Neurite outgrowth in PC12-Tet-On cells induced by the variants of receptor tyrosine kinase TrkC

Neurotrophins are a group of proteins that determine the growth, survival and proper development of nerve cells. These neurotrophins are: NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT3 (neurotrophin 3) and NT4 (neurotrophin 4/5) that bind with high affinity to the Trk family receptor tyrosine kinases. NGF is a ligand for TrkA, BDNF and NT4 for TrkB, and NT3 for TrkC. NT3 can also binds with lower affinity to TrkA and TrkB. In the central nervous system, the presence of TrkB and TrkC on the same nerve cells is quite common, which subsequently justifies the search for differences in the specificity of neurotrophin receptors. Trk proteins contain an extracellular domain that determines proper ligand binding, a trans-membrane domain and a cytoplasmic domain, containing the tyrosine kinase core and regulatory sequences. Trk exists as a dimer and the binding of the neurotrophin to the receptor leads to a conformation change in the cytoplasmic domains that allow the mutual phosphorylation of tyrosine residues in the cytoplasmic domains. This results in the formation of docking sites for cytoplasmic adapter proteins and the enzyme phospholipase Cγ1 (PLCγ1) containing the PTB and SH2 domains, triggering intracellular signaling cascades such as Ras / Raf / MEK / ERK, PI3K/Akt and PLCγ1 leading to second messengers in signal transduction formation. The recruitment of PLCγ1 to a phosphorylated tyrosine residue in the C-terminal region of the Trk is essential for the phosphorylation and activation of PLCγ1 by Trk. In order to verify weather the difference of only two amino acid residues in the C terminal regions of TrkB and TrkC and more specifically in the -3 (S/T) and -1 (V/I) positions immediately before the PLCγ1 binding site is sufficient to induce differential neurite growth following activation of the receptors as well as the importance of the tyrosine residue in the recruitment site of PLCγ1 to TrkC, the following TrkC variants were obtained by site-directed mutagenesis: wild-type; with the substitutions T817S I819V prior to the PLCγ1 binding site (with a C-terminus as in TrkB); with the Y820F substitution (with inactivated PLCγ1 binding site).

The aim of this research was to explain the importance of regulatory sequences at the C-terminus of TrkC in TrkC-mediated neuritogenesis: the PLCγ1 binding site and the sequence immediately preceding the PLCγ1 binding site.

Clones (variants) of PC12-Tet-On cells stably transfected with trkC genes under the transcriptional control of the tetracycline promoter were established and characterized. The transcription of the gene encoding the desired TrkC variant in the clones was confirmed by sequencing. The dependence of the *trkC* gene expression on the concentration of doxycycline, which in the Tet-On system induces the expression of genes under the control of the tetracycline promoter, has been demonstrated. The TrkC protein of the expected size was confirmed on the cell surface. Phosphorylation of ERK1/2 protein kinases was demonstrated as a result of exogenous TrkC stimulation by neurotrophin 3. The phosphorylation kinetics of ERK1/2 was similar in the studied clones which is essentially consistent with the results of previous studies by different authors. The results of our work indicate the tendency for a higher level of ERK1/2

phosphorylation in clones with TrkC variants capable of recruiting PLCγ1 than in clones with the TrkC Y820F variant.

Neuritogenesis, the formation of neurite-like structures, was observed in the obtained PC12-Tet-On cell clones following the stimulation of wild-type TrkC-containing cells by neurotrophin 3. Neurotrophin 3 treatment did not induce neuritogenesis or its level was very low in cells containing TrkC Y820F indicating the importance of a tyrosine residue at the PLCγ1 binding site for the neuritogenesis process. In this study statistically significant trend was demonstrated for the neurotrophin 3-induced cell neuritogenesis of the clone containing the TrkC T817S I819V variant compared to the clone containing wild-type TrkC. This would indicate that for the neurotrophin-induced production of neurite-like structures, amino acid residues in the C-terminal region of TrkC located outside the proper PLCγ1 recruitment site are also important despite the key tyrosine residue at the site of PLCγ1 attachment to the neurotrophin receptor.

In summary, both the PLCγ1 docking site at the C-terminal region of TrkC and preceding amino acid residues are significant for TrkC-triggered outgrowth of neurites from PC12-Tet-On cells.