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# Peptide-enhanced cellular internalization of proteins in neuroscience

Gunnar P.H. Dietz *, Mathias Bähr

*Neurologische Universitätsklinik, Waldweg 33, 37073 Göttingen, Germany*

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**Abstract**

Over the last 15 years, many publications described the use of peptide sequences that have been dubbed cell penetrating peptides (CPP), Trojan Horse peptides, protein transduction domains, or membrane-translocating sequences. These mostly positively charged domains bring attached cargo across biological membranes. One of the reasons for the interest in CPP is their potential as delivery tools to enhance the pharmacodynamics of drugs otherwise poorly bioavailable. In particular, the neuroscientist aiming to deliver a protein or other compound into the brain for analytical or therapeutic reasons is faced with the challenge that few drugs cross the blood–brain barrier. CPP are valuable tools to overcome the plasma membrane or the blood–brain barrier in basic research, and in relevant models of neural disease, and will hopefully help to increase the precious few treatments or even cures for people with diseases of the brain and nervous system. Here, we review applications in neuroscience and recent insights into the mechanism of CPP-mediated trafficking.


*Keywords:* Protein delivery; Trojan Horse; HIV-Tat; Antennapedia (penetratin); Arginine-rich peptide; Ischemia

## 1. Introduction

The majority of cell penetrating peptides (CPP) have been designed on the basis of known, naturally occurring protein domains, such as the *Drosophila* Antennapedia (Antp) homeodomain-derivative penetratin [39,40,86,133], or the basic domain from HIV-1 Tat [54,123], or the HSV-1 structural protein VP22 [50]. Over the last decade, many other CPP have been characterized, such as oligoarginines [67,69,185], or transportan [137] (Fig. 1).

The first CPP-mediated cargo delivery was achieved using the homeodomain of Antp [133]. Antp transduces many cells, including neurons [15,84,86,102]. Its third helix, which is a DNA-binding region of the Antp transcription factor, is sufficient for this ability ([40,142], for review) and has been named penetratin-1 [39]. The C-terminal 7 amino acids are sufficient to transduce cultured cells, with the basic residues being the most critical for efficient transduction [56].

Soon after the finding of the Prochiantz laboratory, Fawell et al. showed that a variety of cargoes, chemically linked to amino acids 37–72 or 1–72 of HIV-Tat, was delivered into cells and tissues in vitro and in vivo [54]. Dowdy's group was the first to generate an in-frame Tat bacterial expression vector and to report the purification of recombinant, transducible proteins [123]. They used this approach to deliver large, enzymatically active proteins throughout the body and even into the brain [160].

An extensive review of the various CPP, their mechanisms of transduction and applications, with about 600 references on the topic, has been published last year [43]. Here, we focus on applications in neuroscience and on results of the past months in the growing field of protein transduction technology.

## 2. On the mechanism and limitations of peptide-mediated protein transduction

The uptake of the Antennapedia homeodomain [133], the Tat CPP [180], VP22 [50] and oligoarginines [69] were initially thought to occur at low temperatures and receptor-independently [38], and to be insensitive to typical metabolic or endocytosis inhibitors [169,180]. Even recent studies that claimed to show receptor- and temperature-independence used FACS analysis to assess the kinetics of cellular penetration [30]. However, unless extensive protease treatment before FACS is used and the efficiency of that treatment is clearly demonstrated, such studies only show that the protein under investigation attaches to the outside of the plasma membrane.

* Corresponding author. Tel.: +49 551 39 12836; fax: +49 551 39 14302.
*E-mail address:* gdietz@gwdg.de (G.P.H. Dietz).
*URL:* http://www.baehrlab.med.uni-goettingen.de/Staff_Positions/Dietz/dietz.html.

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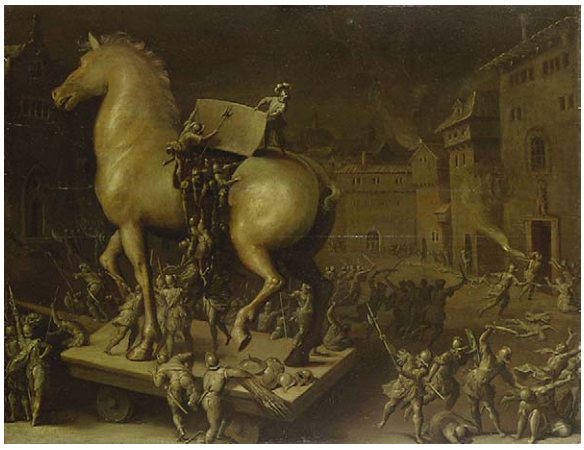

Fig. 1. The Trojan Horse, 17th century, Flemish, oil on panel, Wadsworth Atheneum. Small cationic peptides that mediate the trafficking of proteins and compounds across lipid bilayers have sometimes been called Trojan Horse peptides.

Some of the earlier findings may have suffered from additional artifacts [71]. The reliable proof demonstrating transduction of cells is a quantifiable enzymatic activity within the cells, such as Cre recombinase [77,83,87,90,105,130]. The fixation of cells can lead to a relocalization of soluble proteins and as a consequence to an overestimation of their abundance inside the cell [115]. Tat fusion proteins, when fixed with methanol, show a more diffuse cellular localization as compared to formaldehyde fixed or live cells [121]. Tat and penetratin colocalize with an endocytosis marker in not fixed cells, while after fixation, they can be detected in the nucleus [146]. However, many studies using non-fixated cells have confirmed that CPP do promote membrane passage [157,182,200]. It is becoming more apparent that endocytic mechanisms [24,47,63,139,146] originating from cell membrane lipid rafts are involved in that process [57,58,181], also involving to some degree clathrin-dependent endocytosis [152]. Lipid rafts are membrane microdomains that are enriched in cholesterol and sphingolipids [20, for review]. For instance, intact HIV-1 virions are also translocated through the blood–brain barrier (BBB) via lipid-raft-mediated macropinocytosis [110]. In contrast to clathrin-mediated endocytosis, this pathway is sensitive to cholesterol depletion. The endocytic uptake is followed by the acidification of endosomes and subsequent release of CPP [55].

Part of the contradictions between different publications on membrane penetration are due to the fact that different transduction pathways are not mutually exclusive. Which proportion of the compounds are trafficked in the respective fashion depends on the cargo, the type of CPP employed and the cells to be transduced. A combination of both endocytic and other mechanisms of cell penetration is suggested by cell fractionation experiments, in which the cytosolic compartment was separated from the endocytic compartment [195]. The Tat peptide as well as $YGR_8$ was found primarily in the cytosolic compartment, while $YGK_9$ was mainly located in the endocytic fraction [195]. In this study, only the short CPP, without a cargo, were examined. Penetratin is less sensitive to macropinocytosis inhibitors than the R8 peptide [124].

For CPP cargo of variable size, the mode and efficiency of cell transduction may be different: penetratin [70,134,174] as well as the Tat domain can penetrate artificial membranes at room temperature, i.e. in an energy- and endosome-independent manner [36]. In the latter paper, when linked to a 25 kDa cargo, Tat could no longer pass liposomes, while it could still enter cells in vitro, via endocytic mechanisms. Direct penetration of cationic peptides through the plasma membrane, endocytosis and even receptor-mediated translocation (for instance by LRP for full-length Tat, see [109]) may occur to variable degrees, depending on the CPP, the cargo, the cell type, the culture conditions, or the in vivo application. The analysis of the penetration mechanism can be further complicated by degradation of the CPP [75] or, if fluorophores are used in live imaging, their quenching by binding to cellular molecules, such as heparin sulfates, or by excretion after cleavage from the protein [55].

As a first step of transduction, the positively charged residues of CPP undergo electrostatic interactions with sulfated polysaccharides on the outside of the membrane [34,67,69,111,163,169]. Heparin and heparinsulfates promote peptide aggregation and concentration at their molecular surface [70]. This interaction is also important for full-length Tat internalization [5]. Enzymatic removal of heparan sulfates reduces CPP uptake [200], and in mutant lines deficient in either heparan sulfate or glycosaminoglycan synthesis, uptake is reduced [111]. Particularly important in that interaction seems to be the hydrogen bond of the guanodini moiety with the membrane phospholipids [45,69,143]. The internalization across a membrane is promoted by a peptide sequence with a distribution of positively charged residues along a beta-sheet conformation, as it occurs in penetratin, which enables the anchoring of the peptide in the polar part of the membranes [14].

Delivery into the cytoplasm and nucleus of cargoes less than 1 kDa in size, observed in live, unfixed cells, at least when used at very high concentrations, occurred already after 4 s in some cells; however, other cell types remained unlabeled even after a long exposure to the proteins [200]. However, very high protein concentrations might alter the morphology, integrity, and permeability of the membrane.

In vivo applications of CPP, on the other hand, are apparently well tolerated. Even 16 injections of about 25 nmoles of peptide within 20 days [165] or 14 daily injections of 20 nmoles each [10] showed no obvious signs of toxicity or immunologic response.

## 3. Elucidation of neuronal signal transduction pathways, transmitter release, and long-term potentiation (LTP) in vitro

CPP are a valid alternative to gene transfer technology when studying the structure-function relationship of proteins like G-protein coupled receptors. The third intracellular loop of the angiotensin II type I receptor, fused to the Tat CPP, elicits a response similar to stimulation by angiotensin II on hypothalamus or brainstem neurons, including an increased firing rate, which points out the importance of that domain for the angiotensin signal transduction [179]. In another experi-