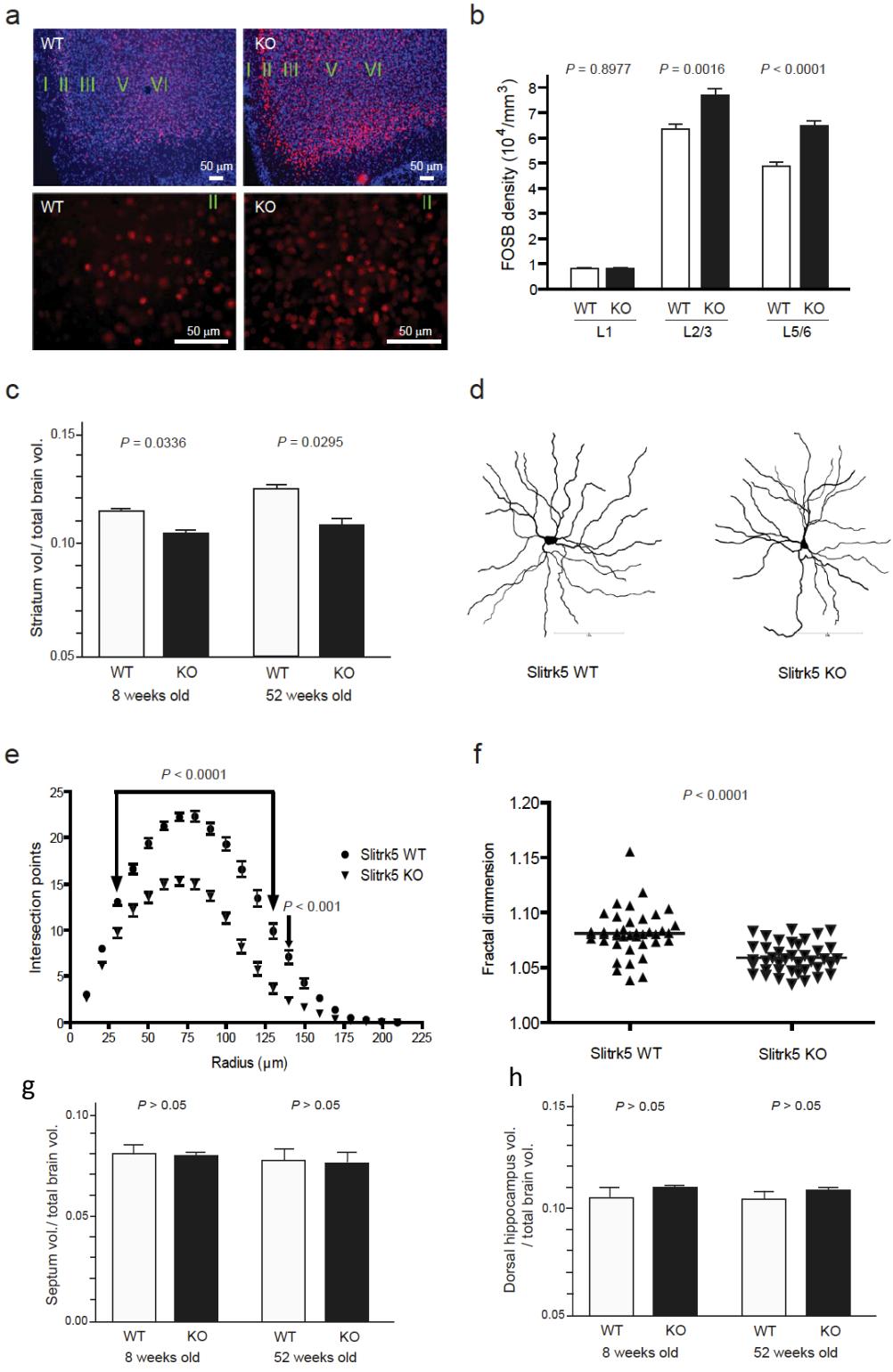

a
WT
KO
I II III V VI
50 µm
II
b
P = 0.8977
P = 0.0016
P < 0.0001
FOSB density (10^4/mm^3)
WT KO
L1
L2/3
L5/6
c
P = 0.0336
P = 0.0295
Striatum vol./ total brain vol.
8 weeks old
52 weeks old
d
Slitrk5 WT
Slitrk5 KO
e
P < 0.0001
P < 0.001
Slitrk5 WT
Slitrk5 KO
Intersection points
Radius (µm)
f
P < 0.0001
Fractal dimmension
g
P > 0.05
Septum vol./ total brain vol.
h
Dorsal hippocampus vol. / total brain vol.

Figure 5. Metabolic changes in the cortex and anatomical defects in the striatum of *Slitrk5*$^{-/-}$ mice. a) Expression of FosB in orbitofrontal cortex by immunostaining for FosB (red) and with DAPI (blue). The top images show the distribution of FosB expression in the various layers of orbitofrontal cortex. The bottom images show a higher magnification of layer II of FosB immunoreactivity in nuclei. **b)** Quantification of FosB expression in all layers of the orbitofrontal cortex. **c)** Cavalieri estimation of striatal volume in *Slitrk5*$^{-/-}$ and WT mice. **d)** Examples of Golgi staining and Neurolucida reconstruction of striatal medium spiny neurons in WT and *Slitrk5*$^{-/-}$ mice. **e)** Sholl analysis of striatal medium spiny neurons in WT and *Slitrk5*$^{-/-}$ mice. All results are presented as means ± s.e.m.; 40 neurons per genotype. **f)** Fractal dimension analysis of striatal medium spiny neurons in *Slitrk5*$^{-/-}$ and WT mice. All results are presented as means ± s.e.m.; 40 neurons per genotype. **g) h)** Cavalieri estimation of septal and dorsal hippocampal volume in *Slitrk5*$^{-/-}$ and WT mice.

Slitrk5$^{-/-}$ mice have impaired cortico-striatal transmission

Because Slitrk family members have been shown to influence neuronal differentiation [113,119], the decreased striatal volume in the *Slitrk5*$^{-/-}$ mice may be accounted for by altered neuronal morphology. We used Golgi staining to visualize individual medium spiny neurons of the striatum in *Slitrk5*$^{-/-}$ mice. There was no difference in striatal cell soma area between *Slitrk5*$^{-/-}$ mice and their wild type littermate controls. Next, we analyzed dendritic complexity in these same neurons. Sholl analysis revealed a decrease in dendritic arbor complexity at 50 µm and greater distances from the soma in *Slitrk5*$^{-/-}$ mice (Figure 5 d,e). We also used fractal dimension analysis to quantify how completely a neuron fills its dendritic field. There was a significant decrease in dendritic complexity of striatal neurons in *Slitrk5*$^{-/-}$ mice (Figure 5f). Although the striatum contains two equally abundant sub-populations of medium spiny neurons, which are classified based on neuropeptides that they produce and the dopamine receptors that they express (D1 and D2), distinguishing between these two types of cells is technically challenging [226]. However, in our

detailed comparative analysis of 40 randomly selected medium spiny neurons in *Slitrk5*$^{-/-}$ mice, we found no evidence for a bimodal distribution in their dendritic complexity. These data suggest that there is no selective deficit of arborization in one sub-population of medium spiny neurons, but rather indicate a general deficit in all medium spiny neurons equally.

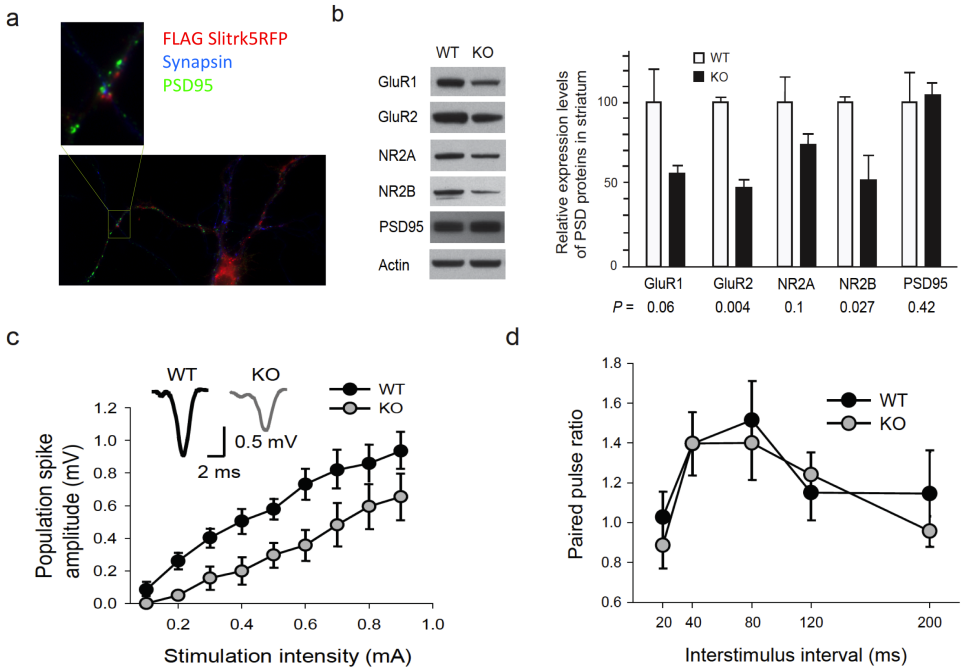


Figure 6. Deficiency in corticostriatal transmission in *Slitrk5*−/− mice is mediated by changes in glutamate receptor composition. **a)** Immunostaining of primary striatal rat neurons (infected with Flag-Slitrk5 lentivirus and transfected with PSD95 fused to mCherry (PSD95-cherry)) in culture with cortical neurons (isolated from transgenic mice that ubiquitously express green fluorescent protein) with Flag-specific antibody (anti-Flag). The arrow points to a magnified area (bottom images) that represents the synapses between cortical and striatal neurons. **b)** Western blot analysis of NMDA and AMPA receptor subunits in the striatum of 5-month-old *Slitrk5*−/− and WT mice. The protein amounts are adjusted to the expression of actin. **c)** Population spike amplitude in *Slitrk5*−/− mice (n = 11, from four mice) and matched WT mice (n = 9, from four mice). The population spike amplitude is

significantly lower in *Slitrk5*−/− mice, $P < 0.01$, repeated-measures analysis of variance. The inset shows examples of corticostriatal population spike amplitudes in *Slitrk5*−/− mice and matched WT mice. **d)** Average paired-pulse ratios of the population spike in *Slitrk5*−/− mice ($n = 17$, from five mice) and matched WT mice ($n = 17$, from five mice). There is no significant difference in the paired-pulse ratio between *Slitrk5*−/− mice and wild-type mice.

We subsequently assessed the cellular localization of Slitrk5 in striatal neurons, and found Slitrk5 in dendritic spines that are positive for PSD95 in co-cultures of cortical and striatal neurons (Figure 6 a). Next, we examined the expression levels of glutamate receptors in the striatum and demonstrated that they are downregulated in old *slitrk5*$^{-/-}$ mice. Indeed, GluN2A, GluN2B, GluA1, and GluA2 protein levels were decreased by 20-60%, with no significant changes in PSD95 levels. We found these changes in both the total lysates (Figure 6b) and also in PSD enriched fractions of synaptosomes (Figure 7).

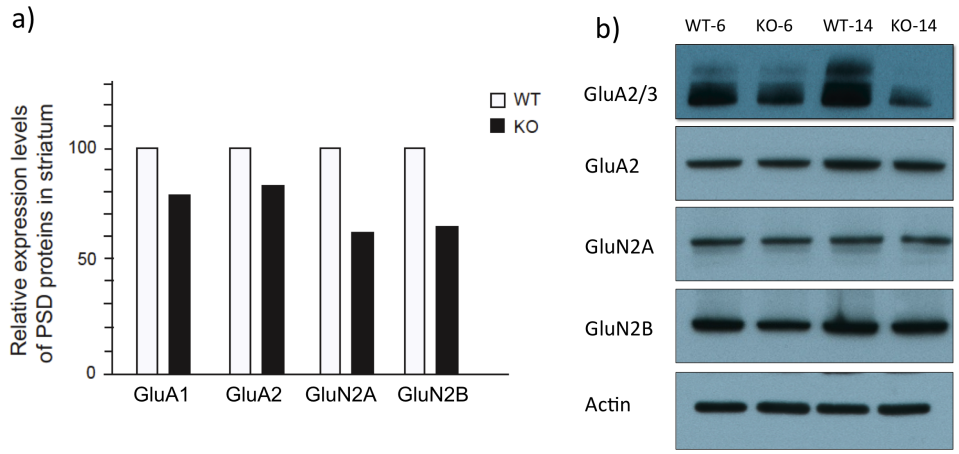


Figure 7. The expression of NMDA and AMPA receptors subunits is downregulated in PSD enriched fractions (synaptosomes) in *Slitrk5* mice. Each column represents data obtained using combined together striatums from 3 mice (*Slitrk5* or wild type controls). The protein levels are adjusted to the expression of actin.

Due to these findings, we investigated whether *Slitrk5*$^{-/-}$ mice have deficiency in cortico-striatal neurotransmission by extracellular recordings in acute striatal slices. We have recorded population spikes from striatum by stimulating the white matter between cortex and striatum. We found significantly reduced population spike amplitude in S*litrk5*$^{-/-}$ mice (Figure 6 d). We did not observe any difference in paired pulse ratios (PPR) of the population spike in *Slitrk5*$^{-/-}$ mice and their wild type littermates, suggesting that the presynaptic mechanisms involved in paired pulse facilitation is not responsible for the observed difference in population spike amplitude (Figure 6e).

Slitrk5 modulates surface expression of AMPARs

Considering the temporal and spatial distribution of Slitrk5 it is surprising to find a phenotype at such a late developmental stage. The functions this protein plays in younger mice as well as in other brain structures are unknown. To gain insight into the developmental expression of the *Slitrk5*$^{-/-}$ mouse phenotype, we decided to perform whole-cell patch clamp recordings in the striatum of younger (P20) mice. Surprisingly we found that AMPA receptor currents were increased in the young *Slitrk5*$^{-/-}$ mice (Figure 8 a) whereas NMDA receptor currents were indistinguishable between *Slitrk5*$^{-/-}$ and wild type littermates (Figure 8 b). Moreover, we also measured non-NMDA spontaneous excitatory post-synaptic activity in *Slitrk5*$^{-/-}$ and, even though we did not observe differences in EPSC frequency, the non-NMDA amplitude was enhanced in the *Slitrk5*$^{-/-}$ (Figure 8 c). The increase in AMPAR amplitude was not due to pre-synaptic changes since PPR was normal in these mice, thus suggesting that in the absence of Slitrk5 there is an overall increase in the synaptic pool of AMPARs. Moreover, it implies that the behavioral defects observed in the older *Slitrk5*$^{-/-}$ are a consequent of a development deregulation of glutamate receptors.

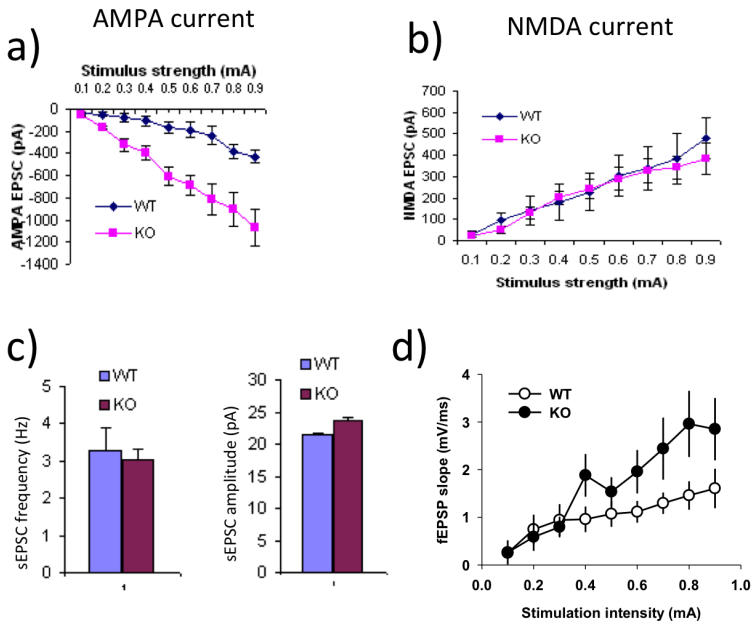


Figure 8. Electrophysiological analysis of young *Slitrk5*$^{-/-}$ mice. a) b) Whole-cell patch-clamp recordings were made from a single striatal neurons of P20 *Slitrk5*$^{-/-}$ mice and wt littermate controls. **c)** Average mEPSC frequency and amplitude of striatal medium spiny neurons of *Slitrk5*$^{-/-}$ mice (n = 5) and wild-type littermates (n = 5). The mEPSC amplitude but not frequency was significantly higher. **d)** Input–output curves of the CA3–CA1 fEPSPs in the 2 month old *Slitrk5*$^{-/-}$ mice (n= 6) and wt littermate controls (n = 6).

To directly investigate if Slitrk5 modulates surface expression of AMPARs, we assessed the surface levels of these receptors in cultured striatal neurons in which Slitrk5 levels were manipulated. We hypothesized that Slitrk5 may act to stabilize intracellular pools of AMPARs or simply reduce surface delivery of these receptors. In order to test this, striatal neurons were transduced with lentivirus carrying FlagSlitrk5 (or GFP as a control) and surface protein levels were measured with surface biotinylation. We observed that Slitrk5 expression led to a decrease in surface AMPAR levels, specifically surface GluA2 was reduced to 40% (figure 9 a).

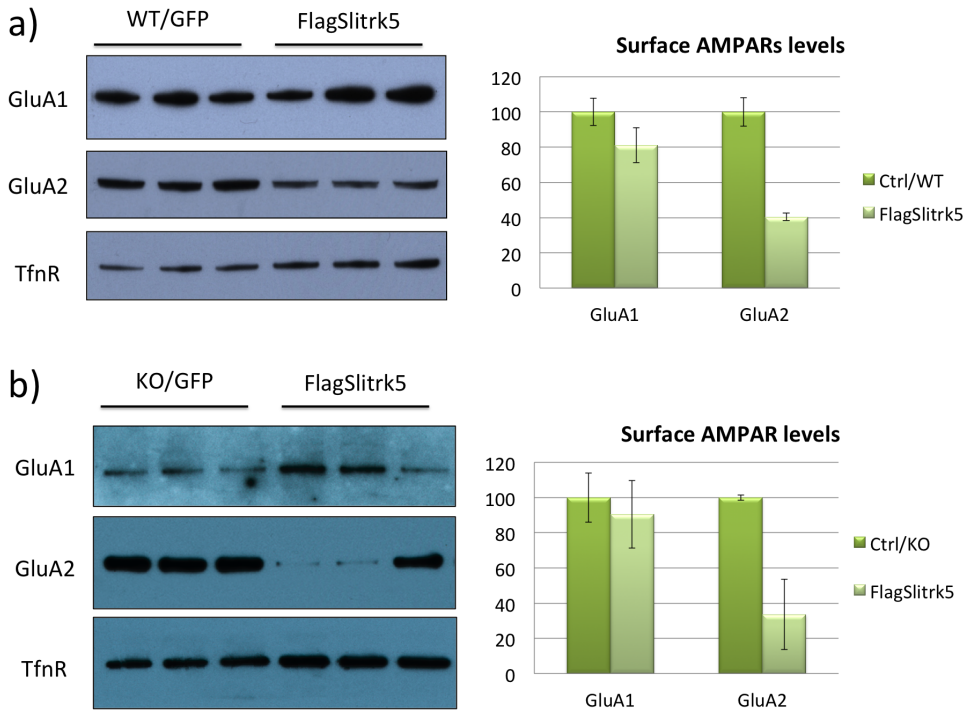


Figure 9. Surface levels of AMPARs upon Slitrk5 expression. **a)** Striatal neurons from wt mice were grown in culture. Slitrk5 levels were manipulated by lentiviral delivery of FlagSlitrk5 cDNA, control cells were transduced with GFP cDNA. Surface biotinylation allowed assessment of surface levels of AMPARS. a) Western blot of GluA1 and GluA2 quantified by normalization to transferrine receptor (TfnR) levels. **b)** Similar surface biotinylation experiment using hippocampal neurons from *Slitrk5*$^{-/-}$ mice, therefore in this condition the control has no expression of Slitrk5.

Slitrk5 is highly expressed throughout the brain including in the striatum, hippocampus and cortex (Figure 1). Therefore it is plausible to think that this protein plays key roles for development and function of brain regions other than the striatum. Glutamate receptor activity in the hippocampus has been intensely studied

and AMPAR-mediated hippocampal plasticity has been widely associated with memory formation and learning [151,156]. To assess if Slitrk5 also modulates surface AMPAR expression in the hippocampus, we repeated the previous experimental setting utilizing hippocampal neurons from *Slitrk5*$^{-/-}$ mice in which Slitrk5 expression was rescued through lentiviral delivery of FlagSlitrk5. We found a striking reduction in the expression of surface GluA2 upon Slitrk5 delivery. Surface levels of GluA1 appeared unchanged (Figure 9 b).

To test if the differential expression of AMPARs in hippocampal neurons was overall reflected in hippocampal function, we performed electrophysiological analysis of this brain region in young (2 month old) *Slitrk5*$^{-/-}$ mice. Consistent with a higher surface expression of AMPARs, these measures revealed that basal synaptic neurotransmission in CA3-CA1 were increased (Figure 8 d). In fact, analysis of Slitrk5 expression in murine hippocampus across development revealed that this protein is expressed as early as embryonic day 17 (Figure 10). Slitrk5 levels are stable until P5, after which they increase to reach a peak at P15, which is the period when synapse development is occuring. After this, Slitrk5 levels remained stable throughout adulthood (Figure 10). Interestingly, GluA1, GluA2 and TrkB followed a similar expression pattern, peaking at P15 and being maintained thereafter throughout adulthood.

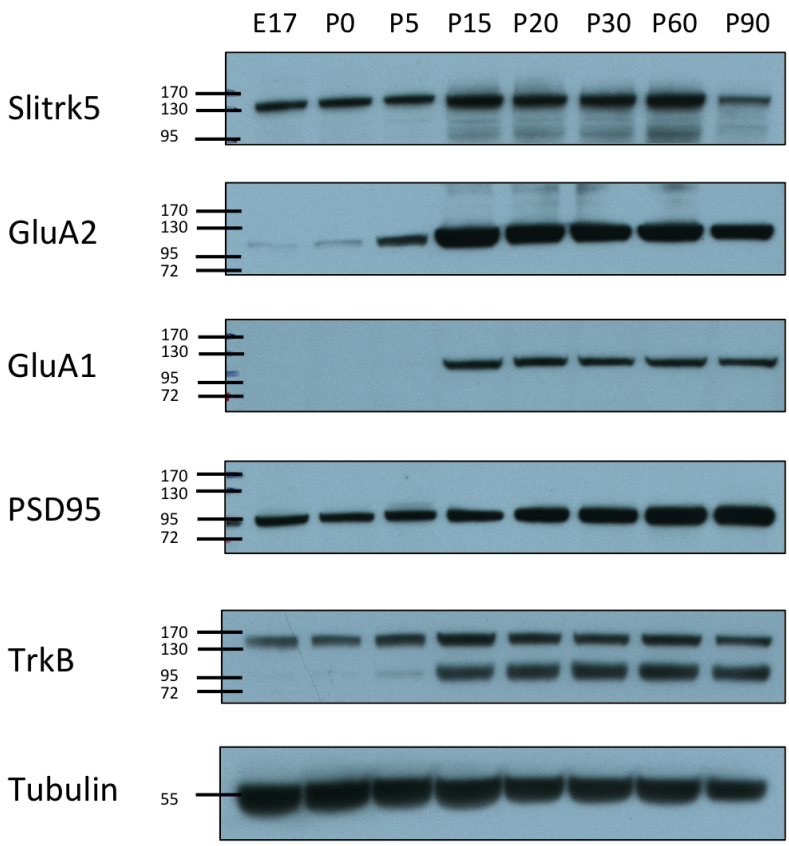


Figure 10. Analysis of Slitrk5 expression across development and comparison with other developmentally regulated proteins. WT mouse hippocampus protein extracts from the indicated developmental stages (E17 to P90) were analyzed by western blot with Slitrk5, GluA1, GluA2, PSD95 and TrkB antibodies and tubulin as a control. In order to obtain sufficient amounts of protein, several hippocampi were combined for the following developmental stages: E17, P0 and P5.

Slitrk5 interacts with PICK1, a known modulator of GluA2 trafficking

Few proteins have been described to interact with AMPARs and modulate its location and traffic. Pick1 is a cytoplasmic protein originally identified as a PKC binding protein, that has been shown to bind GluA2 and GluA3 subunits [227]. In

hippocampal neurons, activity-dependent phosphorylation of GluA2 at Ser880 enhances the affinity of this subunit for Pick1, promoting endocytosis of GluA2 receptors, increasing the proportion of GluA1 homomers at the synapses, that are Ca^{2+} permeable [228,229].

Since we observed a selective reduction of GluA2 from surface hippocampal neurons upon Slitrk5 expression, and Pick1 has been described to affect GluA2 trafficking and not GluA1, we hypothesized that Slitrk5 may alter GluA2 trafficking through Pick1 interactions. In order to test this, HEK293 cells were co-transfected with cDNA of FlagSlitrk5 and MycPICK1. Indeed we observed that immunoprecipitation of Pick1 pulled down Slitrk5 (Figure 11 a). Even though this data suggests that Slitrk5 interacts with GluA2 through Pick1, it does not exclude the possibility that Slitrk5 may directly interact with GluA2. Therefore, we co-expressed FlagSlitrk5 and Myc-tagged GluA2 in heterologous cells. We found that in this system Slitrk5 and GluA2 interacted (Figure 11 c). The interaction between Slitrk5 and GluA2 could either be direct or mediated by Pick1 (which is endogenously expressed in 293 cells). To examine if Pick1 regulates Slitrk5-GluA2 interaction we tested this interaction in the presence or absence of co-transfected Pick1 (Figure 11 c). We observed that expression of Pick1 did not enhance Slitrk5-GluA2 interaction, suggesting that this interaction may be direct.

Pick1 protein is composed of two acidic domains, a BAR and a PDZ domain that interacts with GluA2 but not GluA1 [228]. In order to map the domain of interaction with Slitrk5, we used two deletion constructs in which either N-terminal portion of the protein (containing an acidic and PDZ domain) or the C-terminal region (containing another acid and the BAR domain) were removed. We observed that removal of the BAR domain, which mediates interactions with membrane phospholipids, did not alter the interaction with Slitrk5. However, when PDZ and the acidic domain were truncated, the interaction was lost (Figure 11 b). This suggests that one or both of these domains mediate the interaction with Slitrk5.

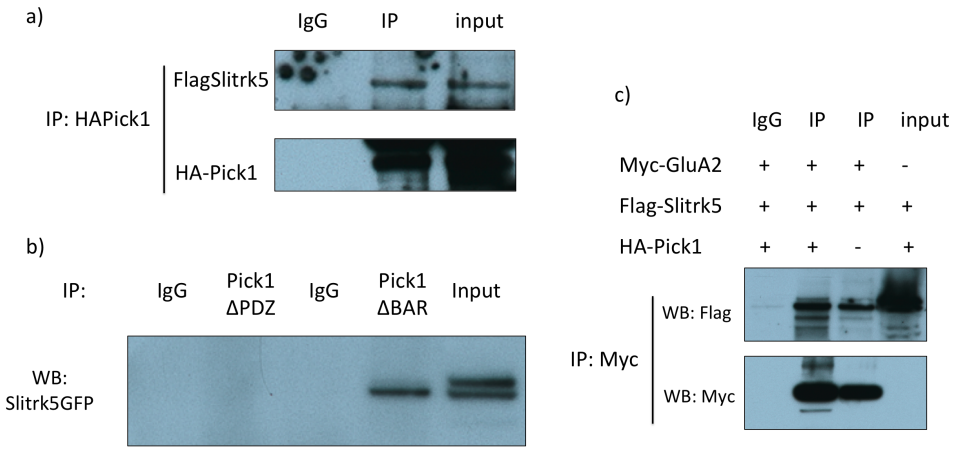
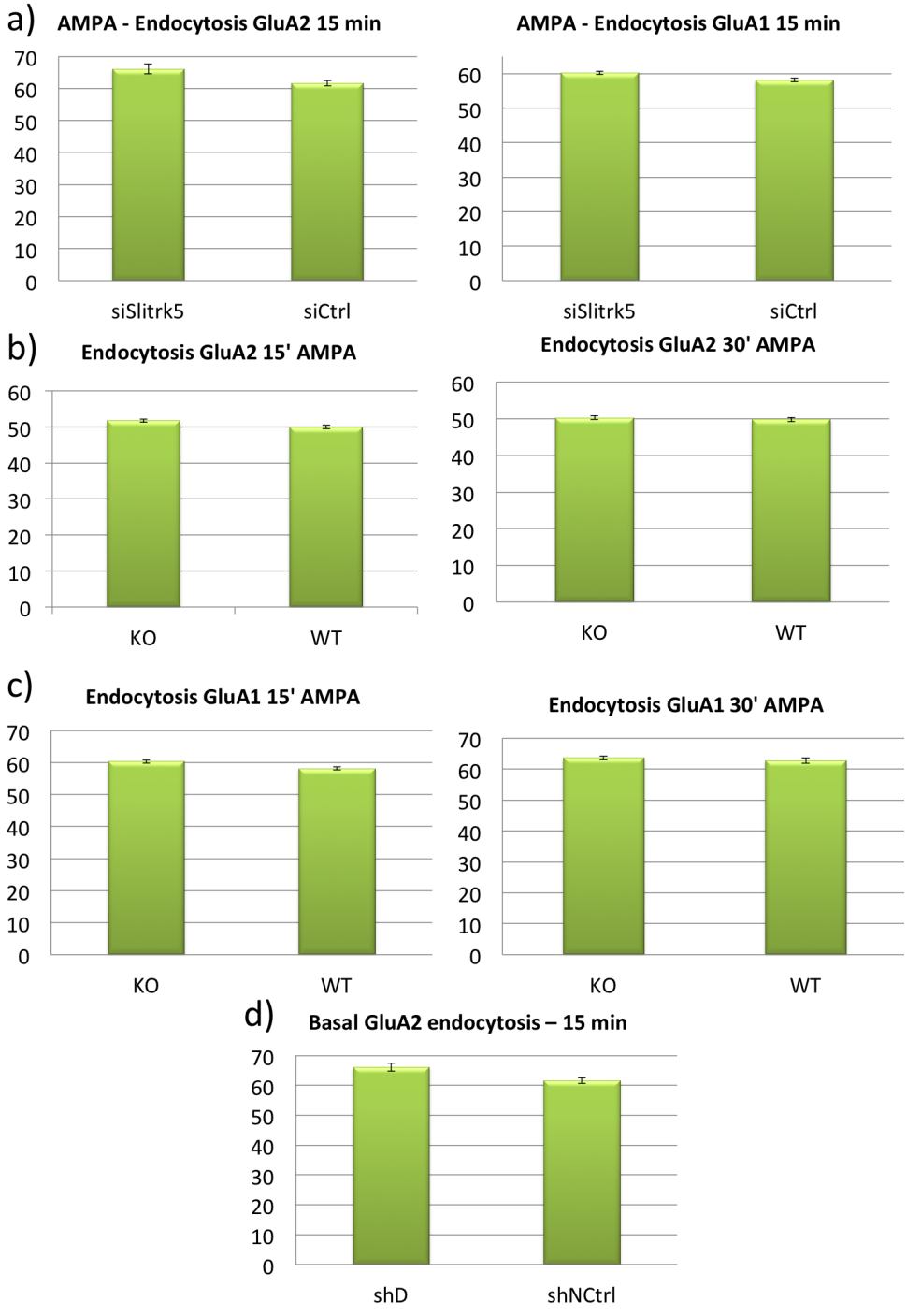


Figure 11. Slitrk5 interaction with Pick1 and GluA2 subunit. a) HEK293 co-transfected with FlagSlitrk5 and HAPick1 were subjected to immunoprecipitation with HA antibodies. Western blot reveals Slitrk5 and Pick1 interaction. **b)** HEK293 were co-transfected with Slitrk5GFP and either one of two Pick1 deletion constructs (ΔPDZ which also lacks the N-terminal acidic domain and ΔBAR, also lacking N-terminal acidic domain). BAR domain is dispensable for interaction with Slitrk5. **d)** Testing if Pick1 enhances Slitrk5 interaction with GluA2. Co-transfection of Myc-GluA2 and FlagSlitrk5 into 293 cells and immunoprecipitation showed that these two proteins interacted in this system. Addition of HAPick1 did not enhance Slitrk5 and GluA2 interaction.

Slitrk5 interaction with Pick1 and GluA2 may explain the reduction in GluA2 surface receptors. The underlying mechanisms are still unclear; however it could involve either a) increase AMPAR endocytosis; b) decrease AMPAR recycling; c) decrease ER-forward trafficking of newly synthesized AMPARs; or lastly, d) Slitrk5 could simply act as a intracellular scaffolding protein such as ABP/GRIP, that stabilize AMPARs in intracellular storage compartments [230,231]. To begin to test these hypotheses, we started by measuring endocytosis of GluA1 and GluA2 by modulating

Slitrk5 levels. In order to accomplish this we developed shRNA against mouse Slitrk5. Two of the shRNA sequences tested led to a 50% knock-down of Slitrk5 when co-expressed in HEK293 cells. Quantification of endocytosis in mouse hippocampal neurons from wt mice co-transfected with the shRNA and Myc-GluA1 or HA-GluA1 revealed that knocking down Slitrk5 did not affect AMPAR endocytosis (data not shown). We wondered if the lack of an effect could be due to the exogenous expression of AMPARS. Therefore, we developed an assay to measure endogenous GluA1 and GluA2 endocytosis. AMPA-mediated as well as basal and NMDA-dependent endocytosis occurred normally in hippocampal neurons transfected with shRNA for Slitrk5 or control (Figure 12). We then asked if these results could be due to a low efficiency of shRNAs. Therefore, we compared AMPAR endocytosis using cultured hippocampal neurons from *Slitrk5*$^{-/-}$ and WT mice (from heterozygous crosses). Also in this setting, AMPAR endocytosis occurred with a similar rate in both groups, irrespective of the stimulation condition or the time point used (Figure 12). These data strongly suggests that the mechanism by which Slitrk5 leads to downregulation of surface AMPARs is not through modulating their endocytosis. Future studies will be required to elucidate if Slitrk5 affects the recycling of AMPARs, the forward ER-trafficking of newly synthetized proteins or stabilizes intracellular pools of AMPAR.


a)
AMPA - Endocytosis GluA2 15 min
70
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shNCtrl

Figure 12. Endocytosis of AMPARs occurs normaly upon Slitrk5 kockdown. Endocytosis of endogenous GluA2 and GluA1 was measured using an immuno-cytochemistry technique. Hippocampal neurons from wt animals (a and c) were transfected with shRNA for Slitrk5 (siSlitrk5) or a scrambled shRNA (siCtrl); or neurons from wild type (WT) or Slitrk5 knockout (KO) were used. Surface GluA1 or GluA2 were labeled with an antibody recognizing the N-terminal portion of this protein, live at 37°C. Neurons were then either stimulated with AMPA, or basal endocytosis was analyzed. Following endocytosis, cells were fixed and the remaining surface pool of AMPARs was measured with a fluorescent secondary antibody. Subsequent permeabilization and staining with another fluorescent antibody labeled the internalized pool of AMPARs. An average of 25 images per condition were analyzed using ImageJ software and endocytosis was measure as percent-internalized pool divided by the sum of internalized and surface (%Int = Int/(Int+Surf)). Error bars represent s.e.m..

Slitrk5-deficient mice are impaired in spatial reference memory

It is widely accepted that AMPARs mediate synaptic plasticity involved in learning and memory. Hippocampal-dependent learning and memory tasks are dependent on AMPA receptor function [156]. Considering the altered expression of AMPA receptors in the *Slitrk5*$^{-/-}$ mice and the altered basal synaptic transmission in CA3-CA1, we hypothesized that hippocampal-dependent spatial learning and memory could be impaired. We tested the *Slitrk5*$^{-/-}$ mice in the object placement task, a spatial episodic hippocampal-dependent memory task. This task explores the natural exploratory activity for novel object locations. The animals are allowed to explore two identical fixed objects for 5 minutes. After a 15-minute inter-trial interval one of the objects was moved to a new location. Given the natural exploratory activity of the mice, wild type mice tend to spend more time exploring the object that is associated with a new context, than the object left in the same location. When we tested the Slitrk5-deficient mice on this task, along with their wild type and heterozygous littermates. We observed that wild type mice spent approximately 60% of the time exploring the displaced object. On the other hand,

Slitrk5-deficient mice were unable to detect the object had been moved and spent an equal amount of time exploring both objects (Figure 13 a).

To further investigate the role of Slitrk5 for spatial memory we examined the *Slitrk5*$^{-/-}$ mice and their wild-type and heterozygous littermate controls in a spatial reference memory task the Morris Water Maze (MWM). The mice were allowed to swim for 60 seconds or until they found a hidden platform that was located in the center of an arbitrarily chosen quadrant. *Slitrk5*$^{-/-}$ mice exhibited significantly higher latency than their wt or het littermates to find a hidden platform during the acquisition phase of MWM task (Figure 13 c). Additionally, we performed a probe test 24 hours after the fourth training day where the platform had been removed from the pool and the mice were allowed to swim freely. The percent time spent on each quadrant was recorded. Slitrk5$^{-/-}$ mice spent approximately 25% of the time on the target quadrant (that corresponds to chance), whereas their wild-type littermates spent 47% of the time in the target quadrant, a difference that was significant (Figure 13 b). Together, the behavioral analyses show that Slitrk5 is essential for normal expression of hippocampal spatial-reference memory tasks.

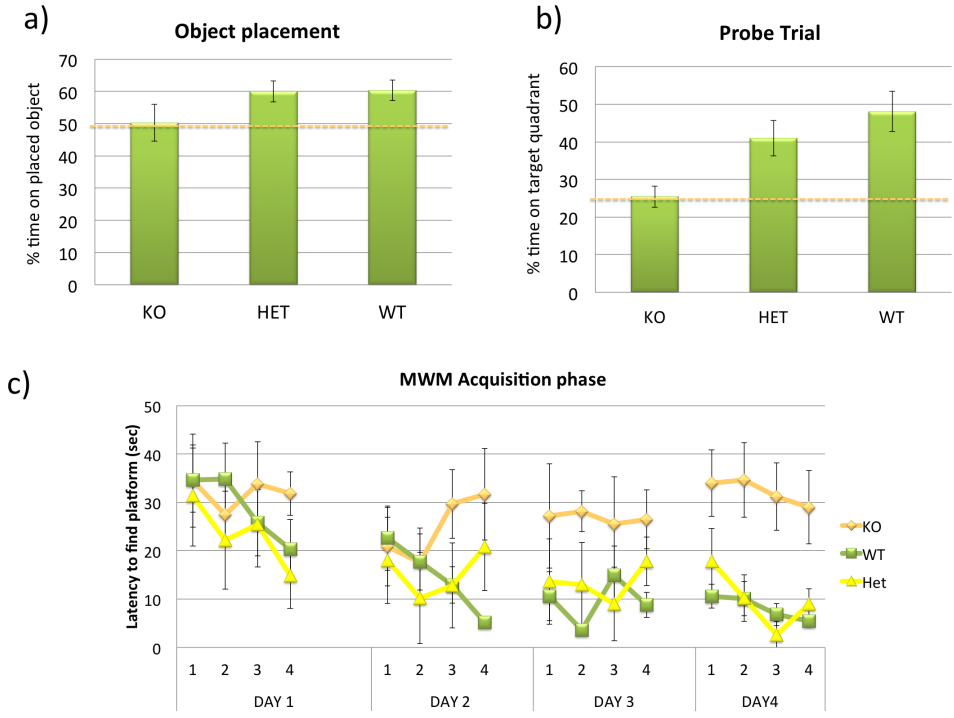

Figure 13. *Slitrk5*$^{-/-}$ mice exhibit spatial reference learning and memory deficits in the object placement and morris water maze tasks a) The mean object exploration time during test phase (when object has been moved to a new context) calculated as a ratio of time spent exploring novel placed object over the total time exploring both objects. Dashed line represents chance. An n of 10 animals per genotype was analyzed; error barrs represent s.e.m. (T-test comparing WT and KO is 0.05; WT and het is 0.9). **b** and **c)** Morris water maze (MWM) task. **c)** Acquisition phase of MWM task showing the mean latency to find a fixed-location hidden platform was averaged across trials. Animals received 4 trials per day during 5 days with a 15 min inter-trial interval, n = 7-10 (statistical analysis using repetitive measures ANOVA p = 0.007). **b)** Probe trial of MWM was performed 24h after 4 days of training, dashed line represents chance. Shown is the percent time spent exploring the target quadrant (where hidden platform was previously located) for the duration of 60 seconds. Error bars represent s.e.m.. (T-test comparing WT and KO was 0.0026, comparing WT and Het was 0.3).

FUTURE DIRECTIONS, SLITRK5 A MULTITALENTED PROTEIN

Slitrk5 induces synapse formation

AMPARs, together with NMDARs are structural components of excitatory synapses. The extracellular domain of GluA2 plays a critical role in maintaining the stability of excitatory synapses [232]. LRRTM2 is another LRR-containing protein that modulates surface levels of AMPARs, namely GluA1. In addittion, LRRTM2 can also induce synapse formation in hippocampal neurons [98]. Recently, a study performing a screen for new synaptogenic proteins identified Slitrk2 as a positive candidate [83]. The extracellular domain of Slitrk2 shares 52% identity with Slitrk5 (Table 2). In all, given its structural characteristics, it is reasonable to speculate that Slitrk5 could also induce synapse formation. To address this question, we performed a coculture assay in which COS cells transfected with Slitrk5 were cultured with hippocampal neurons and synapse formation was quantified by immunostaining of a pre-synaptic marker. Neuroligin was the first synaptogenic protein identified using this method, therefore it was used as a positive control [233]. Human Slitrk5 cDNA was cloned fused with GFP or RFP (FL-Slitrk5). A construct in which Slitrk5 lacked the intracellular domain was also used to test if the extracellular domain was sufficient to induce synapse formation (ECD-Slitrk5) (Figure 14). To obtain a quantitative measure of each protein's ability to instruct presynaptic differentiation, we measured the amount of a pre-synaptic protein, synapsin, clustering associated with transfected COS cells and not associated with MAP2-positive dendrites, to exclude interneuronal synapses (Figure 14 b). Synapsin intensity was also quantified (Figure 14 c). Robust synaptogenic activity for FL-Slitrk5, ECD-Slitrk5 and Neuroligin was observed. Interestingly, in some occasions FL-Slitrk5 formed clusters that would juxtapose to synapsin staining (Figure 14 a). Intracellular FL-Slitrk5 appeared in a puncta-like shape, characteristic of early endosomes. ECD-Slitrk5 did not form clusters and had a more diffuse distribution compared to FL-Slitrk5, suggesting that

even though the extracellular domain is sufficient to induce pre-synaptic differentiation, the cytoplasmic domain is critical for proper cellular targeting. In contrast to Slitrk5 and Neuroligin, GFP or RFP did not induce robust synaptogetic specification. (Figure)

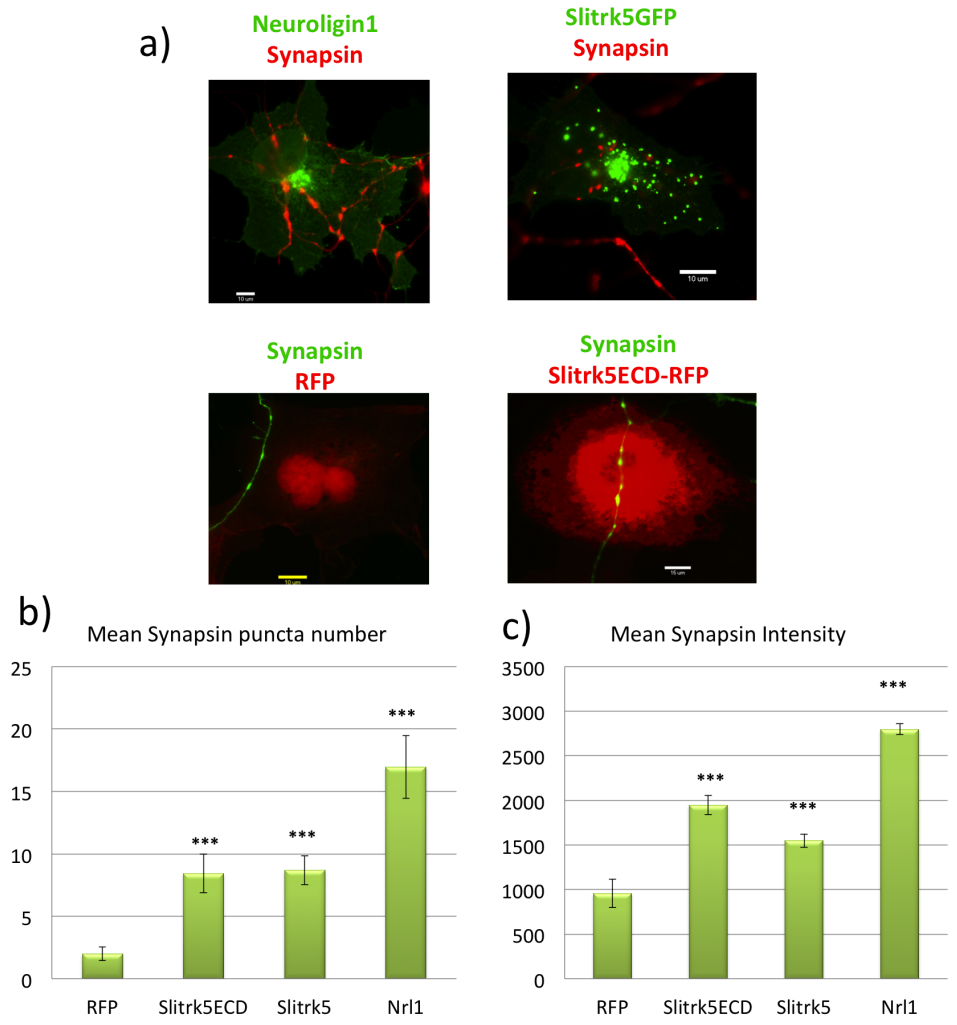

Figure 14. Slitrk5 induces synapse formation. a) COS7 cells transfected with the indicated constructs cultured with hippocampal neurons were immunostained with a pre-synaptic partner synapsin (shown either in green or red as indicated). b) Quantification of synapsin number clustering with transfected cells that were not juxtaposing MAP2 staining

(not shown). c) Quantification of synapsin intensity of clusters contacting transfected COS7 cells. An average of 30 cells per condition was quantified and 3 independent experiments were performed. Error bars represent s.e.m.. RFP (red fluorescent protein); Slitrk5ECD (truncated Slitrk5 containing only the extracellular domain); Nrl1 (Neuroligin 1).

Slitrk5 modulates TrkB trafficking and signaling

Considering the shared structural characteristics between the Slitrk family and Trks such as the LRR domains, the intracellular tyrosines, as well as the similarities between the Slitrk5 knockout mouse and the BDNF knockout mouse (both show selective atrophy of the striatum), together with the previously described associations between Slitrk6 and neurotrophin system [117], we were curious to see if Slitrk5 and TrkB (the Trk member most highly expressed in the brain) crosstalk. We started by looking at their cellular localization. Since our custom-made antibody is improper for immunocytochemistry analysis, we transfected cortical neurons with FlagSlitrk5. We added antibodies to the extracelluar domain of TrkB live to neurons as well as a Flag antibody and stimulated the neurons with BDNF to induce TrkB internalization. After a short incubation period we observed that these proteins co-localized (Figure 15). In fact, the fraction of TrkB co-localizing with Slitrk5 upon BDNF treatment (65.9 ± 2.7%) is almost double than with EEA1 (39.2% ± 3.3%) at the similar time point (Chapter 2). It is possible that the phenotype observed in the Slitrk5 knockout mouse may be in part mediated by deficient neurotrophin signaling, and that would explain the similarities with BDNF knockout mice. Moreover, the fact that Slitrk5 localizes mainly to endosome-like compartments and it can modulate trafficking of AMPARs, may indicate that Slitrk5 can also modulate the endocytic trafficking of TrkB. To test this we performed a TrkB degradation assay with cortical neurons transduced with FlagSlitrk5 or GFP. We assessed single BDNF treatment time point (180 min) that had previously been shown to induce significant degradation of TrkB (Chapter 2). Indeed, we observed

that over-expression of Slitrk5 delayed BDNF-mediated TrkB degradation (Figure 16). Treatment of control neurons with BDNF led to an average of 45.1 ± 8.9% degradation index, as compared with the untreated control. However, overexpression of Slitrk5 led to a degradation of only 72.1 ± 3.4%. Thus, Slitrk5 slows BDNF induced degradation of TrkB receptor by a still unknown mechanism (p value = 0.03).

We showed in chapter 2 that NT4 delays TrkB degradation and this is reflected in prolonged signaling of the lingering receptor. We then asked if over-expression of Slitrk5 would also lead to prolonged signaling. To our surprise, higher levels of Slitrk5 were associated with reduced phosphorylation of Akt and Erk1/Erk2, upon BDNF treatment. In the absence of BDNF, in both conditions (Slitrk5 and GFP over-expression) the basal phosphorylation levels of these proteins were undetectable. Thus, our data suggests that Slitrk5 co-localizes with TrkB to the same compartment, where they seem to interact. Moreover, we observed that Slitrk5 modulates BDNF mediated trafficking of TrkB and it impairs its signaling. Considering the similarities of the BDNF and Slitrk5 null mice we would expect that Slitrk5 would promote TrkB signaling. It is possible that, as it has been observed with other proteins of the trafficking machinery, manipulating the levels of these proteins either by over-expression or down-regulation, has a similar effect in delaying the trafficking flow. Slitrk5 might sort TrkB to an alternate compartment not coupled with the lysosomal degradation neither with signaling partners. Analysis of the endocytic trafficking of TrkB in a Slitrk5 knockout background will help us elucidate these questions.

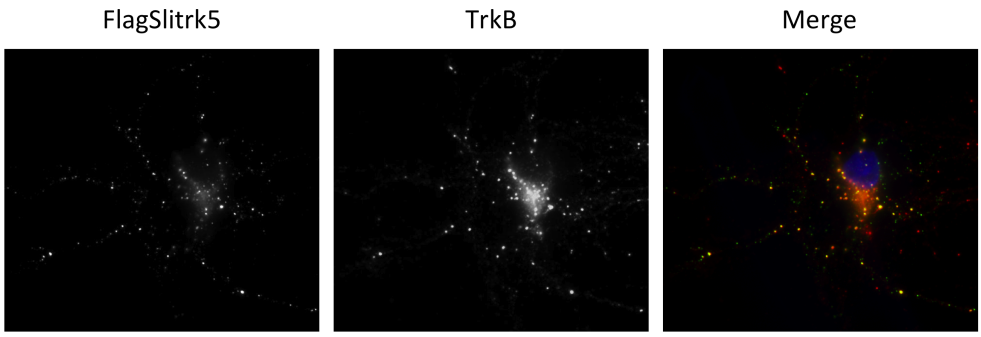

Figure 15. Co-localization of Slitrk5 with TrkB. DIV5 cortical neurons were transfected with FlagSlitrk5. Antibodies against Flag and TrkB were live fed to cortical neurons to label surface fraction of these proteins. Neurons were then treated with BDNF for 15 min prior fixation and immunocytochemistry. Quantification of co-localization was made with Metamorph by initially counting total TrkB puncta, followed by TrkB puncta that co-localized with Slitrk5. This analysis showed that 65.9 ± 2.7% of TrkB co-localizes with Slitrk5.

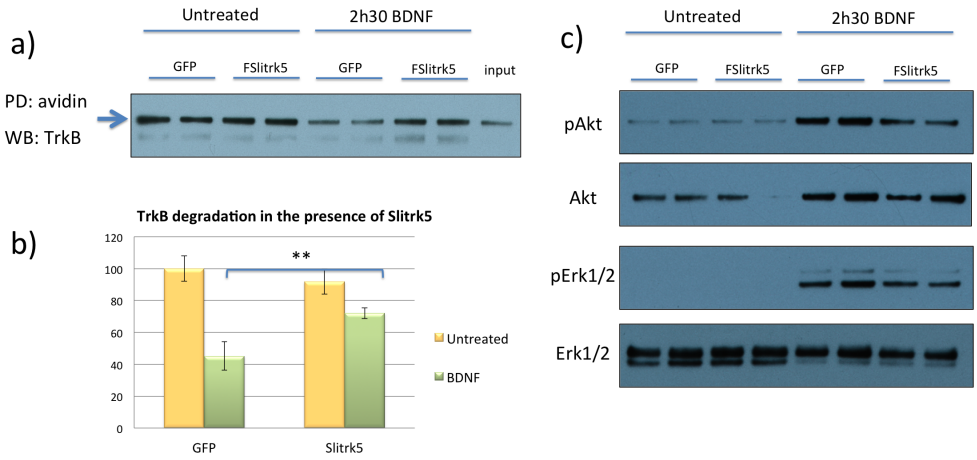

Figure 16. Slitrk5 slows BDNF induced degradation of TrkB receptor. Degradation assay of TrkB receptor with cortical neurons transduced or not with FlagSlitrk5, control neurons were transduced with GFP. Neurons were treated or not (untreated) with 50 ng/ml of BDNF for 180 min. Untreated cells were also surface biotinylated an incubated for the

same amount of time without neurotrophin. **a)** Western blot showing TrkB after avidin pull down. **b)** Quantification of an average of 2 experiments was done with ImageJ. Error bars represent s.e.m. Treatment of control neurons with BDNF led to an average of 45.1 ± 8.9% degradation index, as compared with the untreated control. However, overexpression of Slitrk5 led to a degradation of only 72.1 ± 3.4%. Thus, Slitrk5 slows BDNF induced degradation of TrkB receptor in a significant manner (p value = 0.03). **c)** Signaling mediated by BDNF in the presence or absence of Slitrk5 over-expression. Lysates from extracts of degradation assay were analyzed by western blot for phosphorylation of Akt and Erk1/Erk2.

DISCUSSION

Taken together, our data demonstrate that targeted inactivation of *Slitrk5* in mice leads to OCD-like behavioral phenotypes, including overgrooming with elements of self-mutilation. Mechanistically Slitrk5 decreases surface expression of AMPARs leading to altered hippocampal activity that was reflected in impaired spatial reference memory. Slitrk5 also induces synapse formation in hippocampal neurons.

We found increased neuronal activity in the orbitofrontal cortex of *Slitrk5*$^{-/-}$ mice, which is consistent with functional imaging findings in humans with OCD that implicated deregulation of the CSTC circuitry [223,234]. In addition, *Slitrk5*$^{-/-}$ mice have anatomical deficits in the striatum, such as reduced striatal volume, as well as decreased dendritic complexity of striatal medium spiny neurons. Although this region has not been consistently found to be altered anatomically in people with OCD [126,220,222] emerging literature suggests that striatal dysfunction may underlie behavioral deficits in individuals with OCD [234]. In this context, it has recently been postulated that striatal defects, in the presence of orbitofrontal cortex over-activation, could lead to deficits in thalamic filtering or imbalance in the direct and indirect pathways of the basal ganglia (figure 2 Chapter 1) [235]. Given the ubiquitous neuronal expression of Slitrk5, we were surprised by the selective effect observed on the orbitofrontal cortex and on striatal neurons. Analysis of the caudate putamen and thalamus did not show upregulation of FosB expression. Moreover, fractal dimension analysis of dentate granular neurons in the hippocampus did not show significant dendritic arborization defects in this region. On one hand this selectivity, is reminiscent of the effect of other proteins such as huntingtin, which is also widely expressed in the CNS, but alterations in the huntingtin protein result in functional defects predominantly in striatal and cortical neurons, directly leading to Huntington's disease pathology [236]. On the other hand, it is possible that Slitrk5 may form a signaling complex with corticostriatal-specific proteins, which could

explain these region-specific effects. These findings prompt us to look more carefully not only at other brain regions but also at the developmental expression of the *Slitrk5*$^{-/-}$ mouse phenotype. We started by analyzing young *Slitrk5*$^{-/-}$ mice and then expanded our studies to different brain structures that also highly express Slitrk5.

Electrophysiological analysis of the striatum of young *Slitrk5*$^{-/-}$ mice (P20), revealed increased basal synaptic transmission that was selectively mediated by AMPARs, and not NMDARs, suggesting that even though subtle, the *Slitrk5*$^{-/-}$ mice already show some brain abnormalities early in development. It is possible that overall activation of the brain due to an increase in surface expression of AMPARs led to cytotoxicity that was reflected in a smaller brain at 5 month old and an even smaller striatal volume leading to OCD-like behaviors. The elevated AMPAR currents in the striatum of young *Slitrk5*$^{-/-}$ mice suggest that Slitrk5 somehow stabilizes AMPARs intracellularly, leading to a decrease in the surface pool of these receptors. We tested this hypothesis by overexpressing Slitrk5 cDNA in striatal cultured neurons and quantifying the surface pool of AMPARs. Indeed we observed that lentiviral delivery of FlagSlitrk5 led to a reduction in surface AMPARs, complementing the electrophysiological findings. Interestingly, over-activation of the glutamatergic system within the CSTC pathway has been implicated in humans with OCD [152]. Research groups that investigated the actions of anti-glutamatergic drugs, namely riluzole, alone or in combination with SSRIS, found that inhibiting this pathway contributed to alleviate the symptoms of OCD [237,238].

Changes in hippocampal volume have been associated with patients suffering of OCD [239]. Considering the high expression level of Slitrk5 in this region we decided to investigate the mechanism of action of Slitrk5 in this brain structure. We analyzed mice at 2 months, before the development of over-grooming behavior, and observed that basal synaptic transmission in the hippocampus was enhanced. Similarly to the striatum, this correlated with the biochemical studies showing that rescuing expression of Slitrk5 in hippocampal neurons cultured from *Slitrk5*$^{-/-}$ mice,

led to a decreased AMPAR surface expression. Intriguingly we found a selective reduction in the GluA2 subunit, with no effects on GluA1 surface levels. Of all AMPAR subunits, GluA2 is the one that has the strongest impact on the biophysical properties of oligomeric AMPARs. This is due to the presence of an arginine in the pore-forming sequence of GluA2, whereas the other subunits contain a glutamine, conferring impermeability to bivalent cations, such as Ca^{2+} [240]. As a result, GluA2-lacking AMPARs are strongly inwardly rectifying and Ca^{2+} permeable [230]. An interesting theory is that neurons may utilize Slitrk5 to regulate the GluA2 composition of surface AMPARs (synaptic and extra-synaptic) within specific contexts and thus change the cell's output. Naturally occurring GluA2-lacking AMPARs appear to be inexistent in the hippocampal CA1 region. A recent study that employed a molecular quantification of subunit composition of AMPARs at CA1 synaptic and extra-synaptic sites showed that virtually all surface receptors contain GluA2, with the majority comprising of GluA1GluA2 heteromers and, the remaining consisting of GluA2GluA3 [241]. However, studies have shown that in certain conditions GluA1 homomers can appear. Analysis of the GluA2-knockout mouse have shown that in the absence of this subunit, GluA1 and GluA3 homomeric receptors can form in hippocampal neurons [242]. Moreover, prolonged AMPAR blockade in cultured hippocampal neurons, leads to an increase in GluA1 expression and GluA1 homomeric receptors at synapses [243]. Together, and given the particular biophysical properties of AMPARs lacking GluA2, these studies suggest that in specific contexts neurons may utilize mechanisms, such as those mediated by Slitrk5, to modulate the GluA2 content of surface AMPARs.

In trying to understand the mechanisms underlying Slitrk5-dependent selective decrease of GluA2 subunit surface expression, we found that Slitrk5 could interact with a known modulator of GluA2 traffic, Pick1. Interestingly, a truncated form of Pick1 protein lacking the PDZ domain (which mediates interaction with GluA2) and the N-terminal acidic domain, was unable to interact with Slitrk5,

suggesting that the binding may be mediated by the PDZ domain and that this interaction may be mutually exclusive. GluA2 receptors are anchored at surface and intracellular membranes by ABP/GRIP but undergo cycling between these membranes in association with Pick1 [229,244]. Binding of Pick1 to Slitrk5 in intracellular compartments might prevent Pick1 interaction with GluA2 and thus favor GluA2 binding to ABP/GRIP, stabilizing AMPARs at intracellular compartments.

Future electrophysiological studies should elucidate the precise subunit composition of AMPARs in the hippocampus of the *Slitrk5*$^{-/-}$ mouse; however, our studies suggest that by modulating both expression and availability of Slitrk5, neurons can selectively regulate the biophysical properties of AMPARs.

The extracellular domain of GluA2 plays a critical role in maintaining the stability of excitatory synapses, and also contributes to spine size and density [232,245]. Recent studies employing screens in order to identify new proteins that can induce synapse formation have found a prevalence of LRR-containing proteins with this capability (Table 1 Chapter 1) [83]. Slitrk2 was also identified in this screen as a new synaptogenic protein [83]. The extracellular domain of Slitrk2 shares 52% identity with Slitrk5 (Table 2). In all, these observations led us to hypothesize that Slitrk5 may also induce synapse formation. Indeed we observed that when expressed in COS7 cells, which were cocultured with hippocampal neurons, Slitrk5 instructs the accumulation of pre-synaptic markers at cell contacts. We also found that, even though the extracellular domain of Slitrk5 was sufficient to achieve this function it altered the cellular targeting of the protein. Future studies should elucidate if Slitrk5 acts like other LRR-containing synaptic proteins, such as LRRTM or NGL, binding to a pre-synaptic partner in order to induce synapse formation. Ongoing studies in our laboratory are aiming to answer this question by performing mass spectromic analysis of Slitrk5 binding proteins.

Considering the crucial function Slitrk5 plays for efficient surface expression of AMPARs, proper hippocampal functioning and for synapse formation, we

hypothesized that in the absence of Slitrk5, hippocampal-dependent learning and memory tasks would be compromised. We tested the *Slitrk5*$^{-/-}$ mice on two distinct hippocampal-dependent tasks: the novel object placement and the Morris water maze task. The novel object placement task allows measurement of the memory for spatial location within a familiar arena where the objects were previously explored. This spatial episodic memory task has been shown to depend on an intact hippocampus [246]. Indeed we observed that the *Slitrk5*$^{-/-}$ mice were unable to recognize a displaced object associated with a new context. At 2 month old the heterozygous mice showed a performance similar to wild types, however, at 3 month old, they behaved like *Slitrk5*$^{-/-}$ (data not shown), suggesting that Slitrk5 dosage is important and that the defects are developmentally regulated. We then tested the *Slitrk5*$^{-/-}$ mice on an additional hippocampal-dependent spatial learning task the Morris water maze [247]. The *Slitrk5*$^{-/-}$ mice were significantly impaired during the acquisition phase as wells as on a 24 hour probe trial in this task, reinforcing the importance of Slitrk5 for spatial learning and memory. Interestingly, patients with OCD, also display learning and memory impairments [248,249].

Collectively these studies suggest that Slitrk5 plays critical roles for CNS development and function. In the absence of Slitrk5, there is an aberrant enhancement of surface AMPARs, which was reflected in altered basal synaptic transmission of key CNS structures. Moreover, Slitrk5 acts as a synaptogenic protein critical for hippocampal-dependent learning and memory. These developmental abnormalities were reflected in behaviors that recapitulate the hallmarks of OCD-like phenotypes: repetitive overgrooming that is ameliorated with SSRI treatment; enhanced orbitofrontal cortical activation; deficient cortico-striatal transmission and morphological changes in the medium spiny neurons of the striatum.

Future genetic studies with OCD patients will be determinant to ultimately link Slitrk5 and OCD. Previous reports on Slitrk1 have revealed the difficulties of associating a rare genetic variant with a psychiatric disorder [9]. Their low frequency,

together with the inherently variability associated with human psychiatric studies that are caused both by symptomatic variability and ethnic heterogeneity, contribute to the difficulties of associating a single genetic variant with a disorder. Moreover, given the complexity of human psychiatric disorders, a combination of genetic factors rather than a single variant might be the cause of a given condition. It is possible that mutations in Slitrk5 will be associated with a selective subset of OCD patients with specific behavioral symptoms. OCD is a heterogeneous disorder composed of a multitude of symptoms [128]. Even though the Diagnostic and Statistical Manual of Mental Disorders offers a single definition for OCD, it is established amongst researchers and clinicians that there are different subtypes of OCD that differ not only in symptomology but also in susceptibility to pharmacological treatments [250]. Age of onset in OCD has a bimodal distribution and evidences suggest that early onset OCD is distinct from late onset OCD [125,251]. Based on our results with the Slitrk5$^{-/-}$ mice, it would be interesting to know if Slitrk5 is associated with a specific subtype of OCD with adult onset in which cognitive impairments are also observed.

In all, we provide a new mouse model of OCD-like behaviors, involving a previously uncharacterized neuronal transmembrane protein that modulates region-specific glutamatergic neurotransmission. This model can be used to further dissect the role of Slitrk5 in molecular pathways underlying the pathogenesis of obsessive-compulsive behaviors and cognitive impairments.

Chapter 4 - General Discussion

GENERAL DISCUSSION

The aim of this thesis was to study the cell biology of protein families involved in CNS function and disease. We started by investigating a well-established protein family that is essential for nervous system development and function: the neurotrophin family of ligands and receptors. Since the discovery of NGF as a secreted molecule that could sustain survival of sensory neurons and induce differentiation, research on this growth factor family soared [3,18]. Subsequent studies attempting to find a similar function for this factor in the CNS actually revealed the scarceness of NGF in this region, therefore, the search for a molecular counterpart of NGF in the brain began. Strenuous efforts led to the purification of BDNF from the pig brain and, interestingly, this new neurotrophic factor was capable of sustaining survival of neuronal populations irresponsive to NGF [11]. Due to the conserved sequences of these two proteins, the identification of two additional members: NT3 and NT4, was facilitated. The expression profile of neurotrophins and their receptors is spatially and developmentally regulated. NGF and its receptor TrkA are highly expressed in the PNS but their presence and function in the CNS is limited. On the contrary, TrkB and its two ligands, BDNF and NT4, are widely expressed in the CNS. TrkC and NT3 are expressed in both systems [2,28,29]. The hallmark mechanism of neurotrophins actions involves the secretion of limiting amounts of neurotrophins by target cells, which ensures survival of responsive cells that bind, internalize and, allow retrograde trafficking of the ligand-receptor complex through a signaling endosome. Studies leading to the description of these mechanisms were mainly performed with peripheral neurons [65]. In sensory neurons, retrograde trafficking of the signaling endosome triggers activation of signaling proteins along the axon as well as in the soma. In addition, it also modulates transcription of critical genes [65]. Recent studies have suggested that, as opposed to the PNS, neurotrophins are not essential for survival of neurons in the CNS, but rather play important roles for neuronal differentiation, function and, plasticity [27]. Another major difference

between these two systems is the ligand heterogeneity and specificity. While in the periphery NGF-TrkA are the predominant axis, in the CNS system two ligands, BDNF and NT4, have been described to bind with similar affinities to the same receptor, seemingly fulfilling different functions. However, the mechanisms mediating these differential effects are still unknown and were one of the focuses of this thesis. We discovered that, even though BDNF and NT4 can efficiently activate TrkB and promote endocytosis, they sort the receptor to distinct endocytic fates. Prolonged neurotrophin treatments revealed that BDNF leads to fast down-regulation of TrkB whereas NT4 does not. Thus, NT4 leads to longer lasting signaling mediated through TrkB which, most likely explains previous studies suggesting that NT4 is a stronger trophic factor (supporting survival of more sensory neurons; promoting synaptic function and maturation; reversing monocular deprivation, to name a few [176,183,184]). Hence, even though NT4 is expressed at much lower levels than BDNF in the brain, it induces sustained and prolonged activation of TrkB whereas BDNF triggers fast signaling [28].

Previous work analyzing the endocytic trafficking of Trk receptors focused their attention on specific domains of the receptor and did not address ligand variability. Analysis of the endocytic trafficking of TrkA and TrkB showed that upon NGF treatment, TrkA predominantly recycles back to the surface (80%) whereas BDNF-activated TrkB is mostly sorted to the degradative pathway (only 40% recycles). However, only NGF and BDNF were analyzed in this study, NT3 and NT4 were not investigated [63]. A fraction of TrkA is also sorted to the degradative pathway and binding to a specific E3 ligase, Nedd4-2, was found to be modulated by phosphorylation of cytoplasmic tyrosines [62]. The authors then sought to investigate which region in TrkA would be responsible for the ligand-dependent recycling. A series of mutation analysis led to the identification of a stretch of amino acids near the juxtamembrane region as being key for this process. This sequence is different from TrkB and when transplanted into TrkB, it redirected this receptor into

recycling versus degradative pathway [63]. Subsequent studies showed that an isoform of TrkB lacking the intracellular domain TrkB.T1, which is highly expressed in the brain, predominantly recycles to the membrane upon BDNF treatment by a default mechanism (NT4 was not tested). In contrast, full length TrkB recycled in a sequence-directed manner and it was dependent on interaction with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) [252]. Hrs was initially established as a key molecular player recognizing and sorting ubiquitinated cargo to the lysosome, promoting its degradation [253]. Subsequent studies revealed a surprising role for Hrs in mediating sequence-directed recycling of the β_2 adrenergic receptor [254]. This study was noteworthy since it introduced a new level of regulation into the endocytic trafficking field previously disregarded. Prior studies, mainly using EGFR and TfnR as models, coined the idea that sorting to the late endosome and lysosome is sequence directed, whereas in the absence of specific target information, receptors are transported with the bulk of the membrane and recycle to the surface [54,255]. The finding that TrkA recycling is sequence-dependent suggests that this receptor also recycles through a specific sorting molecule.

In our studies we asked the question if sorting to different endocytic compartments could be achieved by modulating the system from a ligand perspective. Our finding that BDNF induces similar endocytosis of TrkB receptor as NT4 but sorts the receptor to a degradative pathway confirmed our hypothesis. Considering the much slower degradation curve elicited by NT4 we believe this ligand is sorting the receptor to the recycling pathway, therefore, one could ask if NT4 recruits Hrs in a more efficient manner than BDNF. However, our mass spectrometry analysis shows that both ligands recruit Hrs efficiently (Table 1 Chapter 2). An alternate hypothesis is that the acidic pH of the early endosome that negatively modulates NT4-mediated ubiquitination might sort the receptor to the recycling pathway rather than to degradation (Figure 1). Experiments testing NT3

and NGF induced phosphorylation of TrkA at different pHs, showed that in acidic environments mimicking the early endosome compartment (pH = 6), NT3 could no longer maintain TrkA phosphorylation, whereas NGF could. This suggests that NT3 uncouples from TrkA in the early endosome leading to recycling of the receptor [202]. Indeed NT3 is unable of eliciting retrograde trafficking of TrkA containing signaling endosomes [30]. These experiments were performed using concentrations at which both ligands elicit similar TrkA phosphorylation at pH=7 (NT3 was 10 fold more concentrated than NGF) [202]. In the case of NT4 and BDNF, the affinity for TrkB is similar at pH=7, however, upon acidic wash it was shown that the dissociation was much faster for NT4 [168]. Similar experiments with EGFR ligands have shown that ligand-receptor uncoupling in the early endosome promotes recycling as opposed to degradation [57]. In the case of BDNF and NT4, we think that a similar mechanism is in place. We observed that BDNF induces more efficient ubiquitination of TrkB than NT4 at acidic pHs, whereas phosphorylation was similar. Thus these neurotrophins probably sort the receptor to different microdomains within the early endosome, with BDNF probably allowing a conformation that recruits a specific E3 ligase or other players involved in sorting ubiquitinated cargo to the lysosome, more efficiently, thus leading to faster degradation (Figure 2). NT4 might promote more efficiently recruitment of molecules mediating recycling or retrograde trafficking. The identification of TrkB E3 ligase will greatly contribute to elucidate these pathways, as we will be able to directly assess the recruitment of this protein upon BDNF or NT4 treatment. Unfortunately, analysis of candidate molecules based on our mass spectrometry analysis, did not lead to fruitful outcomes suggesting that the binding of E3 ligase to TrkB might be transient and labile.

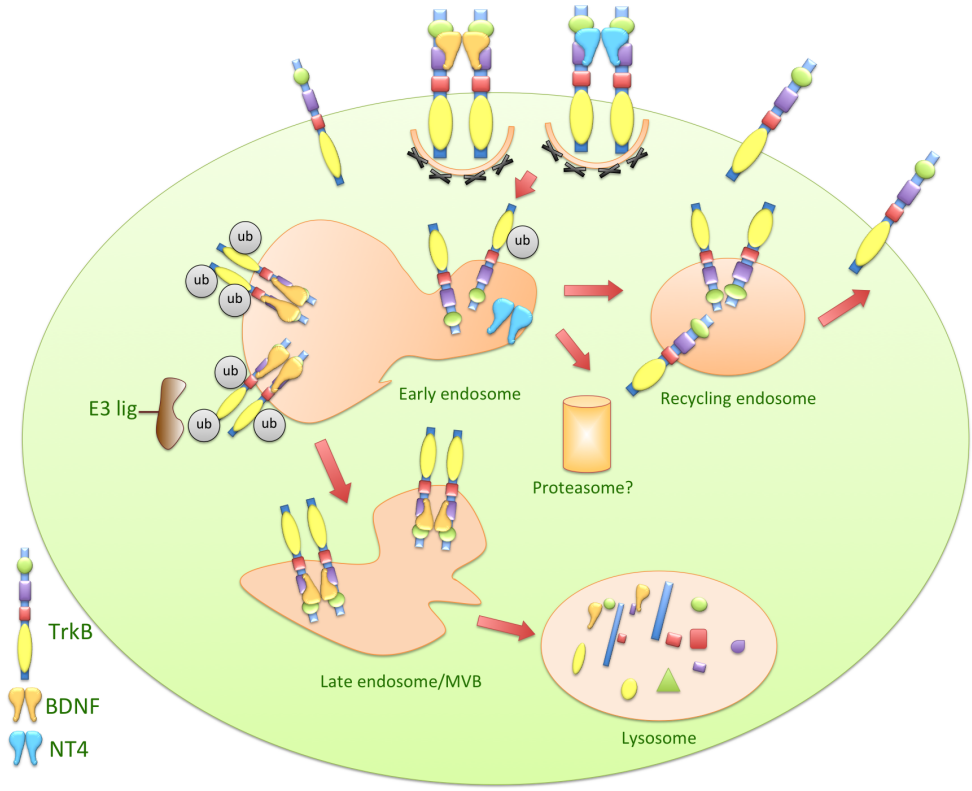

Figure 1: Model of endocytic trafficking of TrkB receptor elicited by BDNF or NT4. BDNF and NT4 bind to TrkB receptor with the same affinity and induce equivalent phosphorylation and endocytosis, in a clathrin-dependent way. The acidic environment of the early endosome reduces the affinity of NT4 to TrkB and ligand-receptor uncoupling promotes recycling of the receptor. On the other hand, BDNF and TrkB remain bound. This leads to different receptor conformations elicited by BDNF and NT4. Most likely, BDNF allows a more efficient recruitment of a specific E3 ligase, as supported by the higher ubiquination state achieved with this ligand at acidic pHs, sorting the receptor to the degradative pathway. NT4 sorts the receptor either to a separate compartment that is not linked to the degradative pathway and thus allows sustained signaling.

The studies depicted here clearly show the importance of regulating the endocytic trafficking of Trk receptors, and how modulation of this system can lead to

opposite biological outcomes. In addition to regulating the sorting of the receptor, the neurotrophins themselves follow different sorting pathways. Neurotrophins are initially synthetized as precursors or pro-neurotrophins, which are cleaved to produce the mature proteins [256]. The pro-domain of BDNF contains sequences unique to this protein that allows binding of the sorting molecule sortilin. Sortilin sorts BDNF to the regulated secretory pathway and, disruption of this binding site reroutes BDNF to the constitutive secretory pathway, without affecting NT4 [257]. NT4 and BDNF protein sequences are most divergent in the pro-domain. Therefore it was not surprising that sortilin did not bind NT4. Identification of the molecules that regulate NT4 sorting will be critical do understand how this process can be regulated. Humans carrying a common Val/Met genetic variant in the pro-domain of BDNF have high anxiety and hippocampal deficits [171,258,259]. The finding that BDNF with the Met allele bound less efficiently to sortilin and as a consequence was sorted to the constitutive secreted pathway, as opposed to the activity-dependent secreted pathway, reinforces the importance of finding similar molecules regulating the trafficking of NT4 [257].

It is interesting to note that neurotrophins and their receptors do not exist in Drosophila melanogaster or Caenorhabditis elegans, both invertebrate organisms with a nervous system, strengthening the idea that these proteins are not absolutely necessary for the development of neural circuits [18]. Instead, they appeared in evolution to allow complex systems to engage in higher order cognitive functions. In this line of thought it is curious that NT4 is the less conserved amongst species and, homologues of this protein have not been found in neither fish nor birds, that do express BDNF and NGF homologues, implying that it is the latest “addition” to the neurotrophin family [260]. According to phylogenetic analysis the four vertebrate neurotrophins were generated by gene duplication of the NGF/NT3 and BDNF/NT4 groups after the lamprey split from the common vertebrate lineage. Despite this major duplication that was conserved up to mammals, other rounds of duplications

occurred. For instance, fish express Neurotrophin 6 and 7 and the acquisition of specific different functions correlates with the increase in complexity of the vertebrate lineage [260,261]. Considering these findings, NT4 may have emerged with the increase in complexity of the nervous systems across evolution, as an extra member in the neurotrophin family was necessary to allow more variability and sustain higher order complex behaviors. Besides expansion of existing families, throughout evolution new gene families have arisen as a way to allow more complex organisms to adapt. Studies investigating the evolution of mammalian genes infer a gain of 689 new genes since the split from chimpanzees and a loss of only 86 genes [262]. Moreover, expansions in the human lineage for gene families with brain-specific functions had larger than expected changes, confirming the need for new molecules in order for a more complex system to develop [262].

With a continued interest of understanding how protein families can modulate key aspects of CNS development and function, we focused on another protein family that also appeared late in evolution, are highly expressed in the brain and most importantly, share structural similarities with Trks: the Slitrk family. Orthologs of Slitrk genes are thought to be present in fish species but not in lower organisms. However, a thorough examination of Slitrk orthologs across species has not been done so far. Similar to Trks, Slitrks also contain extracellular LRR domains, an NPXY domain in the cytoplasmic juxtamembrane region and putative phosphorylated tyrosines at the C-terminus. We selected the Slitrk5 member as a model to study the Slitrk family, considering that it contained all the domains conserved in the family and its expression was one of the highest in the brain [118]. Interestingly we found that BDNF activated TrkB co-localized with Slitrk5 in cortical neurons at higher ratios than with EEA1, suggesting that these two proteins share the same endocytic compartment. Moreover, we found that over-expression of Slitrk5 delayed BDNF-induced degradation of TrkB in cortical neurons, discovering a new potential mechanism to modulate TrkB endocytic trafficking.

To further investigate the Slitrk5 functions we developed a complete Slitrk5 KO mouse. Intriguingly, initial analysis of this mouse revealed some similarities with the conditional BDNF null mouse such as striatal atrophy with reduced striatal volume and a marked decrease in dendritic complexity of medium spiny neurons [27], further strengthening a connection between these two protein families. A crosstalk between Slitrks and neurotrophins was implied on the very first set of studies that led to the discovery of the Slitrk protein family [8]. In trying to dissect the functions of Slitrks, these investigators used cultured PC12 and Neuro2a cells (as a model of neuronal cells), and quantified neurite number and length upon Slitrk over-expression. Interestingly, different results were obtained if the transfected cells were treated with NGF or not. In the case of Slitrk1, it was observed that in the absence of NGF, over-expression of Slitrk1 in PC12 cells led to an increase of neurite number per cell, as compared with cells transfected with control cDNA. However, upon NGF treatment, which normally induces robust neurite sprouting in PC12 cells, the Slitrk1 transfected cells had less neurites per cell on average, as compared with the controls, suggesting that in this system Slitrk is antagonizing NGF signaling [8].

In comparison with our Slitrk5$^{-/-}$ mouse, the observations obtained with the Slitrk6 null mouse also advocates for a trophic function for Slitrks. Slitrk6 expression is tightly regulated and very high in the sensory epithelia of the inner ear. Analysis of the development of the vestibular and auditory organs revealed pronounced reduction in cochlear innervation, as well as loss of neurons in the spiral and vestibular ganglia. Moreover, sensory epithelia from Slitrk6 null mice had reduced ability in promoting neurite outgrowth of spiral ganglion neurons. Interestingly, it was found that expression of both BDNF and NT3 mRNA, as well as TrkB and TrkC protein, were decreased in the inner ear. Considering that these two neurotrophins are key for the proper development of this organ, the results argue for a crosstalk between the Slitrk and neurotrophin system [117].

In addition, the Slitrks belong to a family of proteins that similarly to BDNF are associated with psychiatric disorders in humans [9]. Variants in Slitrk1 gene have been associated with Tourrete's syndrome, trichotillomania and OCD. Re-sequencing of X-chromosome genes found two missense variants in Slitrk2 in patients with schizophrenia and in their affected siblings [9]. Our own studies describe how the lack of Slitrk5 expression in the mouse leads to OCD-like phenotypes that were ameliorated with selective serotonin re-uptake inhibitors, the major pharmacological treatment for human patients. Therefore the crosstalk between the Slitrk and the neurotrophin families might go way beyond what our current knowledge allow us to predict.

Based on the findings described in detail on chapter 3, we found that Slitrk5 modulates brain morphology, interacts with TrkB and modulates its endocytic trafficking; induces synapse formation in hippocampal neurons and is key for high order cognitive functions in the sense that its absence leads to OCD-like symptoms and impaired spatial reference memory in the mouse. Figure 2 depicts three possible scenarios that may explain the molecular functions of the Slitrk5 protein as a synaptogenic protein and TrkB partner. One hypothesis is that in a similar way to other LRR containing proteins (see Table 1 Chapter 1), Slitrk5 acts mainly as a post-synaptic protein that induce synapse formation through interactions with a pre-synaptic protein of still unknown identity. In addition, the results obtained with the Slitrk5$^{-/-}$ mouse, as well as previous data on Slitrk1 and Slitrk6, suggest a trophic role for this protein. In this scenario Slitrk5 could act as a transmembrane receptor itself that would be activated by a soluble ligand, leading to activation of downstream signaling cascades that could overlap with the neurotrophin system. Alternatively or in addition, Slitrk5 can directly interact with Trk receptors and modulate Trk mediated signaling. Given the similarities of the Slitrk5 and BDNF null mice, where both show morphological defects in the striatum, it would be interesting to address if the neurotrophic system is compromised in the Slitrk5 null mice. Perhaps Slitrk5

acts as a positive modulator of TrkB function in the absence of which, this receptor does not signal efficiently. P75, another neurotrophin receptor, has been shown to interact with Trks and positively modulate their selectivity to BDNF and NGF. During development of sympathetic neurons, differential expression of p75 and TrkA promotes cell competition. Selective cell survival is dependent on the regulated expression of these receptors and their binding to different neurotrophins [263]. In the CNS however, p75 is barely detectable in normal conditions, thus leading us to speculate that, in the brain, Slitrk5 may play a similar role as p75. Tight regulation of RTK signaling is critical for the overall cell function; moreover, besides BDNF and NT4, also NT3 is expressed in the brain. Therefore, the idea that an analog of p75, such as Slitrk5, would modulate the selectivity of TrkB for these neurotrophins, adding another level of flexibility and regulation is interesting.

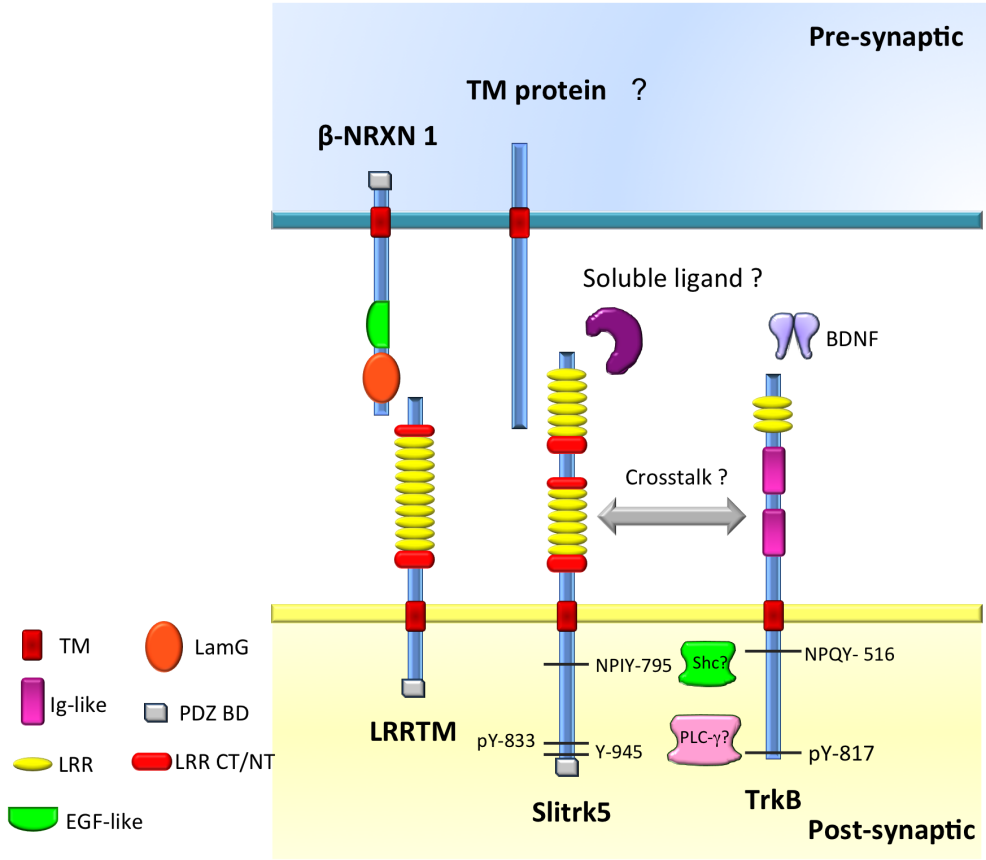

Figure 2: Model of Slitrk5 function. Three hypotheses regarding the mechanisms of Slitrk5 action are displayed: synapse formation hypothesis; soluble ligand hypothesis and transmembrane interactor hypothesis. Synapse formation hypothesis: Slitrk5 can induce synapse formation when expressed in COS7 cells cultured with hippocampal neurons, possibly by interacting with a pre-synaptic partner of still unknown identity. Discovery of such a protein would allow to a complete understanding of Slitrk5 function. As an example, an LRR-containing protein with an established function role as a synaptogenic molecule: LRRTM2, is shown. LRRTM2 localizes to the post-synaptic cell and binds both α and β Neurexins (NRXN) through its LRR domains on the pre-synaptic site.Soluble ligand hypothesis:, Given the structural similarities with a transmembrane receptor, the extracellular LRR domains and putative signaling motifs in the cytoplasmic domain, it would be interesting to find if Slitrk5, in a similar way as Trk receptors, is activated by a secreted

ligand. Binding of soluble ligand to Slitrk5 would drive endocytosis and activation of downstream signaling cascades. Slitrk2, 3 and 5 contain an NPXY motif at the juxtamembrane region. Phosphorylated NPXY serves as docking site for phosphotyrosine bindig proteins such as Shc that links activated receptors to intracellular signaling pathways. Transmembrane interactor hypothesis: The phenotype of the Slitrk5$^{-/-}$ as well as previous data on Slitrk1 and Slitrk6, all converge to a hypothetical trophic role for the Slitrk family. One possible scenario is that Trks and Slitrks act synergistically to achieve efficient downstream signaling in order to maintain basic neuronal functions.

TM, transmembrane domain; Ig, immunoglobulin-like domain; LRR, leucine rich repeat; EGF, epidermal growth factor-like domain; LamNT, laminin N-terminal domain; LamG, laminin G domain.

In addition, we show that by manipulating Slitrk5 levels, the surface expression of GluA2 subunit of AMPARs was drastically changed. The finding that Slitrk5 can modulate trafficking of probably the two most important plasticity protein families in the brain (neurotrophin family and glutamate receptor family), mediating both development and also higher order cognitive functions, strengthens the importance of clearly delineating the molecular mechanisms of this protein family [19,264]. It is still unclear however how Slitrk5 modulates both GluA2 and TrkB trafficking (figure 3). Upon BDNF activation, TrkB is internalized to an endocytic compartment where it co-localizes and interacts with Slitrk5. This interaction leads to differential TrkB trafficking reflected in slower degradation and inefficient signaling. Considering that 66% of endosomes containing TrkB co-localize with Slitrk5 whereas only 39% are also immunopositive to EEA1, its plausible to think that Slitrk5 redirects TrkB to an alternate endocytic compartment, less efficiently linked with ubiquitin-binding proteins (that promote degradation) as well as signaling proteins (as observed by the reduces Akt and MAPK activation) (Figure 3). On the other hand, Slitrk5 can reduce surface levels of GluA2. Our studies show that Slitrk5 can interact with Pick1, which is a key regulator of GluA2 trafficking. Interestingly we observed

that Pick1 binds Slitrk5 and GluA2 through the same domain, suggesting that these interactions may be mutually exclusive. Thus, Slitrk5 may promote intracellular stabilization of GluA2 receptors by favoring GluA2 interaction with ABP/GRIP versus Pick1. We also observed that Slitrk5 can interact with GluA2 in heterologous cells, hence, it could regulate the trafficking of this protein directly. Our results show that Slitrk5 does not alter AMPAR endocytosis; therefore, two additional pathways remain: recycling and degradation. Slitrk5 might prevent GluA2 trafficking to the recycling compartment or promote more efficient degradation through the lysosome, thus leading to reduced surface expression of GluA2 (Figure 3).

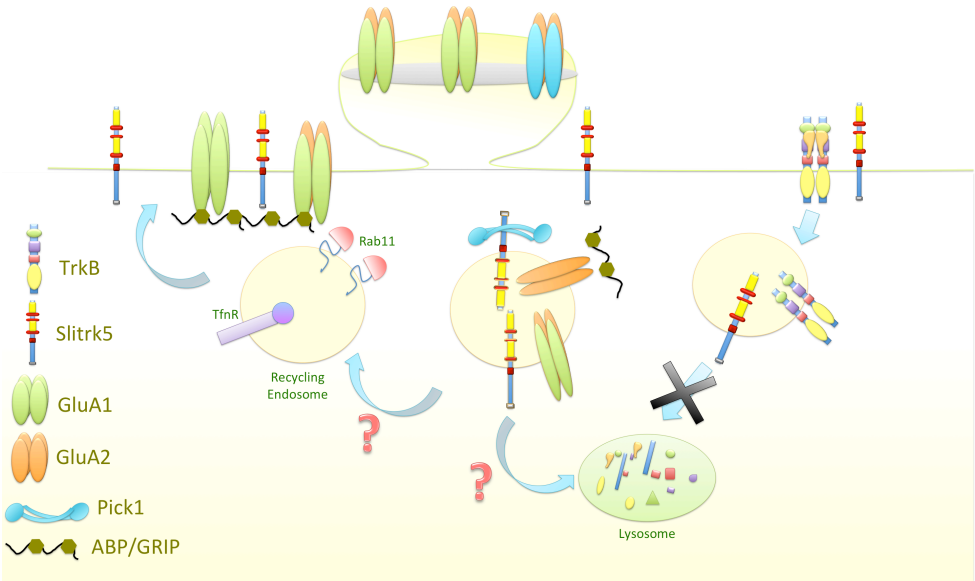

Figure 3: Model of Slitrk5 as a modulator of AMPARs and TrkB trafficking. TrkB trafficking: upon BDNF stimulation TrkB is internalized to an intracellular endocytic compartment that also contains Slitrk5. Interaction with Slitrk5 within this compartment alters TrkB trafficking by reducing receptor degradation, perhaps by stabilizing TrkB receptor. The reduced BDNF-induced Akt and MAPK signaling upon Slitrk5 overexpression suggest that this compartment is not efficiently linked to signaling proteins. AMPARs trafficking: Slitrk5

overexpression leads to a decrease in surface GluA2, an effect that is potentially mediated by Pick1. Slitrk5 can interact with Pick1, which is known to induce GluA2 cycling both from surface as well as from intracellular membranes. Slitrk5 may promote intracellular stabilization of GluA2 receptors by favoring GluA2 interaction with ABP/GRIP versus Pick1. Binding of Slitrk5 to Pick1 may prevent Pick1 interaction with GluA2 and thus favoring receptor anchorage through scaffolding molecules (ABP/GRIP). On the other hand, Slitrk5 can interact with GluA2 (in heterologous cells) and thus regulate the trafficking of this protein directly. Slitrk5 might prevent recycling of GluA2 by impairing trafficking to the recycling compartment or promote its degradation by enhancing sorting to the lysosome.

TrkB and GluA2 have not been found in the same compartments, therefore, the differential expression of Slitrk5 and the interaction with different partners might regulate these proteins differently. The majority of the experiments described in this thesis analyzing the crosstalk between TrkB and Slitrk5 were done using cultures DIV5-6 in vitro, whereas in the studies performed with Slitrk5 and AMPARs more mature cultures (DIV10-15) were employed. In fact it was noted that, the more mature the cultures, the larger impact Slitrk5 expression had in decreasing surface AMPARs. Perhaps the interaction of Slitrk5 with TrkB and GluA2 are temporally regulated so that early in development Slitrk5 would associate more with TrkB and later, when synapses are being maintained and modulated by environmental cues, Slitrk5 would have a more prominent role in the glutamate receptor system. Supporting this view is the analysis of the developmental expression of Slitrk5, TrkB, GluA1 and GluA2 in the hippocampus (Figure 10 Chapter 3). Slitrk5 is highly expressed at embryonic day 17 (E17), peaking post-natally at P15. TrkB is also expressed both pre and post-natal and in parallel with Slitrk5 it peaks at P15. GluA2 expression at E17 was barely detectable, but interestingly, if followed the same pattern as Slitrk5 and TrkB, peaking at P15 and stabilizing throughout adulthood. Future studies will be necessary to further understand the mechanisms employed by Slitrk5 in regulating the trafficking of AMPAR and TrkB.

The Slitrk family has six members and so far, few studies have been performed on 3 members of this family. Little is known about the functions of Slitrk2, Slitrk3 and Slitrk4. Future studies addressing other members of the Slitrk family will elucidate us regarding the main function these proteins are playing in the brain. The fact that these genes show high conservancy across species strengthens the idea that they are critical for proper overall function of the CNS. However, their major function is still unknown. It is plausible to speculate that similarly to other protein families such as the Neuroligin family and the LRRTM family, some redundancy exists. Neuroligin proteins were the first shown to instruct pre-synapse formation when expressed at the surface of non-neuronal cells, in contact with neurons [233]. The finding that the pre-synaptic partner of neuroligin, the neurexins, could instruct post-synapse formation in neurons, complemented this set of cell adhesion-like molecules that bridge the two synaptic sites [265]. Subsequent studies revealed the prominent role neurexin-neuroligin proteins play as central organizing molecules for both inhibitory and excitatory synapse formation. However, despite all the loss of function and gain of function studies illustrating a dramatic effect in synapse formation, the in vivo validation of the relevance of this proteins was still lacking mainly due to functional redundancy of this system [266]. The generation of a triple neuroligin knockout was necessary to obtain a clearly noticeable phenotype [267]. The fact that a single Slitrk gene knockout had such dramatic effects in the morphology of CNS and behavior, suggests that in the Slitrk family, functional redundancy is reduced. However, it will be very interesting to analyze a Slitrk1/Slitrk5 knockout, for example, and assess if the psychiatric phenotype is exacerbated. Moreover, while our studies indicate that overexpression of Slitrk5 in COS7 cells is sufficient for synapse formation, loss of function experiments will be needed to address if it is absolutely necessary. Moreover, it is still unknown if Slitrk5 selectively induces excitatory synapses or if it could also be implicated in inhibitory, GABAergic synapses.

Our studies point to a multitalented transmembrane protein capable of interacting with the neurotrophin system through TrkB, inducing synapse formation and modulating AMPAR trafficking (Figure 2 and Figure 3). Clearly the finding of more Slitrk5 binding partners will contribute to our understanding of this protein's function. Moreover, it will be interesting to address if Slitrk5 acts as a receptor to an unidentified soluble ligand mediating specific signaling cascades affecting both the glutamatergic and the neurotrophic system.

Thus, the findings described in this thesis highlight the importance of meticulously investigating the molecular mechanisms underlying protein families involved in CNS function and development. Considering the complexity of this system, changes that at first sight might seem to induce a small effect, can have significant consequences when integrated into this broader and elaborated arrangement. Small changes in protein traffic can have extremely different outcomes at the cellular level, not to mention at the scale of a whole system. The experiments performed here, analyze discrete events occurring within a limited time-scale, whereas protein trafficking occurs continuously and is crucial for the cell's maintenance. Hence, even if a small fraction of a given set of proteins is mis-sorted in each round, these abnormalities accumulate and in the long range significantly affect the cell's function. In addition, this study strengthens the notion that a complete understanding of the "brain's proteome" is crucial to the identification and development of new molecular targets in disease. Further studies with patients with OCD or OCD-spectrum disorders will possibly elucidate the exact role Slitrk5 plays for these disorders. With the advances of genetics and medicine we are entering an era of "personalized medicine" where treatments are starting to be tailored to the individual's specific traits. Psychiatric disorders are multi-symptomatic, complex and can either be idiopathic or familial. Therefore, multiple combinations of genetic variants, completely different sets of locus, or even environmental cues, might be associated with the origin of a similar condition. As genetic research generates

enormous amounts of new information, the basic cell biology studies will provide a powerful approach to pinpoint the molecular pathways and the targets to be addressed.

Outstanding question box:

1. What is the E3 ligase which ubiquitinates TrkB?
2. Does NT4 sort TrkB to an alternate endocytic compartment?
3. Does NT4 induce retrograde trafficking of TrkB-containing signaling endosomes whereas BDNF promotes local signaling?
4. Which sorting molecules regulate NT4 trafficking?
5. Is Slitrk5 mutated in patients with a particular subset of late onset OCD as well as hippocampal deficits?
6. What are the main molecular partners interacting with Slitrk5?
    a. Is Slitrk5 a transmembrane receptor activated by a soluble ligand and implicated in specific intracellular signaling cascades?
    b. Does Slitrk5 interact with other Slitrk members?
    c. Does Slitrk5 interact with a presynaptic protein maintaining synapse structure?
7. Does Slitrk5 stabilize intracellular pools of GluA2-containing AMPARs or does it promote GluA2 recycling?
8. Will Slitrk5 as well as other Slitrk members turn out to be the main components of the endocytic trafficking pathway or synaptogenic molecules?

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