Neurotoxins exhibiting shortened biological activity

[0001] The present invention relates to the pharmaceutical field. Specifically, it contemplates a polynucleotide encoding a neurotoxin polypeptide exhibiting a reduced duration of the biological effect in a subject, wherein said polypeptide comprises at least one E3 ligase recognition motif in the light chain, wherein said E3 ligase recognition motif is preferably a binding motif for the E3 ligase MDM2. The invention further pertains to polypeptides encoded by the polynucleotides of the invention as well as polypeptides comprising one or more amino acid substitutions. Further encompassed by the present invention are vectors and host cells comprising the said polynucleotides, polypeptides encoded thereby and antibodies specifically binding to the polypeptides. Moreover, the invention relates to medicaments comprising said polynucleotides and polypeptides, as well as specific therapeutic applications thereof. Furthermore, the present invention contemplates methods for the manufacture of the polypeptides and medicaments.

[0002] Clostridium botulinum and Clostridium tetani produce highly potent neurotoxins, i.e. botulinum toxins (BoNTs) and tetanus toxin (TeNT), respectively. These Clostridial neurotoxins (CNTs) specifically bind to neuronal cells and disrupt neurotransmitter release. Each toxin is synthesized as an inactive unprocessed approximately 150 kDa single-chain protein. The posttranslational processing involves formation of disulfide bridges, and limited proteolysis (nicking) by the bacterial protease(s). Active neurotoxin consists of two chains, an N-terminal light chain of approx. 50 kDa and a heavy chain of approx. 100 kDa linked by a disulfide bond. CNTs consist of three domains, i.e. the catalytic light chain, the heavy chain encompassing the translocation domain (N-terminal half) and the receptor binding domain (C-terminal half), see Krieglstein 1990, Eur. J. Biochem. 188, 39; Krieglstein 1991, Eur. J. Biochem. 202, 41; Krieglstein 1994, J. Protein Chem. 13, 49. The Botulinum neurotoxins are synthesized as molecular complexes comprising the 150 kDa neurotoxin protein and associated non-toxic, complexing proteins. The complex sizes differ based on the Clostridial strain and the distinct neurotoxin serotypes ranging from 300 kDa to 900 kDa. The complexing proteins in these complexes stabilize the neurotoxin and protect it against degradation, see Chen 1998, Infect. Immun. 66(6): 2420-2425.

[0003] Clostridium botulinum secretes seven antigenically distinct serotypes designated A to G of the botulinum neurotoxin (BoNT). All serotypes together with the related tetanus neurotoxin (TeNT) secreted by Clostridium tetani, are Zn^{2+}-endoproteases that block synaptic exocytosis by cleaving SNARE proteins, see Couesnon, 2006, Microbiology, 152, 759. BoNTs cause the flaccid muscle paralysis seen in botulism, see Fischer 2007, PNAS 104, 10447.

[0004] Despite its toxic effects, Botulinum toxins have been used as therapeutic agents for a large number of diseases or disorders. Botulinum toxin serotype A was approved for human use in the United States in 1989 for the treatment of strabism, blepharospasm, and other disorders. It is commercially available as a Botulinum A neurotoxin with complexing proteins, for example, under the tradename BOTOX (Allergan Inc.) or under the tradename DYSPORT (Ipsen Ltd.). An improved, complex-free neurotoxin A polypeptide preparation is available under the tradename XEOMIN (Merz Pharmaceuticals LLC). The effect of Botulinum toxin is only temporary, which is the reason why repeated administration of Botulinum toxin may be required to maintain a therapeutic effect.

[0005] The Clostridial neurotoxins weaken voluntary muscle strength and are effective therapeutics for strabism, focal dystonia, including cervical dystonia, and benign essential blepharospasm. They have been further shown to relief hemifacial spasm, and focal spasticity, and, moreover, to be effective in a wide range of other indications, such as gastrointestinal disorders, hyperhidrosis, and cosmetic wrinkle correction, see Jost 2007, Drugs 67, 669.

[0006] However, weakening muscle strengths and contraction is also desirable for medical conditions or disease such as wound healing, immobilisation for bone and tendon fracture treatment, post surgery immobilization, specifically in connection with haemorrhoidectomy, introduction of dental implants, or hip joint replacement (endoprothesis), knee arthroplasty, ophthalmological surgery, acne, irritable bowel disease, vaginism, lower back pain, or benign prostate hyperplasia. The neurotoxins usually exhibit their biological effect over a time period which is longer than actually needed for efficient treatment of said diseases or conditions. A prolonged muscle paralysis is, however, detrimental or at least less preferable in the therapy of the said medical conditions or diseases. Neurotoxins exhibiting their biological effect only over the desired time period are, however, not yet available.

[0007] Accordingly, the technical problem underlying the present invention can be seen as the provision of means and methods for complying with the aforementioned needs. The

technical problem is solved by the embodiments characterized in the claims and herein below.

[0008] The present invention, accordingly, relates to a polynucleotide encoding a neurotoxin polypeptide exhibiting a reduced duration of the biological effect in a subject, wherein said polypeptide comprises at least one E3 ligase recognition motif in the light chain, wherein said E3 ligase recognition motif is preferably a binding motif for the E3 ligase MDM2. The reduced duration of the biological activity of a, thus, modified polypeptide has been exemplified for BoNT/E-MDM2. In addition, said neurotoxin polypeptide has been further optimized by site-directed mutagenesis of specific amino acid residues in the light chain. To this end, exposed amino acid residues in the neurotoxin light chain located in spatial proximity to the introduced E3 ligase MDM2 recognition motif have been identified by three-dimensional structural analysis. Subsequently, the identified exposed amino acid residues in the neurotoxin light chain have been substituted by lysine residues. This optimization approach resulted in an even faster degradation of the mutated BoNT/E-MDM2 polypeptides, in comparison to non-mutated BoNT/E-MDM2 polypeptides, as demonstrated in the following Examples.

[0009] Accordingly, such modified or mutated neurotoxin polypeptides of the invention are particularly useful for therapy of diseases which require a short or reduced duration of the biological effect of a neurotoxin.

[0010] The term "polynucleotide" as used herein refers to single- or double-stranded DNA molecules as well as to RNA molecules. Encompassed by the said term is genomic DNA, cDNA, hnRNA, mRNA as well as all naturally occurring or artificially modified derivatives of such molecular species. The polynucleotide may be, in an aspect, a linear or circular molecule. Moreover, in addition to the nucleic acid sequences encoding the aforementioned neurotoxin polypeptide, a polynucleotide of the present invention may comprise additional sequences required for proper transcription and/or translation such as 5'or 3' UTR sequences. The polynucleotide of the present invention encodes a modified neurotoxin polypeptide derivable from one of the antigenically different serotypes of Botulinum neurotoxins, i.e. BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, or Tetanus neurotoxin (TeNT). In an aspect of the present invention, the said polynucleotide comprises (prior to the modification of the invention, i.e. modification of the light chain by at least one E3 ligase recognition motif) a nucleic acid sequence as shown in SEQ ID NO: 1 (BoNT/A), SEQ ID NO: 3 (BoNT/B), SEQ ID NO: 5 (BoNT/C1), SEQ ID NO: 7 (BoNT/D), SEQ ID NO: 9 or 81 (BoNT/E), SEQ ID NO: 11 (BoNT/F), SEQ ID NO: 13 (BoNT/G) or SEQ ID NO: 15 (TeNT). Moreover, encompassed is, in an

aspect, a polynucleotide comprising a nucleic acid sequence encoding an amino acid sequence (prior to the modification of the invention, i.e. modification of the light chain by at least one E3 ligase recognition motif) as shown in any one of SEQ ID NO: 2 (BoNT/A), SEQ ID NO: 4 (BoNT/B), SEQ ID NO: 6 (BoNT/C1), SEQ ID NO: 8 (BoNT/D), SEQ ID NO: 10 or 82 (BoNT/E), SEQ ID NO: 12 (BoNT/F), SEQ ID NO: 14 (BoNT/G) or SEQ ID NO: 16 (TeNT). In another aspect, the said polynucleotide is a variant of the aforementioned polynucleotides comprising one or more nucleotide substitutions, deletions and/or additions which in still another aspect may result in an encoded amino acid having one or more amino acid substitutions, deletions and/or additions. Moreover, a variant polynucleotide of the invention shall in another aspect comprise a nucleic acid sequence variant being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical or 100% identical to the nucleic acid sequence as shown in any one of SEQ ID NOs: 1, 3, 5, 7, 9, 81, 11, 13 or 15 or a nucleic acid sequence variant which encodes an amino acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical or 100% identical to the amino acid sequence as shown in any one of SEQ ID NOs: 2, 4, 6, 8, 10, 82, 12, 14, or 16. In an aspect, each of the aforementioned variant polynucleotides encodes a variant polypeptide retaining one or more biological properties and, in another aspect, all of the biological properties of the respective neurotoxin polypeptide, i.e. the BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G or Tetanus neurotoxin (TeNT). Those of skill in the art will appreciate that full biological activity is maintained only after proteolytic activation, even though it is conceivable that the unprocessed precursor can exert some biological functions or be partially active. The term "biological properties" as used herein refers to (a) receptor binding, (b) internalization, (c) translocation across the endosomal membrane into the cytosol, and/or (d) endoproteolytic cleavage of proteins involved in synaptic vesicle membrane fusion. In a further aspect, the variant polynucleotides can encode variant polypeptides having improved or altered biological properties, e.g., they may comprise cleavage sites which are improved for enzyme recognition or may be improved for receptor binding or any other property specified above. In yet a further aspect, the variant polynucleotides shall encode fusion neurotoxin polypeptides comprising a part of at least two neurotoxin polypeptides of different serotypes, e.g., a fusion neurotoxin comprising a heavy chain of BoNT/A and a light chain of BoNT/E or a binding domain of BoNT/E and the translocation domain and a light chain of BoNT/A.

[0011] The term "identical" as used herein refers to sequence identity characterized by determining the number of identical amino acids between two nucleic acid sequences or

amino acid sequences wherein the sequences are aligned so that the highest order match is obtained. It can be calculated using published techniques or methods codified in computer programs such as, for example, BLASTP, BLASTN or FASTA (Altschul 1990, J. Mol. Biol. 215, 403). The percent identity values are, in one aspect, calculated over the entire amino acid sequence. A series of programs based on a variety of algorithms is available to the skilled worker for comparing different sequences. In this context, the algorithms of Needleman and Wunsch or Smith and Waterman give particularly reliable results. To carry out the sequence alignments, the program PileUp (Higgins 1989, CABIOS 5, 151) or the programs Gap and BestFit (Needleman 1970, J. Mol. Biol. 48; 443; Smith 1981, Adv. Appl. Math. 2, 482), which are part of the GCG software packet (Genetics Computer Group 1991, 575 Science Drive, Madison, Wisconsin, USA 53711), may be used. The sequence identity values recited above in percent (%) are to be determined, in another aspect of the invention, using the program GAP over the entire sequence region with the following settings: Gap Weight: 50, Length Weight: 3, Average Match: 10.000 and Average Mismatch: 0.000, which, unless otherwise specified, shall always be used as standard settings for sequence alignments.

[0012] The term "activity", "function", "biological activity", "biological function", or "biological effect" of a neurotoxin as used herein denotes the amount of cellular exocytosis inhibited from a cell per unit of time, such as exocytosis of a neurotransmitter, e.g. acetylcholine, from a target cell, such as a neuron. More specifically, it refers to the biological activity of a mature di-chain neurotoxin polypeptide exhibiting a) receptor binding, b) internalization, c) translocation across the endosomal membrane into the cytosol, and/or d) endoproteolytic cleavage of proteins involved in synaptic vesicle fusion. The term "duration of the biological effect (of the neurotoxin) in a subject" as used herein means the time period of the biological activity of a neurotoxin in a subject to which the neurotoxin has been applied. *In vivo* assays for assessing biological activity include the mouse LD50 assay and the *ex vivo* mouse hemidiaphragm assay as described by Pearce et al. (Pearce 1994, Toxicol. Appl. Pharmacol. 128: 69-77) and Dressler et al. (Dressler 2005, Mov. Disord. 20:1617-1619, Keller 2006, Neuroscience 139: 629-637). The biological activity can also be assessed by a cell-based assay as described, e.g., by Whitemarsh et al. (Whitemarsh et al. 2012, Toxicol. Sci. 126: 426-435). The biological activity is commonly expressed in Mouse Units (MU). As used herein, 1 MU is the amount of neurotoxic component, which kills 50% of a specified mouse population after intraperitoneal injection, i.e. the mouse i.p. LD50. The term "subject" as used herein means a mammal, preferably a human.

[0013] Moreover, fusion polypeptides further comprising detectable marker peptides or tags are encompassed in further aspects of the neurotoxin polypeptide of the invention exhibiting a reduced duration of the biological effect in a subject, due to the at least one E3 ligase recognition motif in the light chain. In one aspect, suitable tags are, e.g., FLAG-tags, Myc-tags, His-tags, HA-tags or GST-tags which also allow for a more efficient purification of the tagged polypeptides. Detectable marker peptides, in an aspect, include fluorescent proteins such as GFP, BFP, YFP and the like. Said fusion polypeptides can comprise additional polypeptide domains in some aspects. For example, the neurotoxin polypeptide of the invention can comprise a peptide domain which mediates cell penetration in order to access the site of action of the neurotoxin light chain, i.e. the cytoplasm of a neuronal target cell. For this purpose, e.g., a poly-arginine peptide can be used for fusion to the neurotoxin polypeptide of the invention which is well known in the art.

[0014] The neurotoxin polypeptide (encoded by the polynucleotide) of the invention further comprises at least one E3 ligase recognition motif in its light chain. As set forth elsewhere herein in more detail and as demonstrated in the following Examples, the duration of the biological effect of a neurotoxin in a subject can be influenced, i.e. altered, by the incorporation of at least one E3 ligase recognition motif into the light chain or the addition of at least one E3 ligase recognition motif to the N- or C-terminus of the light chain. Thus, in one aspect, the neurotoxin polypeptide of the invention comprises at least one internally or terminally introduced E3 ligase recognition motif in the light chain. Such a modification results in a reduced duration of the biological effect of the neurotoxin of the invention in a subject, in comparison to a neurotoxin not comprising an E3 ligase recognition motif. In an aspect of the invention, the said light chain of the neurotoxin polypeptide encoded by the polynucleotide of the invention is obtained by modification from a light chain being encoded by a polynucleotide comprising any one of the aforementioned specific nucleic acid sequences or variants thereof described above. As described above and well known in the art, the light chains of the neurotoxin polypeptides are generated by proteolytic cleavage of a precursor polypeptide. The light chain is the N-terminal portion of the precursor polypeptide which is obtained as a result of said proteolytic cleavage. The amino acid sequences of the light chains of the neurotoxin polypeptides referred to above can be deduced, in an aspect, from the cleavage sites of the wild type neurotoxins indicated in Table 1. In recombinant neurotoxins, cleavage sites (such as a thrombin cleavage site or enterokinase cleavage site) are introduced into the sequence between the two cysteines forming the disulfide bridge between heavy and light chain, preferably into a linker as defined elsewhere herein.

[0015] **TABLE 1:** Neurotoxins and proteolytic cleavage sites which can be used to form

the active di-chain molecule

Neurotoxin (Bacterial Strain)	Accession number	Cleavage site(s)	Native light chain	Sequence including cysteines forming disulfide bridge and cleavage sites (highlighted)
BoNT/A (Hall/62A)	ABD 65472	K438/T439 K448/A449	M1-K438	KLL**C**VRGIITS**K**..TKSLDKGYN**K**..ALN....DL**C**IKV (SEQ ID NO: 17)
BoNT/B (Okra)	BAE 48264	K441/A442	M1-K441	IQM**C**KSV**K**..APG**C**IDV (SEQ ID NO: 18)
BoNT/C1 (C-6814)	BAA 89713	R444/S445 K449/T450	M1-R444	TKF**C**HKAIDG**R**..SYN**K**..TLD**C**RELLV (SEQ ID NO: 19)
BoNT/D	BAA 90661	K442/N443 R445/D446	M1-K442	TKV**C**LRLT**K**..NS**R**..DDST**C**IKV (SEQ ID NO: 20)
BoNT/E (Beluga)	CAA 43999	K419/G420 R422/K423	M1-K419	IRF**C**KNIVSV**K**..G.IR**K**SI**C**IEI (SEQ ID NO: 21)
BoNT/F (NCTC10281)	CAA 73972	R435/K436 K439/A440	M1-R435	VKF**C**KSVIPR**K**..GT**K**..APPRL**C**IRV (SEQ ID NO: 22)
BoNT/G	CAA 52275	K446/S447	M1-K446	IAM**C**KPVMY**K**NTG**K**..SEQ**C**IIV (SEQ ID NO: 23)
TeNT	P 04958	R449 (R455)	M1-A457	IGL**C**KKIIPPTNI**R**..ENLYN**R**..TASLTDLGGEL**C**IKI (SEQ ID NO: 24)

[0016] The term "E3 ligase recognition motif" as used herein refers to (a) modification(s) of the light chain of the neurotoxin polypeptide of the invention which result in accelerated degradation of said neurotoxin polypeptide by endogenous degradation pathways present in the subject to which the neurotoxin has been applied. The E3 ligase recognition motif is a structural motif which allows recognition of the motif and binding to the motif of an E3 ligase. Further peptide degradation signals mediating cellular degradation of proteins are known in the art, and comprise, for example, PEST motifs, WW motifs, or WD40 motifs. The degradation pathway can be a proteasomal degradation pathway or a lysosomal degradation pathway. In another aspect, a degradation pathway may merely result in a partial degradation of the neurotoxin polypeptide of the invention, e.g., by one or more proteolytic cleavage steps. The said E3 ligase recognition motif can be introduced into the light chain, i.e. be located (internally) within the light chain or linked thereto either N- or C-terminally. In the latter case, a neurotoxin polypeptide of the invention can, for example, carry an E3 ligase recognition motif which is interposed between the neurotoxin light chain and the neurotoxin heavy chain. For example, such a construct may in one aspect have the

arrangement, from the N-terminus to the C-terminus: Neurotoxin light chain - E3 ligase recognition motif - neurotoxin heavy chain. Alternatively, the domain arrangement can be, from the N- to the C-terminus: Neurotoxin heavy chain - E3 ligase recognition motif - neurotoxin light chain. In a further aspect, an additional linker, such as, e.g., a poly-Glycine linker, may be used to interconnect said neurotoxin light and heavy chain. Such a construct may be arranged, from the N- to the C-terminus: Neurotoxin light chain - E3 ligase recognition motif - linker - neurotoxin heavy chain. Alternatively, the domain arrangement can be, from the N- to the C-terminus: Neurotoxin heavy chain – linker - E3 ligase recognition motif - neurotoxin light chain. The linker preferably comprises a protease cleavage site, for instance, an enterokinase cleavage site or a thrombin cleavage site. The position of the E3 ligase recognition motif is preferably C-terminal of the first disulfide bridge-forming cysteine of the light chain and N-terminal of the protease cleavage site; see Figure 1. Upon cleavage by the respective protease, the neurotoxin light chain (comprising the E3 ligase recognition motif) is released from the above-mentioned construct. In other aspects, the neurotoxin polypeptide of the invention comprises not only one E3 ligase recognition motif in or attached to the native recombinant light chain, but two, three, four or even more E3 ligase recognition motifs. The term "modified" neurotoxin polypeptide exhibiting a reduced duration of the biological effect in a subject as used herein means that the neurotoxin polypeptide of the invention comprises at least one E3 ligase recognition motif in or attached to the native recombinant light chain, preferably in combination with one or more mutations in the neurotoxin light chain. Even more preferred, said mutation in the neurotoxin light chain is an amino acid substitution as defined elsewhere herein.

[0017] The person skilled in the art is well aware of suitable E3 ligase recognition motifs and how to introduce or link them to the neurotoxin polypeptide's light chain. Moreover, the skilled artisan can generate polynucleotides encoding such neurotoxin polypeptides with the at least one E3 ligase recognition motif by applying recombinant molecular biological techniques or chemical modifications. For example, site directed mutagenesis may be used for introducing the E3 ligase recognition motifs referred to herein. Alternatively, a nucleic acid sequence for the polynucleotide comprising the coding sequences for the neurotoxin polypeptide and the envisaged E3 ligase recognition motif may be designed and the entire polynucleotide may subsequently be chemically synthesised.

[0018] In one aspect, the said E3 ligase recognition motif is at least one internally or terminally introduced E3 ligase-recognition and/or E3 ligase-binding motif. Preferably, the recognition and binding motif for a respective E3 ligase is identical, i.e. said motif is used

both for recognition and binding of the E3 ligase. In this aspect, the E3 ligase recognition motif targets the neurotoxin polypeptide's light chain of the invention to cellular degradation via the ubiquitin-mediated proteasome degradation pathway. Degradation via the ubiquitin-proteasome pathway involves two discrete and successive steps: (i) covalent attachment of multiple ubiquitin molecules to the neurotoxin polypeptide's light chain of the invention to form a poly-ubiquitin chain and (ii) degradation of the, thus, tagged neurotoxin polypeptide's light chain of the invention by the 26S proteasome pathway. As described in the art, ubiquitin, a highly conserved 76-amino acid protein, is conjugated to the target protein by a three-step mechanism. Initially, the C-terminal carboxyl group of ubiquitin is activated by an ubiquitin-activating enzyme (E1). The thioester formed by attachment of ubiquitin to the E1 enzyme is then transferred through a trans-acylation reaction to an ubiquitin-conjugating enzyme (E2). Depending on the E3 ligase involved, the ubiquitin is then either directly transferred to the E3 ligase (HECT E3 ligases) or the E2-ubiquitin complex binds to the E3 ligase (RING E3 ligases). Finally, in both cases the E3 ligase binds specifically to the substrate and catalyses the last step in the conjugation process, which is the covalent attachment of ubiquitin to the substrate, in the present case to the neurotoxin polypeptide's light chain of the invention (Marmor and Yarden 2004, Oncogene 23: 2057-2070). Successive conjugations of ubiquitin to the internal lysines of previously added ubiquitin molecules lead to the formation of poly-ubiquitin chains. The poly-ubiquitinated target protein is then recognized by the 26S proteasome and eliminated (Schrader 2009, Nat. Chem. Biol. 5: 815-22). Preferably, the E3 ligase recognition motif as defined herein mediates irreversible degradation. Such irreversible degradation has been described, for instance, for the peptide degradation signal domain "ALAPYIP" (SEQ ID NO: 25). In particular aspects, a E3 ligase-recognition and/or -binding motif is a peptide or peptidomimetic having a length of less than 50 amino acids residues, a length of less than 40 residues, a length of less than 30 residues, a length of less than 20 residues, or a length of less than 15 residues. Examples of specific E3 ligase recognition motifs are indicated in Table 2 or E3 ligase recognition motifs comprising the amino acid sequence ETFSDLWKLLPE (SEQ ID NO: 26), TSFAEYWNLLSP (SEQ ID NO: 27), LTFEHYWAQLTS (SEQ ID NO: 28), LTFEHWWAQLTS (SEQ ID NO: 29), LTFEHSWAQLTS (SEQ ID NO: 30), ETFEHNWAQLTS (SEQ ID NO: 31), LTFEHNWAQLTS (SEQ ID NO: 32), LTFEHWWASLTS (SEQ ID NO: 33), LTFEHWWSSLTS (SEQ ID NO: 34), LTFTHWWAQLTS (SEQ ID NO: 35), ETFEHWWAQLTS (SEQ ID NO: 36), LTFEHWWSQLTS (SEQ ID NO: 37), LTFEHWWAQLLS (SEQ ID NO: 38), ETFEHWWSQLLS (SEQ ID NO: 39), RFMDYWEGL (SEQ ID NO: 40), MPRFMDYWEGLN (SEQ ID NO: 41), SQETFSDLWKLLPEN (SEQ ID NO: 42) and/or LTFEHNWAQLEN (SEQ ID NO: 78). Preferably, the E3 ligase recognition motif mediates degradation of at least 10, 20, 30, 40,

50, 60, 70, 80, or 90%, more preferably 100% of the neurotoxin polypeptide's light chain of the invention within the cell. Degradation can be determined by assays described in the art, e.g. by, *in vitro* assays like quantitative cell based assays, or *in vivo* assays such as the mouse running assay, digit abduction assay (DAS), or rat grip strength assay.

[0019] **TABLE 2:** In an aspect, the E3 ligase recognition motif comprises or consists of a consensus sequence as shown in the following table (wherein "X" may represent any of the naturally occurring amino acids).

E3 ubiquitin Ligase	**Recognition motif (consensus)**
VBCCul2	ALAPYIP (SEQ ID NO: 25)
MDM2	XXFXXXWXXLXX (SEQ ID NO: 43)
Smurf2	ELESPPPPYSRYPM (SEQ ID NO: 44)
RN181	KVGFFKR (SEQ ID NO: 45)
E3alpha	LLVRGRTLVV (SEQ ID NO: 46)
SCF	DRHDSGLDSM (SEQ ID NO: 47)
Siah	PXAXVXP (SEQ ID NO: 48)
Itch	PPXYXXM (SEQ ID NO: 49)
Nedd4-2	PPXY (SEQ ID NO: 50)

[0020] In another aspect, the E3 ligase recognition motif is a binding motif for the E3 ligase MDM2. The corresponding human nucleic acid and amino acid sequences are shown in Accession Nos. NM_002392 and NP_002383, respectively. Preferably, said binding motif for the E3 ligase MDM2 comprises or consists of an amino acid sequence selected from the group consisting of ETFSDLWKLLPE (SEQ ID NO: 26), TSFAEYWNLLSP (SEQ ID NO: 27), LTFEHYWAQLTS (SEQ ID NO: 28), LTFEHWWAQLTS (SEQ ID NO: 29), LTFEHSWAQLTS (SEQ ID NO: 30), ETFEHNWAQLTS (SEQ ID NO: 31), LTFEHNWAQLTS (SEQ ID NO: 32), LTFEHWWASLTS (SEQ ID NO: 33), LTFEHWWSSLTS (SEQ ID NO: 34), LTFTHWWAQLTS (SEQ ID NO: 35), ETFEHWWAQLTS (SEQ ID NO: 36), LTFEHWWSQLTS (SEQ ID NO: 37), LTFEHWWAQLLS (SEQ ID NO: 38), ETFEHWWSQLLS (SEQ ID NO: 39), RFMDYWEGL (SEQ ID NO: 40), MPRFMDYWEGLN (SEQ ID NO: 41), SQETFSDLWKLLPEN (SEQ ID NO: 42) and/or LTFEHNWAQLEN (SEQ ID NO: 78). Even more preferred, said binding motif for the E3 ligase MDM2 comprises or consists of the amino acid sequence LTFEHNWAQLTS (SEQ ID NO: 32) or LTFEHNWAQLEN (SEQ ID NO: 78). It is also preferred that the length of

the binding motif for the E3 ligase MDM2 is between 9 and 15 amino acid residues, more preferably it is 12 amino acid residues in length. Figure 1 illustrates an example of a neurotoxin polypeptide of the invention comprising a binding motif for the E3 ligase MDM2 (MDM2 motif). Figure 2 shows that the interposition of a binding motif for the E3 ligase MDM2 between the neurotoxin light chain and heavy chain as illustrated in Figure 1 allows recognition and binding of the E3 ligase MDM2 to the, thus, modified neurotoxin light chain, resulting in ubiquitination of surrounding surface exposed lysine residues and faster degradation of the ubiquitinated neurotoxin light chain of the invention by the cellular proteasome system. It is encompassed within the scope of the present application, that the sequence of the indicated binding motifs for the E3 ligase MDM2 can be further modified, for example, by one or more nucleotide substitutions, deletions and/or additions which in still another aspect may result in an encoded amino acid sequence having one or more amino acid substitutions, deletions and/or additions. Said modifications can be carried out in order to alter (e.g. improve) the binding of the E3 ligase MDM2 to the indicated binding motifs, resulting in a still enhanced degradation of the neurotoxin polypeptide of the invention by the ubiquitin-mediated proteasome degradation pathway.

[0021] In a further aspect, said polynucleotide of the invention comprises a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence having a nucleotide sequence as shown in SEQ ID NO: 51 or 79;

b) a nucleic acid sequence encoding a polypeptide having an amino acid sequence as shown in SEQ ID NO: 52 or 80; and

c) a nucleic acid sequence being at least 40%, preferably at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the nucleic acid sequence of a) or b).

[0022] As demonstrated in the following Examples, the neurotoxin polypeptide (encoded by the polynucleotide) of the present invention comprising at least one E3 ligase recognition motif in the light chain exhibits a reduced duration of the biological effect in a subject upon administration, in comparison to an unmodified neurotoxin polypeptide (not comprising one or more E3 ligase recognition motif(s) in the light chain). The reduced duration of the biological activity of a, thus, modified polypeptide has been exemplified for BoNT/E-MDM2. The reduced duration of the biological effect of the neurotoxin polypeptide of the invention is a result of the faster degradation of said neurotoxin polypeptide (i.e. of the catalytic neurotoxin light chain) by the proteasome system in the neuron of the subject.

[0023] The above-mentioned BoNT/E-MDM2 polypeptide has been further improved by the present inventors by site-directed mutagenesis of exposed amino acid residues in the light chain which are located in spatial proximity to the MDM2-recognition motif. "Exposed amino acid residues" as used herein means that the amino acid residues are located at the surface of the neurotoxin's light chain, e.g. the BoNT/E light chain, and the side chains of said amino acid residues are not involved in intra-molecular interactions. Said exposed amino acid residues within the light chain have been first identified by three-dimensional structural analysis and then substituted by lysine residues. This optimization procedure resulted in an even more accelerated degradation of the mutated BoNT/E-MDM2 polypeptides, in comparison to non-mutated BoNT/E-MDM2 polypeptides comprising the E3 ligase recognition motif. It has unexpectedly been found that substitutions at Q53, N72, N378, N379, R394 and/or T400 by lysine resulted in faster degradation of the BoNT/E-MDM2 by the proteasome system. The indicated position of the respective amino acid residue is based on the numbering in the amino acid sequence shown in SEQ ID NO. 52. In particular, BoNT/E-MDM2 mutants in which (i) Q53, N72, N378, N379, R394 and T400 (SEQ ID NO. 57); (ii) Q53, N378 and N379 (SEQ ID NO. 58); (iii) N72, N378 and N379 (SEQ ID NO. 61); or (iv) N378, N379 and T400 (SEQ ID NO. 75) in the light chain, have been substituted by lysine residues showed a reduced biological effect on cultured cortex neurons. In contrast, numerous other mutations did not show any reduced duration of the biological effect, as demonstrated in Figure 3. Accordingly, in a further aspect, the polypeptide of the invention comprises or consists of an amino acid sequence selected from the group consisting of:

a) an amino acid sequence as shown in SEQ ID NO: 57, 58, 61 or 75; and

b) an amino acid sequence being at least 40%, preferably 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of a).

Preferably, the amino acid sequence of b) carries the same set of mutations as SEQ ID NO: 57 (Q53, N72, N378, N379, R394 and T400), 58 (Q53, N378 and N379), 61 (N72, N378 and N379) or 75 (N378, N379 and T400), in the light chain. In another aspect, the invention pertains to the polynucleotides encoding the amino acid sequences of a) or b) above.

[0024] In an aspect, the said biological effect of the neurotoxin polypeptide of the invention observed in the subject causes muscle paralysis, i.e. a (reversible) inactivation of the muscle's capability to contract. In a further aspect, the reduced duration of the biological effect of the neurotoxin polypeptide of the invention in a human subject persist

less than 5, 4, 3, 2 weeks or even less than 1 week. In another aspect, the effects can be tested *in vivo* by the so-called mouse running assay (Keller 2006, Neuroscience 139: 629-637), the digit abduction assay (Aoki 2001, Toxicon 12: 1815-20) or the rat grip strength assay (Torii 2011, Toxicon 57(1): 93-9). The biological effects can be determined by the person skilled in the art without further ado. A reduced duration of the biological effect, in an aspect, refers to a statistically significant reduced duration. Whether the duration of an effect is statistically significant reduced can be determined by those skilled in the art by applying standard statistical tests, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test etc. Preferred confidence intervals are at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.1, 0.05, 0.01, 0.005, or 0.0001. Preferably, the probability envisaged by the present invention allows that the diagnosis will be correct for at least 60%, at least 70%, at least 80%, or at least 90% of the subjects of a given cohort or population. In an aspect, the said reduced duration persists less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, less than 50%, less than 45%, less than 40%, less than 30% or less than 20% of the normal duration, i.e. the duration observed for an unmodified neurotoxin polypeptide (not comprising one or more E3 ligase recognition motif(s) in or attached to the light chain). In an aspect, normal duration persists for approximately 4 months in the case of BoNT/A, 2 months in the case of BoNT/B, approximately 3 to 4 months in the case of BoNT/C or approximately 4 to 6 weeks in the case of BoNT/E (Foran, J. Biol. Chem. 278(2): 1363-1371, Eleopra 1998, Neurosci. Lett. 13, 256(3): 135-138, Eleopra 1997, Neurosci. Lett. 14,224(2): 91-94, Sloop 1997, Neurology 49(1): 189-194, Washbourne 1998, J. Physiol. Paris 92(2): 135-139). It is to be understood that the duration of the effect depends on individual influences in a subject such as genetic background, age, life style etc. Therefore, an approximate duration as meant herein refers to a duration as indicated above for the respective neurotoxin polypeptides (e.g., 4 months for BoNT/A or 4 to 6 weeks for BoNT/E) with a standard deviation of 25% or less, 20% or less, 15% or less, 10 % or less or 5% or less.

[0025] It has advantageously been found in accordance with the present invention that a neurotoxin polypeptide can be modified to exhibit a shortened biological effect in a subject upon administration. This can be achieved by introducing or linking an E3 ligase recognition motif to the light chain of the said neurotoxin polypeptide since it was found that the persistence of the light chain correlates with the duration of the biological effect. The shortened duration of the biological effect elicited by the neurotoxin polypeptides of the invention is beneficial for various medical applications set forth elsewhere herein which require a reduced duration of the biological effect of said neurotoxin. Such a reduced duration is particularly important in case of short term or acute treatments with

neurotoxin, for example, in treatment of surgery wounds, in order to facilitate quick and painless wound healing without scar forming, or in paralyzing the eye lid muscles of patients in artificial coma whose eyes might suffer damages by drying out. Further applications of the neurotoxin polypeptide of the invention are listed elsewhere herein.

[0026] The present invention contemplates a vector comprising the polynucleotide of the present invention.

[0027] The term "vector", preferably, encompasses phage, plasmid, viral or retroviral vectors as well as artificial chromosomes, such as bacterial or yeast artificial chromosomes. Moreover, the term also relates to targeting constructs which allow for random or site-directed integration of the targeting construct into genomic DNA. Such target constructs, preferably, comprise DNA of sufficient length for either homologous or heterologous recombination as described in detail below. The vector encompassing the polynucleotides of the present invention, in an aspect, further comprises selectable markers for propagation and/or selection in a host. The vector may be incorporated into a host cell by various techniques well known in the art. For example, a plasmid vector can be introduced in a precipitate such as a calcium phosphate precipitate or rubidium chloride precipitate, or in a complex with a charged lipid or in carbon-based clusters, such as fullerenes. Alternatively, a plasmid vector may be introduced by heat shock or electroporation techniques. Should the vector be a virus, it may be packaged *in vitro* using an appropriate packaging cell line prior to application to host cells. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host/cells. Moreover, in an aspect of the invention, the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells or isolated fractions thereof in the said vector. Expression of the polynucleotide comprises transcription of the polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in host cells are well known in the art. In an aspect, they comprise regulatory sequences ensuring initiation of transcription and/or poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the lac-, trp- or tac- promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1- or the GAL1-promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Moreover, inducible expression control sequences may be used in an expression vector encompassed by the present invention. Such inducible vectors may comprise tet or lac

operator sequences or sequences inducible by heat shock or other environmental factors. Suitable expression control sequences are well known in the art. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pBluescript (Stratagene), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (Invitrogen) or pSPORT1 (Invitrogen). Preferably, said vector is an expression vector and a gene transfer or targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector of the invention into targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (2001) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1994).

[0028] Moreover, the present invention pertains to a host cell comprising the polynucleotide or the vector of the present invention.

[0029] The term "host cell" as used herein encompasses prokaryotic and eukaryotic host cells. Preferably, the host cell is an isolated prokaryotic or eukaryotic host cell. In an aspect the host cell is a bacterial cell and, in another aspect, a Firmicutes bacterial cell. In one aspect, the said bacterial host cell is an *E. coli* host cell. In another aspect, it is a Clostridium host cell. In a further aspect, the said Clostridium host cell is a Clostridium botulinum host cell, in even a further aspect, a cell of one of the aforementioned seven different serotypes of Clostridium botulinum. In yet another aspect, the bacterial host cell is a Clostridium tetani host cell. In a further aspect, the host cell is a Bacillus host cell and in a particular aspect a Bacillus megaterium host cell. A eukaryotic host cell, in an aspect, is a cell of an animal cell line suitable for production of toxic proteins or a fungal host cell such as a yeast host cell.

[0030] Also encompassed by the present invention is a polypeptide encoded by the polynucleotide of the invention. In an aspect of the polypeptide of the invention, the polypeptide further comprises at least one amino acid substitution in the light chain of the neurotoxin polypeptide (but not in the MDM2 binding motif as defined herein). Preferably, the substitution of the naturally occurring amino acid in the light chain is by lysine. More preferably, said polypeptide comprises at least one of the amino acid substitutions selected from the group consisting of Q53K, N72K, N378K, N379K, R394K and T400K. The

polypeptide can comprise not only one of said amino acid substitutions but also two, three, four, five, six or even more amino acid substitutions. BoNT/E-MDM2 mutants in which (i) Q53, N72, N378, N379, R394 and T400 (SEQ ID NO. 57), (ii) Q53, N378 and N379 (SEQ ID NO. 58), (iii) N72, N378 and N379 (SEQ ID NO. 61) or (iv) N378, N379 and T400 (SEQ ID NO. 75) in the light chain have been substituted by lysine residues are particularly preferred. In other aspects of the polypeptide of the invention, the polypeptide can comprise, in addition to the above-mentioned amino acid sequence substitutions in the light chain, one or more amino acid sequence substitutions in the MDM2 binding motif, for example, in order to improve binding of the E3 ligase MDM2 to the polypeptide of the invention.

[0031] The term "polypeptide" as used herein encompasses isolated or essentially purified polypeptides being essentially free of other polypeptides including the complexing proteins (HA70, HA17, HA33, or NTNH (NBP) of *Clostridium botulinum* or polypeptide preparations comprising other proteins in addition. Moreover, the term includes recombinant and chemically modified polypeptides. Such modifications may be artificial modifications or naturally occurring modifications. As referred to above, the polypeptide of the present invention shall have the biological properties of the neurotoxin polypeptides referred to above. Moreover, it shall exhibit shortened duration of the biological effect in a subject upon administration. Further, the polypeptide of the invention can be composed of a Botulinum toxin with a new binding domain to target other types of cells. The polypeptide of the invention, in an aspect, can be manufactured by a method of manufacturing a polypeptide as described elsewhere herein in more detail. In an aspect of the invention, a polypeptide preparation is also envisaged which comprises a complex of the neurotoxin polypeptide and its complexing proteins.

[0032] Moreover, the present invention relates to an antibody which specifically binds to the polypeptide of the present invention. In a further aspect, the antibody specifically binds to the amino acid sequence shown in SEQ ID NO: 52, 57, 58, 61 or 75.

[0033] Antibodies against the polypeptide of the invention can be prepared by well known methods using a purified polypeptide according to the invention or a suitable fragment derived therefrom as an antigen. A fragment which is suitable as an antigen may be identified by antigenicity determining algorithms well known in the art. Such fragments may be obtained either from the polypeptide of the invention by proteolytic digestion or may be a synthetic peptide. In an aspect, the antibody of the present invention is a monoclonal antibody, a polyclonal antibody, a single chain antibody, a human or humanized antibody or primatized antibody, chimerized antibody or a fragment thereof.

Also comprised as antibodies by the present invention is a bispecific or bispecific single chain antibody, a synthetic antibody, an antibody fragment, such as a Fab, Fv or scFv fragment etc., or a chemically modified derivative of any of these. The antibody of the present invention shall specifically bind (i.e. does not cross react with other polypeptides or peptides) to the polypeptide of the invention. Specifically, the antibody shall also not cross react with the unmodified neurotoxin polypeptide (not carrying a MDM2 binding motif). Further, the antibody shall not cross react with the E3 ligase MDM2. Specific binding can be tested by various well known techniques. Antibodies or fragments thereof can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. Monoclonal antibodies can be prepared by the techniques originally described by Köhler et al. (Köhler 1975, Nature 256: 495) or Galfré (Galfré 1981, Meth. Enzymol. 73) which comprise the fusion of mouse myeloma cells to spleen cells derived from mammals which have been immunized by the antigen, i.e. the polypeptide of the invention or a immunogenic fragment thereof. The antibodies can be used, for example, for the immunoprecipitation and immunolocalization of the polypeptides of the invention as well as for the monitoring of the presence of said polypeptides, for example, in recombinant organisms, and for the identification of compounds interacting with the proteins according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of the protein of the invention (Schier 1996, Human Antibodies Hybridomas 7: 97-105; Malmborg 1995, J. Immunol. Methods 183: 7-13). In another aspect, the antibody of the invention specifically binds to one or more of the amino acid substitutions selected from the group consisting of Q53K, N72K, N378K, N379K, R394K and T400K, with the position numbering corresponding to that of SEQ ID NO: 52.

[0034] The polynucleotide or polypeptide of the invention can be used as a medicament, in general.

[0035] The term “medicament” as used herein refers, in one aspect, to a pharmaceutical composition containing the biologically active neurotoxin polypeptide of the invention or a polynucleotide encoding it as pharmaceutical active compound. The said medicament may be used for human or animal therapy of various diseases or disorders in a therapeutically effective dose. The medicament can be formulated by various techniques dependent on the desired application purposes. Different aspects of a medicament according to the present invention are specified herein below.

[0036] In an aspect, the medicament comprises the biologically active neurotoxin polypeptide of the present invention one or more pharmaceutically acceptable carrier as a pharmaceutical composition. The pharmaceutically acceptable carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and being not deleterious to the recipient thereof. The pharmaceutical carrier employed may include a solid, a gel, or a liquid. Exemplary of solid carriers are lactose, terra alba, sucrose, talc, gelatine, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary of liquid carriers are glycerol, phosphate buffered saline solution, water, emulsions, various types of wetting agents, and the like. Suitable carriers comprise those mentioned above and others well known in the art, see, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania. It will be understood that a carrier might also be a virus or retrovirus suitable for gene therapy, in particular, if the active ingredient of the medicament is the polynucleotide of the invention.

[0037] The medicament, in an aspect, will be dissolved in a diluent prior to administration. The diluent is also selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water or physiological saline. In addition, the pharmaceutical composition or formulation may also include other carriers or non-toxic, non-therapeutic, non-immunogenic stabilizers and the like. Thus, the neurotoxin polypeptide of the invention can be present, in an aspect, in liquid or lyophilized form. In an aspect, it can be present together with glycerol, protein stabilizers (HSA) or non-protein stabilizers such as polyvinyl pyrrolidone (PVP), hyaluronic acid or free amino acids. In an aspect, suitable non-proteinaceous stabilizers are disclosed in WO 2005/007185 or WO 2006/020208.

[0038] In another aspect, the medicament will be provided as a solution comprising the neurotoxin polypeptide. Moreover, the solution can comprise carriers or stabilizers referred to above as well. A stable liquid formulation of the neurotoxin polypeptide can be provided, in an aspect, as disclosed by US 7,211,261.

[0039] The pharmaceutical composition is, in one aspect, administered topically. Conventionally the medicament will be administered intra-muscular or subcutaneous (near glands) depending on the desired medical indication. However, depending on the nature and the mode of action of a compound the pharmaceutical composition may be administered by other routes as well.

[0040] A therapeutically effective dose refers to an amount of the neurotoxin polypeptide or the polynucleotide of the invention which prevents, ameliorates or treats the symptoms

accompanying a condition or disease referred to in this specification. Therapeutic efficacy and toxicity of the compound can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. The medicament of the present invention will comprise, in an aspect, dosage recommendations in the prescribers or users instructions in order to anticipate dosage adjustments depending on the individual recipient.

[0041] The medicament referred to herein are developed to be administered at least once in order to treat or ameliorate or prevent a disease or condition recited in this specification. However, the said medicament may be administered more than one time.

[0042] The medicament according to the present invention may in a further aspect of the invention comprise drugs in addition to the biologically active neurotoxin polypeptide which are added to the pharmaceutical composition during its formulation.

[0043] Moreover, the present invention pertains to the use of the polynucleotide or the polypeptide of the present invention for the preparation of a medicament for the treatment of wound healing, immobilization for bone and tendon fracture treatment, post surgery immobilization, specifically in connection with haemorrhoidectomy, introduction of dental implants, hip joint replacement (endoprothesis), epicondylitis, knee arthroplasty, ophthalmological surgery, acne, irritable bowel disease, vaginism, low back pain, or benign prostate dysplasia.

[0044] The symptoms associated with the aforementioned medical conditions or diseases are well known to the person skilled in the art and are described in standard text books of medicine such as Stedman or Pschyrembel.

[0045] Moreover, the present invention also relates to the use of the polynucleotide or the polypeptide of the present invention for the preparation of a diagnostic medicament for determining whether a subject is susceptible for a neurotoxin therapy.

[0046] The diagnostic medicament referred to above is a neurotoxin polypeptide medicament as referred to above. However, the medicament is to be applied for a time and at a dosage regimen allowing merely the determination of whether a subject responds to the neurotoxin polypeptide at all or the determination of a suitable dosage regimen. Since the above neurotoxin polypeptide – although having therapeutic potential as well - is

pivotally used for a diagnostic purpose rather than for treating or amelioration in this aspect, the medicament comprising it is termed "diagnostic medicament". Thus, such a time-restricted pre-screen with the modified neurotoxin polypeptides of the present invention will assist in selecting subjects susceptible for a therapy using an unmodified neurotoxin as well as in determining a suitable dosage. Potential side effects of a therapy based on an unmodified neurotoxin which would normally persist over a longer time can be reduced due to the reduced duration of the biological effect elicited by the modified neurotoxin polypeptide of the invention.

[0047] The present invention encompasses a method for the manufacture of a neurotoxin polypeptide encoded by the polynucleotide of the invention comprising the steps of:

a) cultivating the host cell of the invention under conditions which allow for the expression of the neurotoxin polypeptide encoded by the polynucleotide of the invention, and
b) obtaining the neurotoxin polypeptide encoded by the polynucleotide of the invention from the host cell culture of a).

[0048] The polypeptide may be obtained from the culture, in an aspect, by all conventional purification techniques including affinity chromatography, ion exchange chromatography, size exclusion chromatography, high pressure liquid chromatography (HPLC) and precipitation techniques including antibody precipitation. In another aspect, the neurotoxin polypeptide may be obtained as a complex comprising in addition to the neurotoxin polypeptide complexing proteins. Moreover, obtaining as used herein, in an aspect, includes activation of the neurotoxin polypeptide. This can be achieved by proteolytic cleavage of the (single-chain) neurotoxin polypeptide precursor either intracellular by an endogenous or exogenous (e.g., recombinant expressed) protease or outside the cell by contacting the neurotoxin polypeptide, e.g., prior, during or after the aforementioned purification, with the protease under conditions allowing for cleavage.

[0049] Furthermore, a method for the manufacture of a medicament is contemplated in accordance with the present invention, said method comprising the steps of the aforementioned method of the invention and the further step of formulating the neurotoxin polypeptide encoded by the polynucleotide of the invention as a medicament.

[0050] It will be understood that such a method for the manufacture of a medicament is carried out according to the GMP standards for medicaments in order to ensure quality, pharmaceutical safety, and efficacy of the medicament. Suitable formulations of the

medicament are described elsewhere in this specification. The person skilled in the art is, however, well aware of how such formulations can be made.

[0051] The invention also encompasses a method for the manufacture of a cosmetic composition comprising the steps of the method of the invention and the further step of formulating the neurotoxin polypeptide as a cosmetic composition.

[0052] "Cosmetic composition" as used herein can be formulated and used as described for a pharmaceutical composition above. For a cosmetic composition, likewise, it is envisaged that the compound of the present invention is in an aspect used in substantially pure form. Impurities, however, may be less critical than for a medicament. Cosmetic compositions are, in a further aspect, to be applied intramuscular. In an even further aspect of the invention, cosmetic compositions comprising the neurotoxin can be formulated as an anti-wrinkle agent.

[0053] The present invention also pertains to such a cosmetic composition and to the use of the polynucleotide or the polypeptide of the present invention for the preparation of a cosmetic composition to be used as an anti-wrinkle agent.

[0054] All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

Figures:

[0055] **Figure 1:** Modified Botulinum neurotoxin harboring an MDM2 binding motif. Illustration of a modified botulinum neurotoxin with a binding motif for the E3 ligase MDM2 interposed between the botulinum neurotoxin light chain and heavy chain, comprising, from the N- to the C-terminus, a botulinum neurotoxin light chain, a MDM2 binding motif, a linker comprising a protease cleavage site and the botulinum neurotoxin heavy chain. The botulinum neurotoxin light chain and heavy chain are interlinked by a disulfide bridge.

[0056] **Figure 2:** Introduction of MDM2 binding motifs. Interposition of a binding motif for the E3 ligase MDM2 between the botulinum neurotoxin light chain and heavy chain as illustrated in Figure 1 allows recognition and binding of the E3 ligase MDM2 to the, thus, modified botulinum neurotoxin, resulting in ubiquitination of the indicated surrounding surface exposed lysine residues and faster degradation of the ubiquitinated botulinum

neurotoxin by the cellular proteasome system. Illustrated is the MDM2 binding consensus motif XXFXXXWXXLXX (SEQ ID NO: 43, with "X" representing any of the naturally occurring amino acids).

[0057] **Figure 3:** Generation and analysis of the duration of the biological activity of BoNT/E-MDM2 mutants. The effect on the duration of the biological activity of BoNT/E-MDM2 mutants in frontal cortex cells of the mouse is shown, in comparison to non-mutated BoNT/E-MDM2 (SEQ ID NO. 52). Indicated are the corresponding amino acid residues which have been substituted by lysine, respectively. As a result, BoNT/E-MDM2 mutants in which (i) Q53, N72, N378, N379, R394 and T400 (SEQ ID NO. 57), (ii) Q53, N378 and N379 (SEQ ID NO. 58), (iii) N72, N378 and N379 (SEQ ID NO. 61) or (iv) N378, N379 and T400 (SEQ ID NO. 75) in the light chain have been substituted by lysine residues showed a reduced duration of the biological effect on cortex neurons. Consequently, the introduction of lysine residues in the BoNT/E-MDM2 neurotoxin at the indicated positions resulted in a quicker degradation of the mutant light chain in cortex neurons. In contrast, numerous other tested amino acid substitutions and combinations thereof in the light chain did not show an effect on the duration of the neurotoxin's biological activity. The abbreviation "n.d." means not (yet) determined.

[0058] **Figure 4:** Activation of purified BoNT/E-MDM2 (SEQ ID NO: 52) by protease cleavage. Purified single-chain BoNT/E was cleaved with Thrombin according to Example 2, the protease was removed and the activated BoNT/E was analyzed by SDS-PAGE and Commassie staining. Lane 1: purified single-chain BoNT/E-MDM2; Lane 2: activated BoNT/E-MDM2; Lane 3: activated BoNT/E-MDM2, Thrombin removed.

[0059] **Figure 5:** Comparison of BoNT/E-MDM2 (SEQ ID NO: 52) with the Recombinant Wild Type BoNT/E (rWT) (SEQ ID NO: 82) in frontal cortex neurons. Frontal cortex neurons were incubated with equipotent doses of rWT (SEQ ID NO: 82) and BoNT/E-MDM2 (SEQ ID NO: 52). At defined time points, the ratio of cleaved to total SNAP-25 was analyzed which is a measure of the presence of the light chain in the nerve cell.

[0060] **Figure 6:** Comparison of BoNT/E-MDM2 (SEQ ID NO: 52) with the Recombinant Wild Type BoNT/E (SEQ ID NO: 82) in the Digit Abduction Score (DAS) Assay. Equipotent doses of rWT (SEQ ID NO: 82) and BoNT/E-MDM2 (SEQ ID NO: 52) were injected into the gastrocnemius muscle of mice and the paralysis was analyzed in the DAS assay.

[0061] **Figure 7:** Activation of purified BoNT/E-MDM2 (SEQ ID NO: 80) by protease cleavage. Purified single-chain BoNT/E was digested with Thrombin according to Example 6, the protease was removed and the activated BoNT/E was analyzed by SDS-PAGE and Coomassie staining. Lane 1: purified single-chain BoNT/E-MDM2; Lane 2: activated BoNT/E-MDM2; Lane 3: activated BoNT/E-MDM2, Thrombin removed.

[0062] **Figure 8:** Comparison of BoNT/E-MDM2 (SEQ ID NO: 80) with the Recombinant Wild Type BoNT/E (SEQ ID NO: 82) in the Foto Digit Abduction Score (DAS) Assay. Equipotent doses of rWT (SEQ ID NO: 82) and BoNT/E-MDM2 (SEQ ID NO: 80) were injected into the gastrocnemius muscle of mice and the paralysis was analyzed in the Foto-DAS assay. In contrast to the DAS Assay (see Fig. 5), the effect in this assay was shown as the difference 1 – ratio of width to length of the injected paw.

Examples:

[0063] The invention will now be illustrated by the following Examples which shall, however, not be construed as limiting the scope of the invention.

[0064] **Example 1:** Construction, expression and purification of recombinant BoNT/E comprising a MDM2 recognition motif (BoNT/E-MDM2; SEQ ID NO: 52)

[0065] The coding sequence of Botulinum neurotoxin type E (BoNT/E) harboring the MDM2 binding motif "LTFEHNWAQLTS" as shown in SEQ ID NO: 32 was gene synthesized and subcloned into an *E. coli* expression vector adding C-terminal purification tags (e.g. His-tag). The protein (with the amino acid sequence shown in SEQ ID NO: 52, BoNT/E-MDM2) was expressed in *E. coli* BL21 using LB medium for 24 h at 16°C. The expressed neurotoxin was purified using a 3-step chromatography protocol (e.g. affinity chromatography employing C-terminal affinity tags such as His-tag, ion exchange chromatography and/or size exclusion chromatography). The tags were afterwards removed by protease cleavage employing a C-terminal protease cleavage site (e.g. Thrombin cleavage site) and the purity of the protein was analyzed by SDS-PAGE.

[0066] **Example 2:** Activation of purified BoNT/E by protease cleavage

[0067] Purified Botulinum neurotoxin (see Example 1) with the amino acid sequence as shown in SEQ ID NO: 52 harbouring a Thrombin cleavage site in the linker between light and heavy chain was incubated with biotinylated Thrombin at 20°C O/N. Biotinylated Thrombin was removed by affinity chromatography (e.g. incubation with streptavidin

agarose) and the activated toxin was analyzed by SDS-PAGE followed by Coomassie staining (see Figure 4) and immunoblotting. The final concentration of the activated neurotoxin was determined by ELISA using a rabbit anti-BoNT/E antibody for capture and a guinea pig anti-BoNT/E antibody for detection. Potency testing was carried out using the hemidiaphragm assay (HDA).

[0068] **Example 3:** Determination of the persistence *in vitro*

[0069] Frontal cortex tissue was harvested from embryonic day 15-16 mice. Cells were suspended in Neurobasal™ medium at a density of $0.5x10^6$ cells per mL and 2000 µL were seeded onto 6-well plates. Cultures were incubated at 37°C in a 4% CO_2 atmosphere for 3.5 weeks. The cultures were treated with 5-fluaro-2'desoxyuridine (25µM) and uridine (63µM) to prevent further glial proliferation. The cultures were then treated with either 10 pM of wildtype BoNT/E (SEQ ID NO: 82) or BoNT/E comprising a MDM2 recognition motif (SEQ ID NO: 52; Example 2) for precisely 18 h and washed afterwards with conditioned cell culture medium. At this time point and after 3 days, 7 days, 10 days, 14 days and 21 days, cells were harvested and the ratio of cleaved to total SNAP25 in the Western Blot was determined applying a mouse monoclonal antibody (Synaptic Systems #111111). It was found that the cleavage ratio in the cell cultures treated with the BoNT/E comprising the MDM2 recognition motif (SEQ ID NO. 52) reached the 50% ratio (t_{50}) in a time about 25% shorter compared to the wild type BoNT/E (SEQ ID NO: 82); see Figure 5. This shows that the persistence of the light chain in the neuronal cells was reduced by 25% which demonstrates that the duration of the biological effect was reduced by 25%.

[0070] **Example 4:** Determination of the recovery *in vivo*

[0071] BoNT/E-MDM2 as described in Example 2 (SEQ ID NO: 52) was analyzed in the digit abduction assay (DAS) (Aoki, 2001 Toxicon. (12):1815-20). An equipotent dose of wild type and the mutant BoNT/E comprising the MDM2 motif were injected into the gastrocnemius muscle of 10 mice. The mice were scored according to the scale described in Aoki 2001 Toxicon. (12):1815-20. The recovery time of the mice treated with the BoNT/E-MDM2 (Example 2, SEQ ID NO. 52) was reduced by about 20%, compared to wild type BoNT/E (SEQ ID NO: 82); see Figure 6.

[0072] **Example 5:** Construction, expression and purification of recombinant BoNT/E comprising a MDM2 recognition motif (BoNT/E-MDM2; SEQ ID NO: 80)

[0073] The coding sequence of Botulinum Neurotoxin type E (BoNT/E) harboring the MDM2 binding motif "LTFEHNWAQLEN" as shown in SEQ ID NO: 78 was gene synthesized and subcloned into an *E. coli* expression vector adding C-terminal purification tags (e.g. His-tag). The protein (with the amino acid sequence shown in SEQ ID NO: 80, BoNT/E-MDM2) was expressed in *E. coli* BL21 using LB medium for 24 h at 16°C. The expressed neurotoxin was purified using a 3-step chromatography protocol (e.g. affinity chromatography employing C-terminal affinity tags such as His-tag, ion exchange chromatography and/or size exclusion chromatography). The tags were afterwards removed by protease cleavage employing a C-terminal protease cleavage site (e.g. Thrombin cleavage site) and the purity of the protein was analyzed by SDS-PAGE.

[0074] **Example 6:** Activation of purified BoNT/E by protease cleavage

[0075] Purified Botulinum Neurotoxin (Example 5) with the amino acid sequence as shown in SEQ ID NO: 80 harbouring a Thrombin cleavage site in the linker between light and heavy chain was incubated with biotinylated Thrombin at 20°C O/N. Biotinylated Thrombin was removed by affinity chromatography (e.g. incubation with streptavidin agarose) and the activated toxin was analyzed by SDS-PAGE followed by Coomassie staining (see Figure 7) and immunoblotting. The final concentration of the activated neurotoxin was determined by ELISA using a rabbit anti-BoNT/E antibody for capture and a guinea pig anti-BoNT/E antibody for detection. Potency testing was carried out using the hemidiaphragm assay (HDA).

[0076] **Example 7:** Determination of the persistence *in vitro*

[0077] Frontal cortex tissue was harvested from embryonic day 15-16 mice. Cells were suspended in Neurobasal™ medium at a density of $0.5x10^6$ cells per mL and 2000 µL were seeded onto 6-well plates. Cultures were incubated at 37°C in a 4% CO_2 atmosphere for 3.5 weeks. The cultures were treated with 5-fluaro-2'desoxyuridine (25µM) and uridine (63µM) to prevent further glial proliferation. The cultures were then treated with either 10 pM of wildtype BoNT/E (SEQ ID NO: 82) or BoNT/E comprising a MDM2 recognition motif (SEQ ID NO: 80; Example 6) for precisely 18 h and washed afterwards with conditioned cell culture medium. At this time point and after 3 days, 7 days, 10 days, 14 days and 21 days cells were harvested and the ratio of cleaved to total SNAP-25 in the Western Blot was determined applying a mouse monoclonal antibody (Synaptic Systems #111111). It was found that the cleavage ratio in the cell cultures treated with the BoNT/E comprising the MDM2 recognition motif (SEQ ID NO. 80) reached the 50% ratio (t_{50}) in a time about 25% shorter compared with the wild type BoNT/E (SEQ ID NO: 82). This

shows that the persistence of the light chain in the neuronal cells was reduced by 25% which demonstrates that the duration of the biological effect was reduced by 25%.

[0078] **Example 8:** Determination of the recovery *in vivo*

[0079] In a slightly modified setup of the DAS assay (see [0071]), the scoring was replaced by calculating the difference between 1 – ratio of width to length of the injected paw. The recovery time of the mice treated with the BoNT/E-MDM2 (Example 6, SEQ ID NO. 80) was reduced by about 25% compared to wild type BoNT/E (SEQ ID NO: 82); see Figure 8.

[0080] **Example 9:** Generation of BoNT/E-MDM2 mutants

[0081] The generation of two different BoNT/E-MDM2 polypeptides (SEQ ID NO. 52 and SEQ ID NO: 80) has been described in Examples 1 and 5. Subsequently, exposed amino acid residues in the BoNT/E light chain located in the spatial proximity of the MDM2-recognition motif have been identified by the analysis of the three-dimensional structure of the BoNT/E light chain. "Exposed amino acid residues" as used herein means that the amino acid residues are located at the surface of the BoNT/E light chain and the side chains of said amino acid residues are not involved in intra-molecular interactions. The exposed amino acid residues have been identified by molecular dynamic (MD) simulations and calculation of the solvent accessible surface area (SASA). A SASA value higher than 60% (mostly) has been chosen as a specific cutoff. The exchange of the exposed amino acid residues at the identified positions by lysine residues as shown in Fig. 3 has been carried out by site-directed mutagenesis using a QuikChange™ mutagenesis kit, in combination with specific primer pairs which have been designed to this end. The substitution by lysine residues of the identified exposed amino acid residues not involved in intra-molecular interactions has been performed because E3 ligases, including MDM2, ubiquitinylate their substrates at lysine residues. To this end, the E3 ligase first binds to the E3 ligase binding motif in the substrate, e.g. the MDM2 binding motif in a BoNT/E-MDM2 protein as described herein, and then "detects" lysine residues in the spatial proximity to the binding motif in the substrate which can be modified by ubiquitin molecules. Accordingly, the efficacy of proteasomal degradation is increased by the introduction of additional lysine residues in the light chain of the Neurotoxin polypeptide of the invention, comprising a MDM2 E3 ligase binding motif. The resulting DNA constructs have been transformed into an *E. coli* expression strain (BL21) and the thus modified recombinant Botulinum Neurotoxins have been expressed. The mutated recombinant Botulinum Neurotoxins have been purified from cell lysates of *E. coli* via

affinity chromatography (e.g. His-tag), ion exchange chromatography and/or size exclusion chromatography. Said mutated Neurotoxins have been activated by proteolytic cleavage using the protease Thrombin and subsequent removal of the protease. In the following, the thus purified and activated mutated Neurotoxins have been analyzed, as shown in the subsequent Example.

[0082] **Example 10:** Analysis of the degradation of BoNT/E-MDM2 mutants in a cell culture system

[0083] To this end, a cell culture of frontal cortex cells of the mouse has been established, as indicated in Example 3. One x 10^6 cells have been treated with 20 pM BoNT/E-MDM2 (SEQ ID NO. 52) and with the respective BoNT/E-MDM2 mutants (see Figure 3) for 18 hours in six well plates. After 18 hours, 3 days, 7 days, 10 days, 14 days, and 21 days, samples have been isolated and analyzed by Western Blot using an antibody which recognizes both SNAP-25 as well as SNAP-25 cleaved by BoNT/E (Synaptic Systems # 111111). The ratio of cleaved to uncleaved SNAP-25 has been taken as measurement for the biological activity of the light chain of the Botulinum Neurotoxin in the frontal cortex cells that is for its concentration. As a result, it has been found that the proteolytic activity and hence the concentration of the light chain of the BoNT/E-MDM2 mutants has been reduced significantly quicker, in comparison to that of BoNT/E-MDM2; see Figure 3. In particular, BoNT/E-MDM2 mutants in which (i) Q53, N72, N378, N379, R394 and T400 (SEQ ID NO. 57), (ii) Q53, N378 and N379 (SEQ ID NO. 58), (iii) N72, N378 and N379 (SEQ ID NO. 61) or (iv) N378, N379 and T400 (SEQ ID NO. 75) in the light chain have been substituted by lysine residues showed a reduced biological effect on cortex neurons. Consequently, the introduction of lysine residues in the BoNT/E-MDM2 Neurotoxins resulted in a quicker degradation of the mutant light chain in cortex neurons. In contrast, numerous other tested amino acid substitutions and combinations thereof in the light chain did not show an effect on the duration of the Neurotoxin's biological activity.

Claims

1. A polynucleotide encoding a neurotoxin polypeptide exhibiting a reduced duration of the biological effect in a subject, wherein said polypeptide comprises at least one E3 ligase recognition motif in the light chain, wherein said E3 ligase recognition motif is a binding motif for the E3 ligase MDM2.

2. The polynucleotide of claim 1, wherein said binding motif for the E3 ligase MDM2 is selected from the group consisting of ETFSDLWKLLPE (SEQ ID NO: 26), TSFAEYWNLLSP (SEQ ID NO: 27), LTFEHYWAQLTS (SEQ ID NO: 28), LTFEHWWAQLTS (SEQ ID NO: 29), LTFEHSWAQLTS (SEQ ID NO: 30), ETFEHNWAQLTS (SEQ ID NO: 31), LTFEHNWAQLTS (SEQ ID NO: 32), LTFEHWWASLTS (SEQ ID NO: 33), LTFEHWWSSLTS (SEQ ID NO: 34), LTFTHWWAQLTS (SEQ ID NO: 35), ETFEHWWAQLTS (SEQ ID NO: 36), LTFEHWWSQLTS (SEQ ID NO: 37), LTFEHWWAQLLS (SEQ ID NO: 38), ETFEHWWSQLLS (SEQ ID NO: 39), RFMDYWEGL (SEQ ID NO: 40), MPRFMDYWEGLN (SEQ ID NO: 41), SQETFSDLWKLLPEN (SEQ ID NO: 42) and/or LTFEHNWAQLEN (SEQ ID NO: 78).

3. The polynucleotide of claim 1 or 2, wherein said biological effect causes muscle paralysis in the subject.

4. The polynucleotide of any of claims 1 to 3, wherein said reduced duration persists less than 5, 4, 3, 2 weeks or less than 1 week, in the subject, preferably a human.

5. The polynucleotide of any one of claims 1 to 4, wherein the light chain of the polypeptide is obtained by the modification from a light chain being encoded by a polynucleotide comprising a nucleic acid sequence selected from the group consisting of:
   a) a nucleic acid sequence having a nucleotide sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 81, 11, 13 or 15;
   b) a nucleic acid sequence encoding a polypeptide having an amino acid sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 82, 12, 14, or 16; and
   c) a nucleic acid sequence being at least 40% identical to the nucleic acid sequence of a) or b).

6. The polynucleotide of any of claims 1 to 5, wherein said polynucleotide comprises a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence having a nucleotide sequence as shown in SEQ ID NO: 51 or 79;

b) a nucleic acid sequence encoding a polypeptide having an amino acid sequence as shown in SEQ ID NO: 52 or 80; and

c) a nucleic acid sequence being at least 40% identical to the nucleic acid sequence of a) or b).

7. A vector comprising the polynucleotide of any one of claims 1 to 6.

8. A host cell comprising the polynucleotide of any one of claims 1 to 6 or the vector of claim 7.

9. The host cell of claim 8, wherein said cell is an *E. coli* cell or a Clostridium or Bacillus cell.

10. A polypeptide encoded by the polynucleotide of any one of claims 1 to 6.

11. The polypeptide of claim 10, further comprising at least one amino acid substitution by lysine in the light chain of the neurotoxin polypeptide.

12. The polypeptide of claim 10 or 11, comprising at least one of the amino acid substitutions selected from the group consisting of Q53K, N72K, N378K, N379K, R394K and T400K.

13. An antibody which specifically binds to the polypeptide of any of claims 10 to 12.

14. A polynucleotide according to any one of claims 1 to 6 for use as a medicament.

15. A polypeptide according to any of claims 10 to 12 for use as a medicament.

16. The polynucleotide of claim 14 or the polypeptide of claim 15, wherein the medicament is for the treatment of wound healing, immobilisation for bone and tendon fracture treatment, post surgery immobilization, specifically in connection with haemorrhoidectomy, introduction of dental implants, or hip joint replacement (endoprothesis), epicondylitis, knee arthroplasty, ophthalmological surgery, acne, irritable bowel disease, vaginism, low back pain, or benign prostate hyperplasia.

17. A method for the manufacture of a neurotoxin polypeptide encoded by the polynucleotide of any one of claims 1 to 6, comprising the steps of:
    a) cultivating the host cell of claim 8 or 9 under conditions which allow for the expression of the neurotoxin polypeptide encoded by the polynucleotide of any one of claims 1 to 6, and
    b) obtaining the neurotoxin polypeptide encoded by the polynucleotide of any one of claims 1 to 6 from the host cell of a).

18. A method for the manufacture of a medicament comprising the steps of the method of claim 17 and the further step of formulating the neurotoxin polypeptide encoded by the polynucleotide of any one of claims 1 to 6 as a medicament.