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(54) 发明名称

通过颅内递送 SIRNA 治疗亨廷顿氏舞蹈症的
组合物、装置和方法

(57) 摘要

本发明提供了治疗神经退行性障碍的装置、小干扰 RNA 和方法, 该方法包括手术植入导管以使该导管的排放部位与脑内预定的注入部位相邻, 并且通过该导管的排放部位排放预定剂量的能够抑制至少一种神经退行性蛋白产生的至少一种物质。本发明还提供了体内治疗亨廷顿氏舞蹈症而不会损伤患者细胞内质网、自发运动活动和自主运动活动的有益的小干扰 RNA 载体、系统和方法。

1. 一种离体双链核酸,包括第一链核酸和第二链核酸,其中第一链含有由序列 SEQ. ID. NO :24 编码的至少 19 个连续核苷酸,第二链与序列 SEQ. ID. NO :24 编码的第一链的至少 15 个连续核苷酸互补。
2. 根据权利要求 1 所述的离体双链核酸,其中所述的第一链通过一种环结构与第二链连接。
3. 根据权利要求 1 所述的离体双链核酸,其中所述的第二链与包含序列 SEQ. ID. NO :24 的第一链至少 19 个连续核苷酸互补。
4. 一种编码权利要求 1 所述的离体双链核酸的载体。
5. 根据权利要求 4 所述的载体,其中所述的载体是一种病毒载体。
6. 根据权利要求 5 所述的载体,其中所述的病毒载体是一种腺相关病毒载体。
7. 一种如权利要求 1 所述的离体双链核酸在制备抑制患者细胞内亨廷顿蛋白的药物中的应用。
8. 根据权利要求 7 所述的应用,其中所述的患者是人类患者。
9. 根据权利要求 8 所述的应用,其中所述的细胞是神经细胞。
10. 根据权利要求 7 所述的应用,其中所述的药物包括在载体内根据权利要求 1 所述的双链核酸,所述载体可与阳离子脂质体复合,或者用脂质体微粒子或微胶囊包装。

通过颅内递送 siRNA 治疗亨廷顿氏舞蹈症的组合物、装置和方法

[0001] 相关申请的交叉引用

[0002] 本申请是 2006 年 8 月 8 日提交的美国专利申请 US11/501,147 的部分继续申请。后者是 2003 年 11 月 25 日提交的美国专利申请 US 10/721,693 的部分继续申请,并要求了 2003 年 2 月 3 日提交的临时申请 60/444,614 的优先权。

[0003] 本申请也是 2004 年 5 月 25 日提交的美国专利申请 US10/852,997 的部分继续申请。后者是 2003 年 11 月 25 日提交的美国专利申请 US 10/721,693 的部分继续申请,并要求了 2003 年 2 月 3 日提交的临时申请 60/444,614 的优先权。

技术领域

[0004] 本发明涉及通过脑内注入小干扰 RNA 或含有编码小干扰 RNA 的 DNA 的载体来治疗神经退行性障碍的装置、系统和方法。

背景技术

[0005] 本发明提供了将小干扰 RNA 递送到脑内靶位点以抑制或阻滞神经退行性障碍发生和发展的新型装置、系统和方法。在几种神经退行性疾病(诸如帕金森氏病、阿尔兹海默氏病、亨廷顿氏舞蹈症、1、2 和 3 型脊髓小脑性共济失调和齿状核-红核-苍白球-路易氏体萎缩(DRPLA))中,参与疾病整个病程的蛋白已经被鉴定。目前,这些神经退行性疾病还无法治愈。这些疾病使患者逐渐虚弱,大部分最终致死。

[0006] 这些神经退行性疾病(特别是阿尔兹海默氏病和帕金森氏病)的进一步问题是其患病率持续增加,因而造成严重的公共健康问题。最近研究指出 α -突触核蛋白(α -synuclein)(帕金森氏病)、 β -淀粉样前体蛋白裂解酶 1(BACE1(包括其变异体,例如 A、B、C 和 D 型变异体))(阿尔兹海默氏病)、亨廷顿蛋白(huntington)(亨廷顿氏舞蹈症)和 1 型共济失调蛋白(ataxin1)(1 型脊髓小脑性共济失调)分别作为这些疾病的发病机理中的主要致病因子。

[0007] 在帕金森氏病和阿尔兹海默氏病中的神经退行性过程以选择性神经细胞群的广泛丢失为特点,伴有突触损伤和星形胶质化。阿尔兹海默氏病的病理学标志包括淀粉样斑形成、神经元纤维缠结和神经毡细丝形成;帕金森氏病的病理学标志包括脑黑质内所谓路易(Lewy)体的神经元内包涵体的形成和多巴胺能神经元的丧失。虽然触发细胞功能障碍和死亡的机制并不清楚,但是主流观点认为神经退行性变是特异性神经元细胞蛋白诸如 α -突触核蛋白(帕金森氏病)和淀粉样前体蛋白(APP)(阿尔兹海默氏病——被 BACE1 加工成 β -淀粉样蛋白(包括其变异体,例如 A、B、C 和 D 型突变体))累积的后续毒性效应的结果。

[0008] α -突触核蛋白被牵连到帕金森氏病是因为它在路易体内被大量发现,其在转基因小鼠中的过量表达导致帕金森氏病样病变,其分子内的突变与家族性帕金森氏病相关。 α -突触核蛋白,属于一个包括 β 和 γ -突触核蛋白的更大分子家族,是一个具有 140 个氨

基酸的非淀粉样突触蛋白,它是在淀粉样斑中发现的一个 35 个氨基酸的非淀粉样组分蛋白的前体。

[0009] 阿尔兹海默氏病是一种进行性大脑退化性疾病,其特点是精神衰退、记忆丧失、精神错乱和定向障碍。在促成这种病变的细胞机制中有两种类型的纤维状蛋白沉积于大脑:由多聚体 tau 蛋白组成的细胞内神经元纤维缠结,以及大量的主要含 β -淀粉样蛋白的细胞外原纤维。 β -淀粉样蛋白,又称 A β ,由淀粉样蛋白前体蛋白(APP)在 β -和 γ -分泌酶裂解位点经蛋白水解加工而成,这种加工引起细胞毒性,并使 A β 的两种主要形式(A β_{40} 和 A β_{42})产生形成淀粉样蛋白的能力。因此,防止 APP 被加工成产生斑块形式的淀粉样蛋白可以极大地影响该病的进展,这使得 BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)成为抑制或阻滞该病的临床靶标。相似报道显示早老素(presenilin)是重新定向异常过程的候选靶标。

[0010] 亨廷顿氏舞蹈症是一种致死性的、遗传性的神经退行性障碍,其特点是非自主性的“颤搐”运动、抑郁和痴呆。病因已经确定,是 DNA 中由过长的 C, A, G, C, A, G, C, A, G 系列核苷酸组成的单基因突变。CAG 重复序列位于该基因中的编码基因产物蛋白的区域。因此,得到的亨廷顿蛋白也是“倍增的(expended)”,含有由氨基酸谷氨酰胺(由“CAG”编码)组成的超长区。就在该突变被精确定位为亨廷顿氏舞蹈症的病因之后不久,又在其它基因中找到相似的 CAG 重复序列倍增并发现其为多种其它致死性、遗传性神经退行性疾病的病因。目前,这些所谓的“多谷氨酰胺”疾病的名单包括至少 11 种,包括:1、2 和 3 型脊髓小脑性共济失调、脊髓性肌肉萎缩症(SBMA 或肯尼迪病)、齿状核-红核-苍白球-路易氏体萎缩(DRPLA)。虽然在每个这些疾病中含有倍增的 CAG 重复序列的具体基因不同,但确是因为脑中倍增的多聚谷氨酰胺蛋白的产生引起每种疾病。症状通常发生于中青年时期,之后 10 到 15 年就会死亡。目前这些致死性疾病还没有有效的治疗。

[0011] 有相当多的证据显示关闭异常蛋白在神经元中的产生即可治疗多聚谷氨酰胺疾病。已知这些疾病的原因是因突变蛋白而获得了新的功能,而不是该蛋白原有功能的丧失。携带着人倍增基因的 1 型脊髓小脑性共济失调(SCA1)转基因小鼠在青年期发生严重的共济失调(Clark, H. et al., Journal of Neuroscience 17:7385-7395(1997)),但相应小鼠基因被敲除的小鼠则没有罹患共济失调或表现出其它异常(Matilla, A., et al., Journal of Neuroscience 18:5508-5516(1998))。有异常的 1 型共济失调蛋白产生但被基因工程改造不能进入细胞核的 SCA1 转基因小鼠则不会发展为共济失调(Klement, I., et al., Cell 95:41-53(1998))。最后,已经构建亨廷顿氏舞蹈症转基因小鼠模型,其中突变的人转基因被工程改造成通过给予四环素,可以被人工“关闭”(正常情况下,小鼠和人在给予这种抗生素时对这种疾病不会产生任何作用)。这些小鼠产生症状之后,通过长期给予四环素关闭异常蛋白的产生会引起其行为的改善(Yamamoto, A., et al., Cell 101:57-66(2000))。这就显示减少异常亨廷顿蛋白在人类的表达可能不仅仅能预防新诊断患者亨廷顿氏舞蹈症的发展,而且可能会改善已经出现症状的患者的生活质量。

[0012] 最近多个小组进行了 siRNA 有效性的研究。Caplen, et al. (Human Molecular Genetics, 11(2):175-184(2002))对多种不同的双链 RNA 对含 CAG 重复序列的人类雄性激素受体基因 mRNA 转录体细胞表达的抑制能力进行了评价。他们的工作发现当 CAG 重复序列的侧翼序列出现在双链 RNA 中时,只有基因特异性抑制作用发生。他们还表明,构

建的双链 RNA 可以拯救诱导的半胱氨酸蛋白酶 -3 的活性。Xia, Haibin, et al. (Nature Biotechnology, 20 :1006-1010 (2002)) 采用构建的重组腺病毒表达靶向编码绿色荧光蛋白 mRNA 的 siRNA, 测定了其表达融合多聚谷氨酰胺 - 荧光蛋白的工程神经 PC12 克隆细胞系的多谷氨酰胺 (CAG) 表达抑制作用。

[0013] 设计和使用与产生特定蛋白的 mRNA 靶点互补的小干扰 RNA 是最近分子生物学家所用的防止特异性 mRNA 翻译的工具。分子生物学家用来干涉翻译的其他工具包括: 使用抗治疗靶点的核酶裂解 mRNA 序列来治疗阿尔兹海默氏病 (见 W001/16312A2) 和帕金森氏病 (见 W099/50300A1 和 W001/60794A2)。然而, 上述专利没有一篇公开能够局部治疗神经退行性疾病的, 特异性、局部递送小干扰 RNA 载体到脑内靶细胞的方法。上述专利没有公开递送装置的使用或任一将小干扰 RNA 载体递送或注入脑部的的方法。例如, 上述专利没有公开或提示通过颅内递送装置将小干扰 RNA 载体递送或注入脑部的的方法。

[0014] 另外, 上述的现有技术既没有公开任何注入小干扰 RNA 载体到脑内的技术, 现有技术也没有公开小干扰 RNA 载体在注入脑部后, 是否可以进入神经元并且产生预想的小干扰 RNA, 该小干扰 RNA 是否可以减少至少一种参与神经退行性障碍发病的蛋白的产生。

[0015] 现有技术描述了核酶的直接系统性递送。这种用于治疗神经退行性障碍的方法看起来既不可行又难如人意。首先, 干扰 RNA 明显不同于核酶; 其次, 系统性递送的小 RNA 分子不能在体内维持到达预期靶点的足够时间, 也不太可能通过血脑屏障。另外, 现有技术采用的方法可能是不实际的, 因为采用这种方法, 为了达到脑内有效量, 可能必须给予大量的小干扰 RNA。即使血脑屏障可以暂时打开, 通过血流递送的寡核苷酸大多数还是会在体内的其它器官系统特别是肝脏损失掉。

[0016] 美国专利 5, 735, 814 和 6, 042, 579 公开了药物注入治疗亨廷顿氏舞蹈症的方法, 但是这些专利中特别鉴定的药物涉及能改变神经元兴奋性的药物, 而没有特别鉴定旨在进入细胞并在细胞内改变蛋白表达的药物。

[0017] 本发明通过使用病毒载体以编码小干扰 RNA 的 DNA 的形式直接递送到小干扰 RNA 到脑部靶细胞, 解决了现有技术中存在的和系统性递送核酸相关的现有问题。直接递送小干扰 RNA 载体到脑部患病区的注入克服了之前的与递送相关的障碍。进一步, 病毒载体的使用使其能够高效进入靶细胞, 并可以高效短期和长期地通过细胞自身机制直接产生小干扰 RNA 药物。最后, 本发明通过定制用于刺激蛋白 mRNA 编码序列中的特异性位点的活性小干扰 RNA, 提供了一种独特的靶向性和选择性的方式。

[0018] 发明概述

[0019] 本发明提供了一种递送小干扰 RNA 用于神经退行性障碍的装置、系统和方法。

[0020] 所述治疗的第一个目的是递送特异性设计的小干扰 RNA 作为治疗帕金森氏病的治疗药物。治疗帕金森氏病的特异性设计的小干扰 RNA 靶向 α - 突触核蛋白 mRNA 以减少神经细胞中 α - 突触核蛋白的产量。在一个相关的实施方式中本发明中提供了特异性接入黑质递送抗 α - 突触核蛋白的小干扰 RNA 的装置。

[0021] 所述治疗的第二个目的是递送特异性设计的小干扰 RNA 作为治疗阿尔兹海默氏病的治疗药物。治疗阿尔兹海默氏病的特异性设计的小干扰 RNA 靶向 BACE1 (包括其变异体, 例如 A、B、C 和 D 型变异体) mRNA 以减少神经细胞中 BACE1 (包括其变异体, 例如 A、B、C 和 D 型变异体) 蛋白的产量, 从而干扰 β 淀粉样蛋白的产生。在一个相关的实施方式中本

发明中提供了特异性接入梅纳特 (Meynart) 基底核和大脑皮质, 递送抗 BACE1 (包括其变异体, 例如 A、B、C 和 D 型变异体) 的小干扰 RNA 的装置。

[0022] 所述治疗的第三个目的是递送特异性设计的小干扰 RNA 作为治疗亨廷顿氏舞蹈症的治疗药物。治疗亨廷顿氏舞蹈症的特异性设计的专用小干扰 RNA 靶向亨廷顿蛋白 mRNA 以减少神经细胞中亨廷顿蛋白的产量。在一个相关的实施方式中本发明中提供了特异性接入尾状核和壳核 (统称为基底核) 递送抗亨廷顿蛋白的小干扰 RNA 的装置。在本发明的不同实施方式中, 治疗亨廷顿氏舞蹈症的 siRNA 或编码这些 siRNA 的载体包含第一链, 该链含有至少 19 个连续核苷酸, 核苷酸由选自序列 SEQ. ID. NO :24 或序列 SEQ. ID. NO :25 的序列编码。

[0023] 所述治疗的第四个目的是递送特异性设计的小干扰 RNA 作为治疗 1 型脊髓小脑性共济失调 (SCA1) 的治疗药物。治疗 1 型脊髓小脑性共济失调的特异性设计的小干扰 RNA 靶向 1 型共济失调蛋白 mRNA 以减少神经细胞中 1 型共济失调蛋白的产量。在一个相关的实施方式中本发明中提供了特异性接入齿状核、栓状核、苍白核和小脑顶核 (统称为小脑深部核) 递送抗 1 型共济失调蛋白的小干扰 RNA 的装置。

[0024] 所述治疗的第五个目的是递送特异性设计的小干扰 RNA 作为治疗 3 型脊髓小脑性共济失调 (SCA3, 也称马查多 - 约瑟夫病 (Machado-Joseph 病)) 的治疗药物。治疗 3 型脊髓小脑性共济失调的特异性设计的小干扰 RNA 靶向 3 型共济失调蛋白 mRNA 以减少神经细胞中型共济失调蛋白的产量。在一个相关的实施方式中本发明中提供了特异性接入齿状核、栓状核、苍白核和小脑顶核 (统称为小脑深部核)、丘脑底区和黑质递送抗 3 型共济失调蛋白的小干扰 RNA 的装置。

[0025] 所述治疗的第六个目的是递送特异性特定小干扰 RNA 作为治疗齿状核 - 红核 - 苍白球 - 路易氏体萎缩 (DRPLA) 的治疗药物。治疗齿状核 - 红核 - 苍白球 - 路易氏体萎缩 (DRPLA) 的特异设计的小干扰 RNA 靶向 1 型萎缩蛋白 mRNA 以减少神经细胞中 1 型萎缩蛋白的产量。在一个相关的实施方式中本发明中提供了特异性接入齿状核、栓状核、苍白核和小脑顶核 (统称为小脑深部核)、苍白球和红核递送抗 1 型萎缩蛋白的小干扰 RNA 的装置。

[0026] 本发明提供了一种用于治疗神经退行性疾病的小干扰 RNA 载体的递送系统, 它允许将小干扰 RNA 或含有编码小干扰 RNA 的 DNA 的载体 (小干扰 RNA 载体) 靶向递送到脑内靶位点, 在较短或较长的时段内给患者治疗。

[0027] 本发明一个主要的实施方式中, 小干扰 RNA 载体被注入到脑内靶位点, 在那里小干扰 RNA 载体被神经元摄取并转运到靶细胞核内。然后小干扰 RNA 载体被宿主细胞机制转录成 RNA, 产生小干扰 RNA, 该小干扰 RNA 能阻止其靶向的神经退行性蛋白的产生。

[0028] 本发明还提供了使用神经外科装置递送治疗性小干扰 RNA 载体到脑部选择性区域的方法。具体而言, 本发明提供了使用手术植入的导管单次、重复或长期递送小干扰 RNA 载体到脑的方法。引入患病细胞的小干扰 RNA 载体具有小干扰 RNA 被细胞转录所需的必要 DNA 序列, 包括启动子序列、小干扰 RNA 序列, 选择性包括允许治疗性小干扰 RNA 已知末端产生的侧翼序列, 以及选择性还包括多聚腺苷化信号序列。

附图说明

[0029] 图 1 显示 1 型共济失调蛋白 mRNA 的分析图 (采用本领域知晓的定量 RT-PCR 方

法)。该 1 型共济失调蛋白 mRNA 取自用含抗 1 型共济失调蛋白的核酶(图 1 上部泳道)或用含抗 1 型共济失调蛋白的 siRNA(图 1 下部泳道)的质粒转染的 HEK293H 细胞。

[0030] 图 2 显示 1 型共济失调蛋白 mRNA 的分析图(采用相同的本领域知晓的定量 RT-PCR 方法)。该 1 型共济失调蛋白 mRNA 取自用含抗 1 型共济失调蛋白的小干扰 RNA 转染的 HEK293H 细胞(下部泳道),与 1 型共济失调蛋白 mRNA 获自用靶向 3-磷酸甘油醛脱氢酶(GAPDH)的对照 siRNA 转染的 HEK293H 细胞作对照。

[0031] 图 3 显示腺相关病毒表达载体 pAAV-siRNA 的构建。

[0032] 图 4 图示了一种实验器械(麦德托尼克公司(Medtronic, Inc.),明尼阿波利斯,明苏里达州,型号 8506),它能皮下植入颅骨上,并提供一种可使治疗性药物通过并递送到脑的通路端口。

[0033] 图 5 图示了一种实验器械(麦德托尼克公司,明尼阿波利斯,明苏里达州,型号 8506 的原理图),它能皮下植入颅骨上,提供一种通路端口,治疗性药物可通过它递送到脑。

[0034] 图 6 示出了文中所述的多种神经退行性疾病间的关系,以及用小干扰 RNA 载体靶向预期基因产物的治疗位置。

[0035] 最佳实施方式的具体描述

[0036] 本发明同时解决了现有技术中的两个问题:(1) 如何治疗因神经元内致病性蛋白的产生所致的神经退行性疾病的问题;以及(2) 递送治疗性小干扰 RNA 到患病神经元的问题。

[0037] 为了更好地理解本发明,下面列出了术语以及对这些术语的理解范围。

[0038] 术语

[0039] “ α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体))、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白”是指,分别由含有下列基因和 / 或人类基因组 DNA 表达和 / 或编码的氨基酸序列的蛋白及其突变蛋白衍生物: α -突触核蛋白(帕金森氏病),以及 β 位-APP 裂解酶(BACE1(包括其变异体,例如 A、B、C 和 D 型变异体))(阿尔兹海默氏病)、亨廷顿蛋白(亨廷顿氏舞蹈症),以及 1 型共济失调蛋白(1 型脊髓小脑性共济失调),3 型共济失调蛋白(3 型脊髓小脑性共济失调或 Machado-Joseph 病),和 / 或齿状核-红核-苍白球-路易氏体萎缩(DRPLA)。

[0040] 此处使用的“细胞”,用其通常的生物学含义,并不指完整的多细胞有机体。该细胞可存在于有机体,例如人,但是优选哺乳动物来源,诸如人、牛、羊、猿、猴、猪、狗、猫等。然而,产生小干扰 RNA 的一些步骤可能也需要使用原核细胞(如细菌细胞)或者真核细胞(如哺乳动物细胞),因此也包括在术语“细胞”的范围。

[0041] 所用“互补”是指含有一个或多个核酸(DNA 或 RNA)的分子可以和其它含有一个或多个核酸的分子通过传统的沃森-克里克(Watson-Crick)配对或其他非传统的方式形成一个或多个氢键。

[0042] 所用 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体))、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白的“等效”DNA,包括与编码 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体))、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白的 DNA,或者与在包括人类、啮齿类、

灵长类、兔、猪和微生物的多种生物中编码与 α -突触核蛋白、BACE1 (包括其变异体,例如 A、B、C 和 D 型变异体))、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白具有相似功能的蛋白的 DNA 具有同源性的 (部分或全部) 天然产生的 DNA 分子。等效 DNA 序列还包括诸如 5' 非翻译区、3' 非翻译区、内含子、内含子-外显子连接区、小干扰 RNA 靶点等,任选地,包括并入感染性病毒的 DNA,诸如腺相关病毒 (AAV)。

[0043] 术语“功能等效”是指与参考序列或蛋白功能相似的任一衍生物。特别地,术语“功能等效”包括有一个或多个核苷酸碱基插入、删除或替换,而对生物学功能没有显著不利影响的衍生物。所用“基因”是指控制 RNA 生产的 DNA 区域。在产生功能性小干扰 RNA 这种语境下,这种定义包括必需的 DNA 序列信息,包括编码小干扰 RNA 的 DNA 序列、非编码调控序列和包括在内的任何内含子。本定义并未排除其它编码蛋白的基因可以和编码小干扰 RNA 的基因联合或串联而发挥作用的可能性。

[0044] 术语“载体”为本技术领域熟知,定义为质粒 DNA、噬菌体 DNA、病毒 DNA 等,它们可以作为 DNA 运载工具,本发明的 DNA 可以插入其中并且可以被转录为 RNA。术语“多个载体 (vectors)”指这些用于递送目标核酸的基于核酸和 / 或病毒的技术中的任何一种。存在着多种类型的载体并为本技术领域所熟知。

[0045] 术语“表达”定义为基因被转录成 RNA 的过程 (转录);RNA 还可进一步加工成成熟的小干扰 RNA。

[0046] 术语“表达载体”定义为如上所述的载体或运载工具,设计成使插入序列在转化宿主后能够表达。克隆基因 (插入序列) 通常置于调控因子序列的调控之下,例如启动子序列。将克隆基因置于这种调控序列的调控之下通常被称作可操作性地连接到调控因子或序列。

[0047] “启动子”是指能够直接或间接地结合到细胞中的 RNA 聚合酶并启动下游 (3' 方向) 编码序列转录的 DNA 调控区。为了本发明的目的,启动子通过其转录起始位点结合在 3' 末端并向上游延伸 (5' 方向) 以包括启动本底水平以上可检测的转录所必需的碱基或因子。在启动子中,可以发现转录起始位点 (通过 S1 核酸酶作图可方便地定义),以及负责结合 RNA 聚合酶的蛋白结合区 (共有序列)。真核细胞启动子还常常,但并不总是,含有 “TATA” 框和 “CCAT” 框。原核细胞启动子含有 -10 区和 -35 区共有序列用于转录启动。

[0048] 所用“同源”是指该两个或多个核酸分子的核苷酸序列部分或全部相同。

[0049] 所用“高度保守序列区”是指靶基因中的一个或多个区域的核苷酸序列从一代到另一代或从一种生物系统到另一种生物系统不会发生显著变化。

[0050] 所用术语“抑制”或“抑制性的”是指和没有小干扰 RNA 时所观察到的相比,靶基因的活性或 mRNA 或编码靶基因的等效 RNA 的水平被降低。优选的抑制为比没有小干扰 RNA 时降低至少 10%、25%、50%,或至少 75%、85%,或至少 95%。

[0051] 所用“抑制表达”是指 α -突触核蛋白、BACE1 (包括其变异体,例如 A、B、C 和 D 型变异体))、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白 mRNA 水平降低,因此相应的蛋白水平降低以在某种程度上缓解疾病或病情中的症状。

[0052] 所用“RNA”是指核糖核酸,一种由核糖核苷酸通过磷酸-核糖 (糖) 骨架连接所组成的分子。所用“核糖核苷酸”是指鸟嘌呤、胞嘧啶、尿嘧啶,或腺嘌呤,或 2' 位带羟基

β -D-核-呋喃糖配基。本领域众所周知,在 DNA 序列中使用碱基胸腺嘧啶,在 RNA 中使用尿嘧啶作为遗传密码。本领域的技术人员知道在核酸序列中如何用尿嘧啶替代胸腺嘧啶将 DNA 序列转换成 RNA 序列,反之亦然。

[0053] 所用“患者”是指有机体,它是外植体细胞的供者或受者或细胞本身。“患者”还指给予本发明的核酸分子的有机体。优选地,患者是哺乳动物或哺乳动物细胞,诸如人、牛、羊、猿、猴、猪、狗、猫等,或者用于移植的这些动物的细胞。更优选地,患者是人或者人的细胞。

[0054] 术语“突触核蛋白”是指 α -突触核蛋白(特别是人或小鼠的)或者 β -突触核蛋白(特别是人或小鼠的)。编码人 α -突触核蛋白的全长核苷酸序列可通过登录号 AF163864(SEQ ID NO:7) 获得。人 α -突触核蛋白序列的两个变异体可通过登录号 NM000345(SEQ ID NO:14) 和登录号 NM_007308(SEQ ID NO:23) 获得。小鼠 α -突触核蛋白可通过登录号 AF163865(SEQ ID NO:10) 获得。

[0055] 术语“BACE1”是指 1 型 β -淀粉样蛋白前体蛋白裂解酶(特别是人或小鼠的)。BACE1 的几种变异体已经被测序,包括 A、B、C 或 D 型变异体。在某些科学文献中,BACE1 也被称为 ASP2 和 Memapsin2。编码人 BACE1 的全长核苷酸序列及其相关的变异体序列可通过登录号 No. NM_138971(SEQ ID NO:20)、登录号 NM-138972(SEQ ID NO:19)、登录号 NM_138973(SEQ ID NO:21) 和登录号 NM_012104(SEQ ID NO:18) 获得。小鼠同源序列可通过登录号 NM_011792(SEQ ID NO:22) 获得。

[0056] 术语“亨廷顿蛋白”指由亨廷顿氏舞蹈症基因(IT-15)(特别是人或小鼠的)编码的蛋白产物。编码人 IT-15 的全长核苷酸序列可通过登录号 AH003045(SEQ ID NO:9) 获得。小鼠序列可通过登录号 U24233(SEQ ID NO:12) 获得。

[0057] 术语“1 型共济失调蛋白”指由 1 型脊髓小脑性共济失调基因编码的蛋白产物(特别是人或小鼠的)。编码人 SCA1 的全长核苷酸序列可通过登录号 NM_000332(SEQ ID NO:15) 获得。小鼠 SCA1 可通过登录号 NM_009124(SEQ ID NO:13) 获得。

[0058] 术语“3 型共济失调蛋白”指由 3 型脊髓小脑性共济失调基因编码的蛋白产物(特别是人或小鼠的)。编码人 SCA3 的全长核苷酸序列可通过登录号 NM_004993(1 型剪接变异体)(SEQ ID NO:16) 和 NM_030660(2 型剪接变异体)(SEQ ID NO:17) 获得。(小鼠同源序列尚不可得)。

[0059] 术语“1 型萎缩蛋白”是指由齿状核-红核-苍白球-路易氏体萎缩(DRPLA)基因编码的蛋白产物(特别是人或小鼠的)。编码人 DRPLA 的全长核苷酸序列可通过登录号 No. XM_032588(SEQ ID NO:8) 获得。小鼠序列可通过登录号 No. XM_132846(SEQ ID NO:11) 获得。

[0060] 术语“修饰”包括与参考序列或蛋白基本相似的衍生物。

[0061] 此处所用的“核酸分子”是指含有核苷酸的分子。核酸可以是单链的、双链的或多条链的,并且可以包括修饰的或非修饰的核苷酸或非核苷酸或不同混合及其组合。根据本发明的核酸分子的一个例子是编码小干扰 RNA 的基因,即便它不一定具有其更普遍的编码蛋白产物的含义。

[0062] 所用“小干扰 RNA”是指在底物结合区与特异的基因靶标具有互补性的核酸分子,它能特异性地引导宿主细胞的酶裂解靶 RNA。亦即,小干扰 RNA 凭借其序列特异性及其与靶

RNA 的同源性,能够引起 RNA 链的裂解,进而使靶 RNA 分子因不能再被转录而失活。这些互补区容许小干扰 RNA 和靶 RNA 充分杂交因而许可裂解发生。对于生物学活性来讲百分之百的互补常常是必要的,因此是优选的,但是在本发明中低至 90% 的互补性也是可用的。本申请所述的特异性小干扰 RNA 其旨不在限制,本领域的技术人员可以清楚,本发明的小干扰 RNA 最重要之处在于它具有特异性的底物结合位点,该位点能与一个或多个靶核酸区域互补。

[0063] 小干扰 RNA 是双链 RNA 制剂,它能与靶 RNA (通常是信使 RNA) 的一个部分互补 (即能进行碱基配对)。通常,这种互补是 100% 的,但如果需要,也可以低至诸如 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% 或 99%。例如,21 个碱基中的 19 个碱基可以碱基配对。在某些情况下,需要在多个等位变异体间进行选择,需要与靶基因 100% 互补以从其它等位序列中识别出靶序列。当在等位靶标中选择时,长度的选择也是一个重要的因素,因为它是涉及互补百分比以及鉴别等位差异能力的另一重要因素。

[0064] 小干扰 RNA 序列需要具有足够的长度,以便小干扰 RNA 能和靶 RNA 通过碱基配对作用结合在一起。本发明的小干扰 RNA 可以具有不同长度。小干扰 RNA 的长度优选大于或等于 10 个核苷酸并且具有和靶 RNA 稳定地相互作用的足够长度;具体地说是 15 ~ 30 个核苷酸,更具体说是 15 ~ 30 之间的整数个核苷酸,诸如 15、16、17、18、19、20、21、22、23、24、25、26、27、28、29 和 30 个。所用“足够长度”是指大于或等于 15 个核苷酸的寡核苷酸,它的长度足够长,在预期条件下能提供预期的功能。所用“稳定地相互作用”是指小干扰 RNA 和靶核酸的相互作用 (例如通过在靶标与互补核苷酸在生理条件下形成氢键)。

[0065] 所用“包含”是指包括,但不限于“包含”这个词之后的任何内容。因此,术语“包含”表示所列出的元素是必需的或强制性的,而其它元素则是可选的,可以存在也可以不存在。

[0066] 所用“由……组成”是指包括且限于短语“由……组成”中的任何内容。因此,短语“由……组成”表示所列元素是必需的和强制性的,而不再存在其它元素。

[0067] 所用“基本上由……设计的组成”是指包括该短语中列出的任何元素,并限于不干扰或有助于在公开内容中详细说明书的活性或作用的其它元素。因此,短语“基本上由……组成”提示所列出的元素是必需的或强制的,但其它元素依赖于他们是否影响所列元素的活性或作用是可选的,可以存在或不存在。

[0068] 本发明提供了通过颅内递送编码小干扰 RNA 的载体来治疗多谷氨酰胺疾病 (诸如亨廷顿氏舞蹈症和 1 型脊髓小脑性共济失调)、帕金森氏病和阿尔兹海默氏病的手段和工具,该小干扰 RNA 设计成能使致病性蛋白或病情恶化蛋白表达沉默,通过一个或多个植入性颅内导管来递送。具体而言,本发明是:(1) 通过颅内递送编码能使亨廷顿蛋白沉默的小干扰 RNA 的载体来治疗亨廷顿氏舞蹈症的方法;(2) 通过颅内递送编码能使 1 型共济失调蛋白沉默的小干扰 RNA 的载体来治疗 1 型脊髓小脑性共济失调的方法;(3) 通过颅内递送编码能使 α -突触核蛋白沉默的小干扰 RNA 的载体来治疗帕金森氏病的方法;以及 (4) 通过颅内递送编码能使 1 型 β -淀粉样前体蛋白裂解酶 (BACE1) 沉默的表达小干扰 RNA 的载体来治疗阿尔兹海默氏病的方法。

[0069] 如前所述,此处所述的小干扰 RNA (或 siRNA) 是长度为 15 ~ 30 个核苷酸的双链 RNA 片段。它用于触发称为 RNA 干扰的细胞反应。在 RNA 干扰中,双链 RNA 被一种细胞内酶——Dicer 酶消化,产生双螺旋 siRNA。该双螺旋 siRNA 与另一细胞内酶复合物结合,该

复合物因此被激活,导向任何与该 siRNA 序列同源的(或互补的)mRNA 分子。激活的酶复合物裂解靶 mRNA,破坏并防止它被用来指导其相应蛋白产物的合成。RNA 干扰过程看起来似乎是自我扩增的,其方式尚未完全清楚。最近有证据显示,RNA 干扰是一种古老的固有的机制,不仅用于对病毒感染的防御(许多病毒都会给细胞引入外源 RNA),而且也是一种十分基础水平的基因调节。已经发现,RNA 干扰在植物、昆虫、低等动物和哺乳动物中都会发生,并且已经发现 RNA 干扰比其它基因沉默技术如反义技术和核酶技术都有效得多。作为一种生物技术,siRNA 涉及在细胞中引入(或促进细胞产生)短的、双链 RNA 分子,这种 RNA 分子相似于 Dicer 酶从入侵的双链 RNA 病毒加工得到的 RNA 分子。而人为触发 RNA 干扰的过程,并随后从那一点继续下去。

[0070] 为了将小干扰 RNA 递送到患者脑部,优选的方法是将编码该 siRNA 的 DNA 而不是该 siRNA 本身引入脑细胞。编码特定的治疗性的 siRNA 的 DNA 序列在知晓下列情况时可以特定化:(a) 靶 mRNA 可获得的一小部分序列(可以在公共的人类基因组数据库中获得);和(b) 如何确定 DNA 的众所周知的科学规则,该 DNA 在被细胞转录时将会产生相应的 RNA。该 DNA 序列,一旦确定,就可以基于实验室供应商订购的合成分子在实验室中构建,并采用标准分子生物学方法插入到几个替代“载体”中的一个中,以递送 DNA 至细胞。一旦递送到患者脑部的神经元中,那些神经元自身通过将插入的 DNA 转录成 RNA 将会产生 RNA,该 RNA 成为治疗性 siRNA。结果是细胞自身产生将沉默靶基因的 siRNA。结果是细胞产生的靶蛋白的量将会减少。

[0071] 小干扰 RNA 和小干扰 RNA 载体

[0072] 根据本发明,针对患病细胞产生的小干扰 RNA 防止神经元中疾病相关蛋白的产生。根据本发明,使用特异设计的载体来递送小干扰 RNA 到靶细胞的。用成功递送到大脑靶细胞来治疗神经退行性疾病来预测的所设计小干扰 RNA 的成功。

[0073] 已经表明,小干扰 RNA 能够靶向人细胞中的特异性 mRNA 分子。可以构建小干扰 RNA 载体来转染人细胞并产生小干扰 RNA,它能使靶 RNA 裂解因而干扰所编码的蛋白的产生。

[0074] 通过抑制神经致病性蛋白本身的产生或者通过抑制参与该神经致病性蛋白产生或加工过程的蛋白,本发明的小干扰 RNA 载体将会防止致病性蛋白的产生。为了改善患者的生活质量,可能需要给患者重复使用治疗性药物来实现足够大量的神经元的改变。然而,在一个个体神经元中,这种改变是长期性的,足以提供治疗性益处。诸如阿尔兹海默氏病、帕金森氏病、亨廷顿氏舞蹈症或 1 型脊髓小脑性共济失调患者的绝境,使得该治疗的益处超过治疗递送和用药的风险成为可能。虽然采用其它治疗药物和给药途径来实现神经致病性蛋白的减少也有可能,但是开发直接体内转染神经元的成功的治疗措施却可提供基于递送小干扰 RNA 载体到靶细胞的最好的方法。

[0075] 递送外源 DNA 到脑神经元优选的载体是腺相关病毒(AAV),诸如重组 2 型血清型腺相关病毒或重组 5 型血清型腺相关病毒。选择性地,其它病毒载体诸如单纯疱疹病毒,也可用于递送外源 DNA 至中枢神经系统神经元。亦有可能,非病毒载体诸如质粒载体也可单独或与脂质体复合物或聚乙烯胺混合使用,递送外源 DNA 到脑部的神经元。

[0076] 重要的是,应当注意此处举例说明的抗-1 型共济失调蛋白小干扰 RNA 和其它用于治疗神经退行性障碍的小干扰 RNA 都只是本发明实施方式的一些例子。在动物上的采用神经科学领域知晓的神经外科学方法的实验可以鉴定候选小干扰 RNA。用这些实证主义方法

鉴定的靶裂解部位和小干扰 RNA,当给予罹患这些神经退行性疾病的患者时,将产生最大的治疗效果。

[0077] 参照本发明的核酸分子,小干扰 RNA 被导向编码靶蛋白的 mRNA 的互补序列,这些序列既可在真正的蛋白编码序列,也可在与 5' 非翻译区或 3' 非翻译区。杂交后,宿主酶可以裂解该 mRNA 序列。小干扰 RNA 需要完全或高度互补才能有效。互补百分比是指核酸分子中可以第二个核酸分子序列形成氢键(如 Watson-Crick 碱基配对)的连续的残基的百分比(诸如 10 个中有 5、6、7、8、9 或 10 个,就是 50%、60%、70%、80%、90% 或 100% 互补)。“完全互补”指一个核酸序列中的全部连续残基都能与第二个核酸分子序列相同数目的连续残基形成氢键。然而,应当注意,siRNA 序列中的单个错配或碱基替代可能会实质上降低小干扰 RNA 的基因沉默活性。

[0078] 靶向 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白中特异性位点的小干扰 RNA 代表了一种在细胞或组织中治疗帕金森氏病、阿尔兹海默氏病、亨廷顿氏舞蹈症、1 型脊髓小脑性共济失调、3 型脊髓小脑性共济失调和齿状核-红核-苍白球-路易氏体萎缩的新方法。

[0079] 在本发明的一个优选的实施方式中,小干扰 RNA 的长度为 15 ~ 30 个核苷酸,在具体的实施方式中,核酸分子的长度是 15、16、17、18、19、20、21、22、23、24、25、26、27、28、29 或 30 个核苷酸。在优选的实施方式中,siRNA 序列的长度可能在 19 ~ 30 个碱基对,更优选的是 21 ~ 25 个碱基对,更优选的是 21 ~ 23 个碱基对。

[0080] 在一个优选的实施方式中,本发明提供了一种生产一类基于核酸的基因抑制剂的方法,它们对预期靶标表现出高度特异性。例如,小干扰 RNA 靶标优选编码 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和 / 或 1 型萎缩蛋白的靶 RNA 的高度保守序列区,以便使用本发明的一种或多种核酸分子可以提供对一种疾病或病情的特异性治疗。另外,通常,小干扰 RNA 序列可通过识别以一对腺嘌呤碱基(AA)开始的靶序列来选择(见实施例)。可以采用适当的转录酶或表达载体体外或体内构建 SiRNA。

[0081] SiRNA 可以使用 DNA 寡核苷酸体外构建。这些寡核苷酸可以构建成含有与沉默子 siRNA(Silencer siRNA)(Ambion 公司的构建试剂盒 1620)中包含的 T7 启动子引物的 5' 末端互补的 8 个碱基序列。每一基因特异性寡核苷酸与所提供的 T7 启动子引物退火,用克伦诺片段(Klenow 片段)的填充反应产生一全长 DNA 模板以转录成 RNA。两个体外转录的 RNA(一个是另一个的反义 RNA)通过体外转录反应生成,然后互相杂交形成双链 RNA。该双链 RNA 产物用 DNA 酶(去除 DNA 转录模板)和 RNA 酶(削平双链 RNA 末端)处理、柱纯化以提供可以被递送入细胞并检测的 siRNA。

[0082] 通常使用 RNA 聚合酶 III 启动子驱动与 siRNA 结构相似的短发夹 RNA 的表达,来构建能在哺乳动物细胞中表达 siRNA 的 siRNA 载体。编码该“发夹”的插入序列被设计成具有由一小段隔离序列分隔的两段反向重复序列。一段反向重复序列与 siRNA 靶向的 mRNA 互补。一串胸腺嘧啶加到 3' 末端作为 pol III 转录终止位点。一段进入细胞,载体组成型表达发夹 RNA。发夹 RNA 被加工成 siRNA,该 siRNA 诱导靶基因表达的沉默,即所谓的 RNA 干扰(RNAi)。

[0083] 目前为止,大多数有记载的 siRNA 表达载体中,三个不同的 RNA 聚合酶 III (pol III) 启动子中的一个被用于驱动小发夹 siRNA (1-5)。这些启动子包括已经明确表征的人类和小鼠的 U6 启动子和人 H1 启动子。选择 RNA 聚合酶 III 驱动 siRNA 表达是因为它能在哺乳动物中表达较大数量的小 RNA,并且它会在遇到与 3 ~ 6 个尿嘧啶串的时候终止转录。

[0084] 构建的核酸分子可以根据需要被外源性递送到特异组织或细胞靶标。选择性地,该核酸分子(如小干扰 RNA)可以由递送至特异细胞的 DNA 质粒、DNA 病毒载体和/或 RNA 逆转录病毒载体表达。

[0085] 被递送到靶细胞或组织的小核 RNA 序列是基于核酸的 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和/或 1 型萎缩蛋白的表达抑制剂(例如翻译抑制剂),用于在细胞或组织中防治包括帕金森氏病、阿尔兹海默氏病、亨廷顿氏舞蹈症、1 型脊髓小脑性共济失调、3 型脊髓小脑性共济失调和齿状核-红核-苍白球-路易氏体萎缩在内的神经退行性疾病,和其它任何与细胞或组织内 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和/或 1 型萎缩蛋白水平有关的疾病或病情。

[0086] 本发明的基于核酸的抑制剂可直接加入,或与阳离子脂质体复合,用脂质体包装,用病毒载体包装,或以其他手段递送至靶细胞或组织。该核酸或核酸复合物(和生物大分子结合或者不和生物大分子结合),可通过注射、输液泵或支架局部给予离体相关组织或体内。在优选的实施方式中,该核酸抑制剂含有某些序列,这些序列具有足够长度和/或与用 SEQ ID NOS:7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22 或 23 标示的互补底物序列稳定地相互作用。这些小干扰 RNA 的例子也在如与 1 型萎缩蛋白有关的 SEQ IDS NOS:1 和 2,3 和 4,和 5 和 6 等序列中显示。

[0087] 另一方面,本发明提供了含有本发明的一个或多个核酸分子和/或表达载体的哺乳动物细胞。该一个或多个核酸分子可以独立地被导向相同或不同的位置。

[0088] 在本发明的另一方面,与靶 RNA 分子相互作用并抑制 α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和/或 1 型萎缩蛋白活性的小干扰 RNA 分子,是由插入到 DNA 或 RNA 载体的转录单元表达的。该重组载体优选为 DNA 质粒或病毒载体。表达小干扰 RNA 的病毒载体,可以基于,但不限于腺相关病毒、逆转录病毒或腺病毒载体序列构建。优选地,表达小干扰 RNA 的重组载体被按上述方式递送并在靶细胞中维持。选择性地,病毒载体可用于提供小干扰 RNA 的瞬时表达。根据需要这些载体可以重复给药。一旦表达,该小干扰 RNA 结合到靶 RNA 并通过宿主机制抑制其表达进而抑制其功能。小干扰 RNA 表达载体,或小干扰 RNA 是通过使用颅内通路装置递送的。

[0089] 本发明的核酸分子,可单独使用或与其它药物协同使用或联合使用来治疗上述疾病或病情。例如,为了治疗与 α -突触核蛋白(帕金森氏病)、1 型 β -淀粉样前体蛋白裂解酶(阿尔兹海默氏病)、亨廷顿蛋白(亨廷顿氏舞蹈症)和 1 型共济失调蛋白(1 型脊髓小脑性共济失调)相关的疾病或病情,可单独使用或联合使用一种或几种适合于该治疗的药物来治疗患者或处理其它适合的细胞,这对于本技术领域的技术人员是显而易见的。

[0090] 在进一步的实施方式中,所述的小干扰 RNA 可以与其它已知的治疗措施合并使用来治疗上述的病情或疾病。

[0091] 在另一个优选的实施方式中,本发明提供了基于核酸的抑制剂(例如小干扰 RNA)及其用于下调或抑制编码参与帕金森氏病、阿尔兹海默氏病、亨廷顿氏舞蹈症、1 型脊髓小脑性共济失调、3 型脊髓小脑性共济失调和齿状核-红核-苍白球-路易氏体萎缩进展或维持的蛋白的 RNA(例如, α -突触核蛋白、BACE1(包括其变异体,例如 A、B、C 和 D 型变异体)、亨廷顿蛋白、1 型共济失调蛋白、3 型共济失调蛋白和/或 1 型萎缩蛋白)表达的方法。

[0092] 本发明还提供了可以在细胞内由已知的真核细胞启动子(例如:Izant and Weintraub,1985, Science,229,345;McGarry and Lindquist,1986, Proc.Natl.Acad. Sci., USA 83,399, Scanlon et al.,1991, Proc.Natl.Acad. Sci. USA,88,10591-5; Kashani-Sabet et al.,1992, Antisense Res.Dev.,2,3-15;Dropulic et al.,1992, J Virol.,66,1432-41;Weerasinghe et al.,1991, J Virol.,65,5531-4;Ojwang et al.,1992, Proc.Natl.Acad. Sci. USA,89,10802-6;Chen et al.,1992, Nucleic Acids Res.,20, 4581-9;Sarver et al.,1990 Science,247,1222-1225;Thompson et al.,1995, Nucleic Acids Res.,23,2259;Good et al.,1997, Gene Therapy,4,45;所有这些文献在此以全文以引文形式并入本文)驱动表达的核酸分子。本领域的技术人员能够认识到,任何核酸分子都可以从适合的 DNA/RNA 载体在真核细胞中表达。这些核酸分子的活性可以因为被核酶从初级转录物释放而增强(Draper et al., PCT WO 93/23569, and Sullivan et al., PCT WO 94/02595;Ohkawa et al.,1992, Nucleic Acids Symp. Ser.,27,15-6;Taira et al., 1991, Nucleic Acids Res.,19,5125-30;Ventura et al.,1993, Nucleic Acids Res.,21, 3249-55;Chowrira et al.,1994, J Biol.Chem.,269,25856;所有这些文献在此以全文以引文形式并入本文)。

[0093] 本发明另一方面,本发明的 RNA 分子优选从插入 DNA 或 RNA 载体的转录单位表达(见,例如 Couture et al.,1996, TIG.,12,5-10)。该重组载体优选 DNA 质粒或病毒载体。表达小干扰 RNA 的病毒载体,可以基于,但不限于腺相关病毒、逆转录病毒、腺病毒或甲病毒载体构建。

[0094] 优选地,能表达核酸分子的重组载体如上述被递送并维持在靶细胞中。选择性地,可以用病毒载体来提供核酸分子的瞬时表达。这样的载体根据需要可重复给药。一旦表达,核酸分子就会结合到靶 mRNA。递送表达核酸分子的载体可以通过使用所述的颅内通路装置单次、多次或长期递送。

[0095] 一方面,本发明描述了一种表达载体,它含有编码至少本发明的核酸分子的至少一个功能片段的核酸序列。编码本发明的核酸分子的核酸序列以一种允许该核酸分子表达的方式可操纵性地被连接。

[0096] 另一方面,本发明描述了一种表达载体,它含有:a) 转录起始区(例如真核 pol I、II 或 III 起始区);b) 编码至少一个本发明的核酸分子的核酸序列;和 c) 转录终止区(例如真核 pol I、II 或 III 终止区);其中所述的序列以一种允许所述的核酸分子表达和/或递送的方式可操作性地连接到所述的起始区和终止区。

[0097] 正如本领域所知晓和理解的,核酸分子序列的转录是由真核细胞 RNA 聚合酶 I(pol I)、真核 RNA 聚合酶 II(pol II) 或真核 RNA 聚合酶 III(pol III) 启动子驱动的。所有这些文献在此并入本文作为参考。一些研究人员表明 RNA 分子可以由这些能在哺乳动物细胞中发挥功能的启动子来表达(例如 Kashani-Sabet et al.,1992, Antisense

Res. Dev., 2, 3-15; Ojwanget al., 1992, Proc. Natl Acad Sci. USA, 89, 10802-6; Chen et al., 1992, Nucleic Acids Res., 20, 4581-9; Yu et al., 1993, Proc. Natl. Acad Sci. USA, 90, 6340-4; L' Huillier et al., 1992, EMBO J, 11, 4411-8; Lisiewicz et al., 1993, Proc. Natl. Acad. Sci. U. S. A, 90, 8000-4; Thompson et al., 1995, Nucleic Acids Res., 23, 2259; Sullenger & Cech, 1993, Science, 262, 1566)。更具体地说, 诸如由编码 U6 小核 RNA (snRNA)、转移核糖核酸 (tRNA) 和腺病毒 VA RNA 驱动的转录单位都可用于在细胞中产生高浓度的目的 RNA 分子诸如小干扰 RNA (Thompson et al., supra; Couture and Stinchcomb, 1996, supra; Noonberg et al., 1994, Nucleic Acid Res., 22, 2830; Noonberg et al., US Pat. No. 5, 624, 803; Good et al., 1997, Gene Ther., 4, 45; Beigelman et al., 国际 PCT 公开号 WO 96118736; 所有这些文献在此以引文形式并入本文)。上述小干扰 RNA 转录单位可以被合并到多种载体引入哺乳动物细胞, 包括但不限于质粒 DNA 载体、病毒 DNA 载体 (如腺病毒或腺相关病毒载体) 或病毒 RNA 载体 (如逆转录病毒或甲病毒载体) (见 Couture and Stinchcomb, 1996, supra 的综述)。

[0098] 重要的是, 应注意到基于利用小干扰 RNA 靶向 1 型共济失调蛋白的 mRNA 以使之减少的, 针对 1 型脊髓小脑性共济失调症的治疗只是本发明的一个实施方式。其他的实施方式包括利用抗亨廷顿蛋白的小干扰 RNA 在人脑部纹状体给药, 以治疗亨廷顿氏舞蹈症, 以及利用抗 α -突触核蛋白的小干扰 RNA 在人脑部黑质给药, 以治疗帕金森氏症。

[0099] 应当注意, 构建本发明中作为治疗药物的小干扰 RNA 的示例性的方法 (即从 DNA 模板体外转录并组装成双链 RNA, 或将编码发夹结构 RNA 的 DNA 克隆到腺相关病毒表达载体中) 只是两种产生治疗性小干扰 RNA 的可能的手段。也可以使用其它更大规模的、更为有效的方法生产用于人类患者治疗的临床级别和临床数量的小干扰 RNA, 而不会改变本发明的实质。

[0100] 本领域技术人员很熟悉在众所周知的和现有的文献中讨论的原理和方法, 如 Remington's Pharmaceutical Science (17th Ed., Mack Publishing Co., Easton, Pa., 1985) 和 Goodman and Gilman's The Pharmaceutical Basis of Therapeutics (8th Ed., Pergamon Press, Elmsford, N. Y., 1990), 二者在此均以引文形式并入本文。

[0101] 在本发明一个优选的实施方式中, 根据适合递送给人类或哺乳动物的药物组合物的标准方法制成了含有本发明的 siRNA 的药物或其前体或衍生物的组合物。通常, 用于静脉给药的组合物是无菌等渗水性缓冲液中的溶液。

[0102] 如有必要, 该组合物还可以包括增溶剂和局麻药来缓解注射部位的疼痛。通常, 这些成分可单独或混合在单位剂型中提供, 例如, 密封容器如安瓿或 sachette 容器中的冻干粉或无水浓缩物, 标明活性成分含量。当组合物用于输液给药时, 它可以用含有药用级的无菌水或盐的输液瓶配药。当组合物用于注射给药时, 可提供一安瓿无菌注射用水或盐水以便其成分在给药前混合。

[0103] 在静脉给药以外的情况下, 该组合物可以含有少量的湿润剂或乳化剂或 pH 缓冲剂。该组合物可以是液体溶液、悬浊液、乳液、凝胶、聚合物或缓释制剂。该组合物可以和本领域知晓的传统的粘合剂和载体一起制成制剂。制剂可以包括标准载体诸如药用级的甘露醇、乳糖、淀粉、硬脂酸镁、糖类化合物的钠盐、纤维素、碳酸镁等在药物生产中功能明确的惰性载体。已知多种递送系统可用于本发明的治疗剂的给药, 包括用脂质体包囊、微粒、微

囊、纳米颗粒和纳米胶囊等。

[0104] 在另一个优选的实施方式中,含小干扰 RNA 或其前体或衍生物的治疗剂可以制成中性或盐的形式。药学可接受的盐包括与游离氨基基团形成的盐,诸如从盐酸、磷酸、醋酸、草酸、酒石酸等衍生的盐,以及与游离羧基基团形成盐诸如衍生于钠、钾、铵、钙、氢氧化铁、异丙胺、三乙胺、2- 乙氨基乙醇、组氨酸、普鲁卡因或类似物的盐。

[0105] 本发明的治疗量是指根据疾病和病情的性质,对一种特定失调或病情是有效的量,这可以用在药物给药方面已经明确的标准临床技术确定。所用制剂的精确的剂量也有赖于给药途径、疾病或病情的严重程度,应当根据操作者的判断以及患者的需求确定。颅内给药剂量的适当范围通常大约为每毫升 $10^3 \sim 10^{15}$ 感染单位的病毒载体,单次注射 1 ~ 3000 毫升。每毫升中载体感染单位的加入量通常含有大约 10^4 、 10^5 、 10^6 、 10^7 、 10^8 、 10^9 、 10^{10} 、 10^{11} 、 10^{12} 、 10^{13} 、 10^{14} 感染单位病毒载体,在大约 10、50、100、200、500、1000 或 2000 毫升中递送。有效剂量可以由体外或体内试验系统用量效关系曲线推定。

[0106] 用本发明的小干扰 RNA 载体治疗神经退行性疾病,可以给特定患者植入具有通路端口的多个导管来完成全程治疗。在一个优选的实施方式中,每个大脑或小脑半球有一个或几个端口和导管系统。一旦神经外科医生完成植入,患者的神经内科医生就可以进行常规治疗,该治疗由在几周到几个月的时间段重复静脉推注小干扰 RNA 表达载体,以及随着时间监测治疗效果组成。在整个疗程中,该装置可保持植入几个月或几年。在确认治疗有效后,通路端口可选择性地拔除,导管可以封闭并放弃或者拔除。装置的材料应不会干扰磁共振成像,并且,当然,小干扰 RNA 制剂必须与通路端口和导管的材料以及任何表面涂层相兼容。

[0107] 除非特别限定,本文中的科学术语和技术术语及命名法具有与本发明所涉及领域的普通技术人员通常的理解相同的含义。通常,细胞培养、感染、分子生物学方法的流程等是本领域常用的方法。这些标准技术可见于参考手册诸如 Sambrook et al. (1989, Molecular Cloning-A Laboratory Manual, Cold Spring Harbor. Laboratories) 和 Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, N.Y.)。

[0108] 构建 siRNA 表达质粒和 / 或病毒载体所用的聚合酶链式反应 (PCR) 按照已知技术实施,见例如美国专利号 4,683,195 ;4,683,202 ;4,800,159 和 4,965,188 (所有这三篇美国专利公开专利文献以引文形式并入本文)。通常,PCR 涉及在杂交条件下,用待测特定序列每一条链的一条寡核苷酸引物处理核酸样品 (如在热稳定 DNA 聚合酶存在时)。每一引物合成的延伸产物与两条核酸链中的每一条互补,该引物和具有特定序列的每条链充分互补、杂交。从每条引物合成的延伸产物还可以作为模板,用相同的引物进一步合成延伸产物。延伸产物的合成经足够的循环数之后,分析样品以测定待测序列是否存在。可以在电泳后,用溴化乙锭染色使 DNA 可见,以检测扩增序列,或按照现有技术使用可检测标签来等方法检测。PCR 技术的综述见 PCR Protocols, A Guide to Methods and Amplifications, Michael et al. Eds, Acad. Press, 1990。

[0109] 装置

[0110] 本发明还提供了使用前述的小干扰 RNA,递送小干扰 RNA 到脑部靶位的装置、系统和方法。可预见的递送途径是通过使用植入的、内在的脑实质内导管,它能提供途径直接注射含 AAV 或其它载体的小体积液体到局部脑组织的途径。这些导管的近端可以连接到一个

植入的、外科方法固定在患者脑内的颅内通路端口,或者连接到位于患者躯干的植入性的药物泵。

[0111] 本发明范围的递送装置的例子包括 8506 型实验器械(麦德托尼克公司,明尼阿波利斯,明苏里达州),它能皮下植入在颅骨上,提供一个通路端口,治疗药物可以通过它被递送给脑。递送通过一个通过立体定向植入的聚氨酯导管来实现。图 4 和图 5 示意性地描述了 8506 型实验器械。能在 8506 型实验器械通路端口工作的两种型号的导管包括:8770 型脑室导管(麦德托尼克公司),用于递送到脑室,公开于美国专利 6,093,180,在此以引文形式并入本文;以及 IPA1 导管(麦德托尼克公司),用于递送到脑组织本身(脑实质内递送),公开于美国专利 09/540,444 和 09/625,751,在此以引文形式并入本文。后者导管在远端具有多个出口以便沿导管方向的多个位置递送治疗药物。

[0112] 在接入和放置过程中,优选放置某些器件来定位导管远端。优选在远端使用接入和放置过程中能被检测到的标记物。如果接入和定位使用某种形式的 x 射线放射完成的,则标记物优选为射线不能透过的。射线不能透过的标记物至少是远端尖部的一部分 x 射线不能透过,使尖端在接入和放置过程中通过荧光镜或通过 x 线能被观察到。

[0113] 在一个有优势的实施方式中,该射线成像标记物包括钽份,它分散在由生物相容性粘合剂(诸如如上讨论的)组成的基质中。其他可能适合作为射线成像标记物的材料诸如钨或铂材料。通常,射线成像标记物需要预先浇铸在导管远端尖部。

[0114] 选择性地,放射成像标记物可以选择那些具有足够放射浓度以便在成像过程中可视,而在导管尖端铸造时以粉状形式分散在导管远端尖部的材料。

[0115] 可选择地,标记物还可以由与核磁共振成像(MRI)相兼容的材料组成以便远端尖部在 MRI 扫描时可被检测到。这种标记物优选的材料是铂,而钨、钽以及相似的材料也可用。不管是否使用放射成像或 MRI,提供这种成像标记物的目的是使术者能精确地检测到尖端的确切位置,有助于放置以及以后验证导管远端的完整性和位置。

[0116] 除了上述装置,根据本发明的小干扰 RNA 载体的递送也可以用包括但不限于美国专利 5,735,814、5,814,014 和 6,042,579 的多种装置完成。所有这些在此以引文形式并入本文。采用本发明,本技术领域的技术人员可以认识到这些以及其它的装置和系统都可以适合递送本发明的小干扰 RNA 载体来治疗神经退行性疾病。

[0117] 在一个优选的实施方式中,该方法还包括在脑外植入泵的步骤,该泵与导管的近端相耦联,操纵泵即可通过导管的排出部位递送预定剂量的至少一种小干扰 RNA 或小干扰 RNA 载体。进一步的实施方式还包括周期性地为脑外泵更新至少一种小干扰 RNA 或 RNA 载体的供给的进一步步骤。脑内预定位置可以通过多种方法定位,例如,对于某些应用,靶区可以通过立体排列方式或大体解剖图谱来定位。在其它实施方式中,当靶区的精确定位非常关键时,例如,当至少部分可逆性基因治疗系统被递送到患者脑内时,可以采用其它定位方法。这些定位方法包括,但不限于,正电子发射体层显像和单光子发射计算机断层显像(分别是 PET 和 SPECT)、药理学磁共振成像(phMRI)、功能性 MRI(fMRI)和对照增强计算机化成像(CT)扫描。

[0118] 在另外一个实施方式中,基于计算机辅助图谱的功能神经手术方法学可用来准确和精确地注射本发明的至少部分可逆性基因治疗系统。这些方法学允许对脑结构的三维显示和实时操纵。因此,在三维正交定向多脑图谱相互预登记的神经外方案是可能

的,可提高神经毒素的注射或植入靶位界限的精确度,通过减少手术泳道的数目减少手术过程的时间,有助于设计更为复杂的轨道。见如 Nowinski W.L. et al., Computer-Aided Stereotactic Functional Neurosurgery Enhanced by the Use of the Multiple Brain Atlas Database, IEEE TransMed Imaging 19(1);62-69;2000.

[0119] 在另外一个实施方式中,定位方法还容许术中验证导管远端的放置。例如,导管远端的放置的验证可以通过术中使用过 MRI 实施,MRI 可使用术中 MR 成像导向系统通诸如 PoleStar® iMRI Navigation Suite 或与之相当的系统。

[0120] 在另外一个例子中,在接入和放置过程中定位远端的方法是:使用暂时性地附着到导管近端部分或外科医生正在使用的将导管插入到患者脑内的手术仪器上的红外光反射小球。手术室中的红外照相机安放在手术台附近,发射和追踪从这些小球反射的红外信号。检测到的反射使得软件和计算机系统(诸如 StealthStation®)能够计算和显示导管远端的位置,在术中实时地叠加到之前捕获的、该具体患者的 MRI 成像。(这是可能的,因为该导管的远端和该导管近端为已知的线性距离,而红外光反射球就是暂时附着在近端的)。

[0121] 在另外一个实施例中,在接入和放置过程中定位远端的方法是:使用暂时性地附着到导管近端部分或外科医生正在使用的将导管插入到患者脑内的手术仪器上的红外发射发光二极管(LED)。手术室中的红外照相机安放在手术台附近,发射和追踪从这些 LED 发射的红外信号。检测到的反射使得软件和计算机系统(诸如 Stealth Station®)能够计算和显示导管远端的位置,在术中实时地叠加到之前捕获的、该具体患者的 MRI 成像。(这是可能的,因为该导管的远端和该导管近端为已知的线性距离,而 LED 就是暂时附着在近端的)。

[0122] 不管是使用被动反射的红外光还是使用主动发射的红外光来计算导管的位置或术者所用的将导管插入患者脑内的手术仪器的位置,使用红外三角法的目的是使术者精确地测定尖端的确切位置,帮助放置和在术中验证导管远端的完整性和位置。

[0123] 因此,本发明包括使用可植入性泵和导管递送小干扰 RNA 载体,如美国专利 5,735,814 和 6,042,579 所揭示的,并且还使用传感器作为注入系统的一部分来调节递送到脑的小干扰 RNA 载体的量,如美国专利 5,814,014 所揭示。根据本发明的方法,还可使用例如美国专利申请号 09/872,698(2001 年 1 月 1 日提交)和申请号 09/864,646(2001 年 5 月 23 日提交)中所公开的其他装置和系统,在此以引文形式并入本文。

[0124] 综上,本发明提供了递送小干扰 RNA 载体到中枢神经系统的方法,因此可通过减少神经元内致病蛋白的产生来治疗神经退行性疾病。

[0125] 本发明旨在治疗神经退行性障碍和/或疾病,包括帕金森氏病、阿尔兹海默氏病、亨廷顿氏舞蹈症、1 型、2 型和 3 型脊髓小脑性共济失调,和/或任何由致病性蛋白产生而引起或加重的神经退行性疾病,或由任何其它突变蛋白获得新的致病性功能而引起的神经退行性疾病。

[0126] 实施例

[0127] 实施例 1

[0128] 靶向人 1 型共济失调蛋白 mRNA 的小干扰 RNA 的构建

[0129] 作为本发明实施方式的一个实施例,我们制成了一种靶向人 1 型共济失调蛋白 mRNA 的小干扰 RNA。该小干扰 RNA 可减少细胞培养物中人细胞中人 1 型共济失调蛋白 mRNA

的量。作为 1 型脊髓小脑性共济失调症 (SCA1) 的治疗手段,使用植入的通路端口和导管,同样的小干扰 RNA 或类似的小干扰 RNA 将被递送到患者脑部的小脑细胞。结果是这些细胞中的 1 型共济失调蛋白量的减少,因此减缓或阻滞了患者 SCA1 病的进展。

[0130] 抗人 1 型共济失调蛋白的小干扰 RNA 基于人 1 型共济失调蛋白核酸序列构建。人 1 型共济失调蛋白核酸序列从 NCBI 提供的公开的核酸数据库检索,NCBI 检索登录号为 NM_000332 (SEQ ID NO:15)。人 1 型共济失调蛋白 mRNA 序列的一部分被鉴定为小干扰 RNA 裂解的潜在位点,并通过 MFOLD 分析预测为单链的。在登录号 NM_000332 (SEQ ID NO:15) 中,构建了三对抗 1 型共济失调蛋白的 siRNA 靶点:

[0131] 1. 靶向 1 型共济失调蛋白 mRNA 序列 945 ~ 965 位的抗 1 型共济失调蛋白 siRNA:

[0132] SEQ ID NO:1:5' -AACCAAGAGCGGAGCAACGAA-3'

[0133] SEQ ID NO:2:3' -GGTTCTCGCCTCGTTGCTTAA-5'

[0134] 2. 靶向 1 型共济失调蛋白 mRNA 序列 1671 ~ 1691 位的抗 1 型共济失调蛋白 siRNA:

[0135] SEQ ID NO:3:5' -AACCAGTACGTCCACATTTCC-3'

[0136] SEQ ID NO:4:3' -GGTCATGCAGGTGTAAAGGAA-5'

[0137] 3. 靶向 1 型共济失调蛋白 mRNA 序列 2750 ~ 2770 位的抗 1 型共济失调蛋白 siRNA:

[0138] SEQ ID NO:5:5' -AAGCAACGACCTGAAGATCGA-3'

[0139] SEQ ID NO:6:3' -CGTTGCTGGACTTCTAGCTAA-5'

[0140] 设计了一系列的 6 个脱氧寡核苷酸片段,从 MWG Biotech 公司定制并购买,寡核苷酸合成定制服务提供的 6 个片段组成了三个靶位点。另外,这些寡核苷酸构建时还包括和 T7 启动子引物(包括在 siRNA 构建试剂盒中,Ambion 公司。货号 1620)5' 末端互补的 8 个碱基序列。每个特异性寡核苷酸都与所提供的 T7 启动子引物退火,用 Klenow 片段补平,产生用以转录成 RNA 的全长 DNA 模板。通过体外转录反应生成了两种体外转录 RNA(其一是另外一个的反义 RNA),然后彼此杂交产生双链 RNA。该双链 RNA 产物用 DNA 酶(以去除 DNA 转录模板)和 RNA 酶(以削平双链 RNA 末端)处理、柱纯化以提供该三种 siRNA,这三种 siRNA 被递送入细胞并在其中检测。

[0141] 实施例 2

[0142] 靶向人 1 型共济失调蛋白 mRNA 的小干扰 RNA 的递送

[0143] 所构建的如实施例 1 所述的 siRNA 分子 1-3 转染到 HEK293 细胞。收获转染细胞产生的 RNA,分析以测定人 1 型共济失调蛋白 mRNA 的量。

[0144] 图 1 显示定量逆转录酶聚合酶链式反应 (qRT-PCR) 分析 HEK 293H 细胞培养物中每微克总 RNA 中 1 型共济失调蛋白信使 RNA (mRNA) 的量的结果。分析了四个细胞群。第一个细胞群是被抗 GAPDH(一种与 1 型共济失调蛋白 mRNA 表达没有已知的相关性的“管家基因”)的 siRNA 瞬时转染的 293H 细胞(抗 GAPDH siRNA 作为标准对照,siRNA 制备和检测商业试剂盒,Ambion,Inc. 提供)。第二个细胞群是被抗 1 型共济失调蛋白 mRNA(抗 1 型共济失调蛋白 mRNA 序列 1671 位)的 siRNA 瞬时转染的 293H 细胞。第三个细胞群是被含有抗 1 型共济失调蛋白 mRNA 的核酶(该核酶在 1 型共济失调蛋白 mRNA 序列的第 1364 位裂解 1 型共济失调蛋白 mRNA)的质粒瞬时转染的 293H 细胞。第四个细胞群是被含有抗 1 型

共济失调蛋白 mRNA 第 0945 位的 siRNA 瞬时转染的 293H 细胞。转染 48 小时后,同时收获所有细胞群得到总细胞 RNA。

[0145] 在照片中的凝胶上, RT-PCR 反应扩增的 DNA 产物被凝胶电泳按分子大小分离,可以看出是不同浓度的条带。所述的每个细胞群都采用一系列平行反应分析,显示为每个凝胶上部或下部的一组泳道。每一组泳道包括每个泳道的两条带。上面一条带是加入到反应中的已知量的 DNA 的 DNA 扩增产物,它和由细胞 mRNA 逆转录而来的内源性 cDNA 竞争。如果在某一泳道的条带的具有相同的浓度,则可以推断原始细胞样本中的细胞 mRNA 的量与加入到反应管中的已知量的 DNA 的量相同。泳道从左到右,加入的 DNA 标准品的量是减少的,显示是皮克级的量。该分析可以通过观察该组泳道来解释,该组泳道中,条带的浓度从 DNA 标准品最亮到位于其下的细胞产物最亮“跨越”,表示此时 DNA 标准品的量比细胞 mRNA 低了。

[0146] 如图 1 所示的凝胶,上面一组泳道来源于用抗 1 型共济失调蛋白 mRNA 的核酶转染的细胞。将来自细胞样本的条带和来自 DNA 标准品的条带相比较,表明这些细胞中 1 型共济失调蛋白 mRNA 的量为 0.505 皮克到 0.303 皮克之间 / 微克总细胞 RNA。下面一组泳道来源于用抗 1 型共济失调蛋白第 0945 位的 siRNA 转染的细胞。对这些泳道的分析表明,这些细胞中 1 型共济失调蛋白 mRNA 的量为 0.303 皮克到 0.202 皮克之间 / 微克总细胞 RNA。

[0147] 如图 2 所示的凝胶,上面一组泳道来源于用对照用抗 GAPDH 的 siRNA 转染的细胞。对这些泳道的分析表明,这些细胞中 1 型共济失调蛋白 mRNA 的量为 0.711 皮克到 0.400 皮克之间 / 微克总细胞 RNA。最后,下面一组泳道来源于用另一抗 1 型共济失调蛋白第 1671 位的 siRNA 转染的细胞。这些泳道表明 1 型共济失调蛋白 mRNA 的量为 0.404 皮克到 0.303 皮克之间 / 微克总细胞 RNA。

[0148] 总之,具体分析结果如下:

[0149]

治疗	1 型共济失调蛋白 mRNA 的量(皮克/微克总细胞 RNA)		
	下限	上限	估计中点
对照 (GAPDH)	0.400	0.711	0.555
核酶 (A1364A)	0.303	0.505	0.404
siRNA(AT1671)	0.303	0.404	0.353
siRNA(AT0945)	0.202	0.303	0.252

[0150] 表 2 抗 1 型共济失调蛋白的 siRNA 对细胞培养物中 1 型共济失调蛋白 mRNA 表达的影响

[0151] 这些数据显示抗 1 型共济失调蛋白的 siRNA AT1671 和 AT0945,在转染后 48 小时,对于减少细胞中的 1 型共济失调蛋白 mRNA 的量都是有效的,并且 siRNA 对于减少 1 型共济失调蛋白 mRNA 比抗 -1 型共济失调蛋白的核酶更加有效。

[0152] 应当注意,用于本发明的治疗的小干扰 RNA 的示例性的构建方法(即通过体外转录产生的寡核苷酸组装和杂交)只是制备该治疗性小干扰 RNA 的可能的手段之一。亦可使用其它生产用于人类患者治疗的临床级别的和临床数量的更大规模、更为有效的方法而不

会改变本发明的主旨或偏离由所附的权利要求定义的本发明的精神和范围。

[0153] 实施例 3

[0154] 使用小干扰 RNA 等位基因特异性减少 1 型共济失调蛋白的表达

[0155] 在杂合体患者中,如果单核苷酸多态性 (SNP) 在突变体与正常长度的等位基因中有所不同,那么适合的 siRNA 则可以选择性地仅仅降低突变的等位基因的表达。我们已经采用自 SCA1 起始密码下游的第 +927 位 (见登录号 NT_007592) SNP 等位基因特异性 RT-PCR 检测了 293、DAOY、SK-N-SH 和 HeLa 细胞。HeLa 细胞表达 927C 而不是 927T 等位基因,而 293 细胞则表达 927T 而不是 927C 等位基因,DAOY 和 SK-N-SH 细胞则表达两种等位基因变异体。我们以该位点为中心,已经生成了等位基因特异性 siRNA。用这些 siRNA 对内源性 SCA1 mRNA 变异体等位基因特异性抑制的检测结果将被给出。

[0156] 实施例 4

[0157] 小干扰 RNA 病毒载体的构建

[0158] 构建了选择性报告质粒 pAAV-U6-Tracer 来克隆 siRNA (见图 3)。构建的质粒 pAAV-U6-Tracer 含有腺相关病毒的反向末端重复序列 (ITR)、U6 RNA 聚合酶 III 启动子侧翼区 (来自 pSilencer, Ambion)、EF1 启动子、绿色荧光蛋白、Zeocin^r 抗性基因和 SV40 polyA (来自 pTracer, Invitrogen)。克隆的基因片段如图 3 所示。表达 siRNA 的寡核苷酸被克隆到紧接 U6 RNA 聚合酶 III 启动子下游 3' 端的多克隆区域。

[0159] HEK293 细胞用 pAAV-siRNA、pHelper 和 pAAV-RC 共转染,得到病毒生产细胞,其中 pAAV-RC 和 pHelper 质粒是这三个质粒 AAV 生产系统的一部分 (Avigen 公司)。在培养基中生长的 293 生产细胞用于分离重组病毒,该病毒用来转染二级细胞:HeLa 细胞、DAOY 细胞和 SK-N-SH 细胞。

[0160] 实施例 5

[0161] 注射序列为 SEQ ID NO:24 的 siRNA 局部显著地降低 HD mRNA 的量

[0162] 为了证实如上公开的 siRNA 序列在体内是有效的,给恒河猴注射 3×10^{11} 含 AAV 载体的病毒颗粒,该 AAV 载体包含序列如表 3 所示的 SEQ ID. NO:24 或 SEQ. ID. NO:25 序列的 siRNA 或对照 siRNA,对照 siRNA 由 CMV 启动子调控的 GFP 序列上游的 U6 启动子调控。

[0163]

序列号 (SEQ. ID. NO)	序列, 5' -3'
24	GGAGTATTGTGGAACCTTAT
25	TGACAGCAGTGTTGATAAA

[0164] 表 3 抗 HD mRNA 的 siRNA 序列

[0165]

动物#	年龄 (岁)	半球	RNA 构建物	靶标
1	6	左	SEQ ID. NO: 24	壳核、尾状核
		右	SEQ ID. NO: 24	壳核、尾状核
2	15	左	对照	壳核
		右	SEQ ID. NO: 24	壳核
3	19	左	SEQ ID. NO: 25	壳核
		右	SEQ ID. NO: 24	壳核

[0166] 表 4 试验设计

[0167] 亨廷顿蛋白 (HD)mRNA 和蛋白自组织环切块或自组织切片激光微束切割的 (LMD) 细胞分离, 分别用 qRT-PCR 或蛋白质印迹法 (Western blot) 定量。

[0168] 注射含有序列 SEQ IDNO :24 的 siRNA 载体引起动物 1 在表达 GFP 的表达在壳核部位与同一半球 (右侧) 非 GFP 表达壳核部位相比, HD mRNA 减少了 37% (用 qRT-PCR 测定组织环切块)。

[0169] 同一动物的左半球, GFP 表达区域与非 GFP 表达区域相比, HD mRNA 的量减少了 65%~70% (用 qRT-PCR 测定 LMD 切片)。

[0170] 另外, siRNA 治疗效果是半球特异性的。在动物 2 中观察到右半球 (注射含序列 SEQ IDNO :24 的载体) 的 GFP 表达区与左半球 (注射了含对照 siRNA 的载体) 的 GFP 表达区相比, HD mRNA 显著下降。

[0171] 因此, 这些数据显示含 SEQ IDNO :24 序列的 siRNA 的病毒构建体, 可以局部地、显著地减少 HD mRNA 的量。

[0172] 实施例 6

[0173] 注射序列为 SEQ IDNO :24 的 siRNA 不会导致大的解剖学异常, 也不会损害转导细胞的内质网

[0174] 动物按照前述实施例的方案注射。用荧光显微镜检测绿色荧光蛋白, 苏木精伊红 (H&E) 染色、荧光显微镜检测亨廷顿蛋白免疫染色, 免疫染色法检测钙联蛋白, 免疫染色法检测蛋白质二硫键异构酶 (PDI), 进行组织病理学分析。这些研究结果显示 HD 抑制不会引起注射部位任何可检测的神经解剖学异常。病毒转导区可观察到外周血管成套的证据, 但是这种成套与 HD 抑制没有相关性。另外, 钙联蛋白和 PDI 染色没有显示转导细胞内质网的任何明显改变。

[0175] 实施例 7

[0176] 注射序列为 SEQ ID NO :24 的 siRNA 不会改变自发活动并趋向改善精细自主活动 (finelocomotor activity)

[0177] 动物按照实施例 5 的方案注射。自发活动和精细运动行为 (fine motor activity) 分别用 EthoVision 和 mMAP 仪测定。EthoVision 是一种商业化的视觉追踪系统 (EthoVision Pro, version 2.2, Noldus Information Technologies, Asheville, NC), 可以测定观察时段内动物运动的距离 (cm) 和整个身体的运动速度 (cm/sec)。mMAP 仪 (称自动猴运动分析仪 mMAP) 是一种用于客观测定恒河猴在从容器室内的平板上拿取食物时小手肌的精细运

动时间。

[0178] 尾状核和壳核 HD 的抑制不会引起动物自发活动的改变。任何动物的精细自主活动也未受到损害。另外,病毒注射后,所有动物的精细运动技巧都趋向改善。

[0179] 虽然本发明参考特别实施方式进行了描述,可以理解,这些实施方式都只是对本发明的原则和应用的举例说明。因此应当理解,可以对这里举例说明的实施方式进行大量的改变和设计其它排列,均不会偏离由权利要求定义的本发明的精神和范围。

[0180] 说明书中引用的所有文献,包括专利文献和非专利文献,都代表着本发明相关的技术领域的技术人员的技术水平。所有这些文献在此全文以引文形式并入本文,其程度相当于每一篇文献都具体地、个别地指出作为文献并入本文。

序列表

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RI 13-MAY-2002

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RI 12-FEB-2001

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VERSION AH003045.1 GI:663286

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OD 16-MAY-2002

DEFINITION Mus musculus dentatorubral pallidolusian atrophy(Dr
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OD 18-JUL-1995

DEFINITION Mus musculus huntingtin(Hd)mRNA, complete cds.

ACCESSION U24233

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RI 31-OCT-2000

DEFINITION Homo sapiens spinocerebellar ataxia 1(olivopontocerebellar ataxia

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RI 31-JUL-2002

DEFINITION Homo sapiens Machado-Joseph disease(spinocerebellar
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olivopontocerebellar ataxia 3,autosomal dominant,at
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ACCESSION NM_030660

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<309>2002-07-31

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<223>ACCESSION NM_012104

VERSION NM_012104.2 GI:21040369

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<222>(1).. (5832)

<223>LOCUS BACE 5832bp mRNA linear PRI 05-NOV-2002

DEFINITION Homo sapiens beta-site APP-cleaving enzyme (BACE), transcript

variant a, mRNA.

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<308>NM_012104

<309>2002-11-05

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RI 05-NOV-2002

DEFINITION Homo sapiens beta-site APP-cleaving enzyme(BACE), tr
anscript

variant b, mRNA.

ACCESSION NM_138972 ;VERSION NM_138972.1 GI :21040365

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<309>2002-11-05

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<223>LOCUS BACE 5700bp mRNA linear P

RI 21-MAY-2002

DEFINITION Homo sapiens beta-site APP-cleaving enzyme(BACE), tr
anscript

variant c, mRNA.

ACCESSION NM_138971 ;VERSION NM_138971.1 GI :21040363

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<309>2002-05-21

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RI 05-NOV-2002

DEFINITION Homo sapiens beta-site APP-cleaving enzyme(BACE), tr
anscript

variant d, mRNA.

ACCESSION NM_138973 ;VERSION NM_138973.1 GI :21040367

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<309>2002-11-05

<313>(1).. (5625)

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OD 07-JAN-2002

DEFINITION Mus musculus beta-site APP cleaving enzyme(Bace), mRNA.

ACCESSION NM_011792 ;VERSION NM_011792.2 GI :6857758

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<309>2002-01-07

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<222>(1).. (1096)

<223>LOCUS SNCA 1096bp mRNA linear P

RI 05-NOV-2002

DEFINITION Homo sapiens synuclein, alpha(non A4component of amyloid

precursor) (SNCA), transcript variant NACP112, mRNA. ACCESSION

NM_007308 :

VERSION NM_007308.1 GI :6806897

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<309>2002-12-05

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<213>Homo sapiens

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用抗 1 型共济失调蛋白的核酶 (A1364A) 和抗 1 型共济失调蛋白的 siRNA (AT0945) 转染的 HEK293H 细胞



图 1

用对照 siRNA (GAPDH) 和抗 -1 型共济失调蛋白 siRNA (AT1671) 转染的 HEK293H 细胞

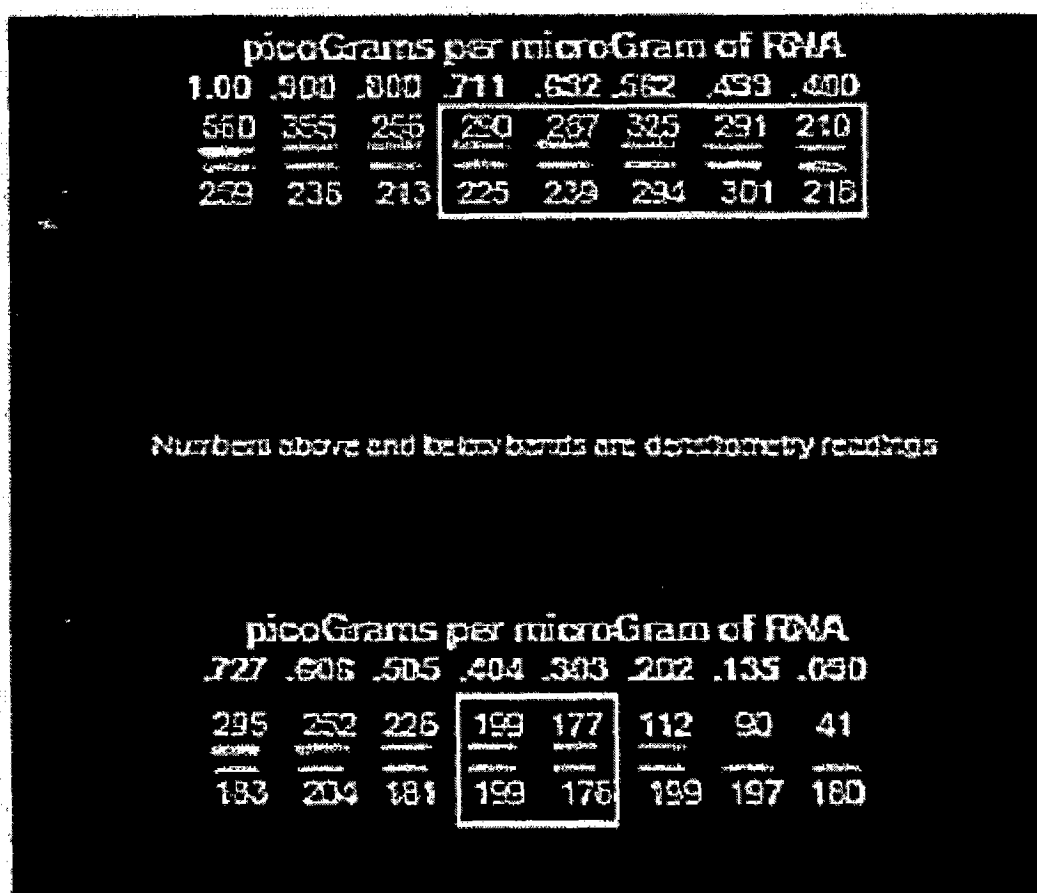


图 2

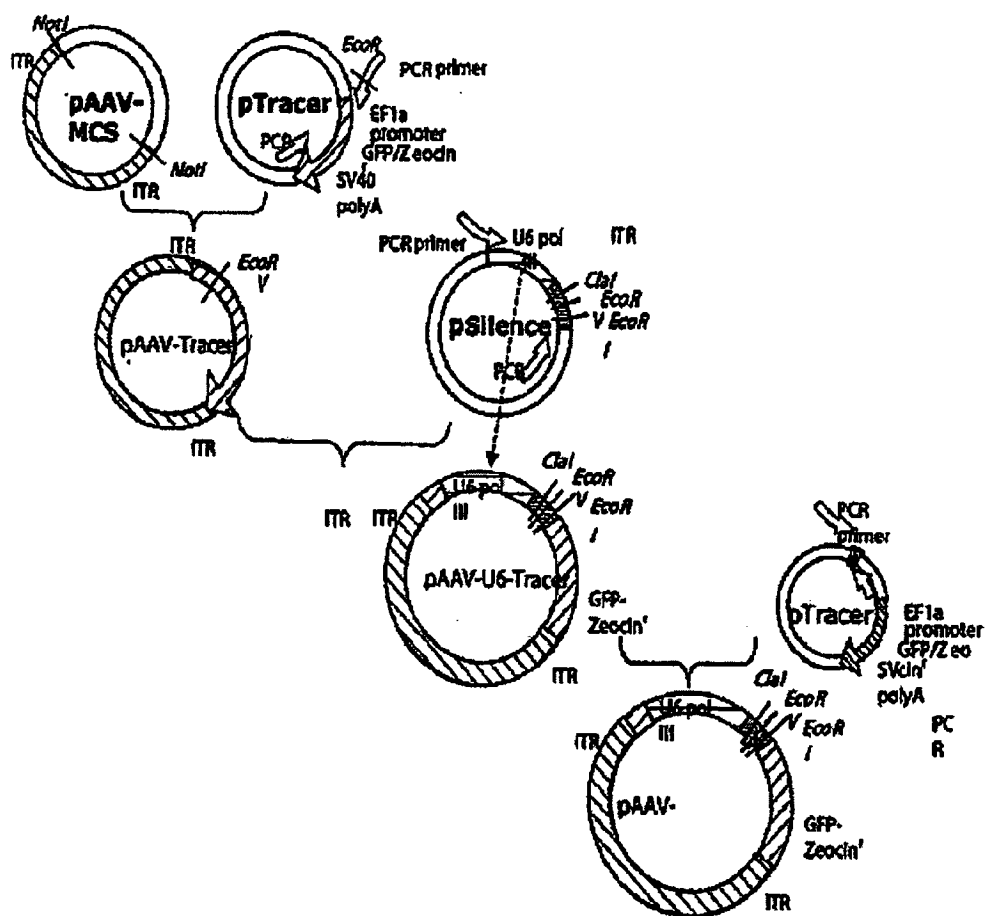


图 3

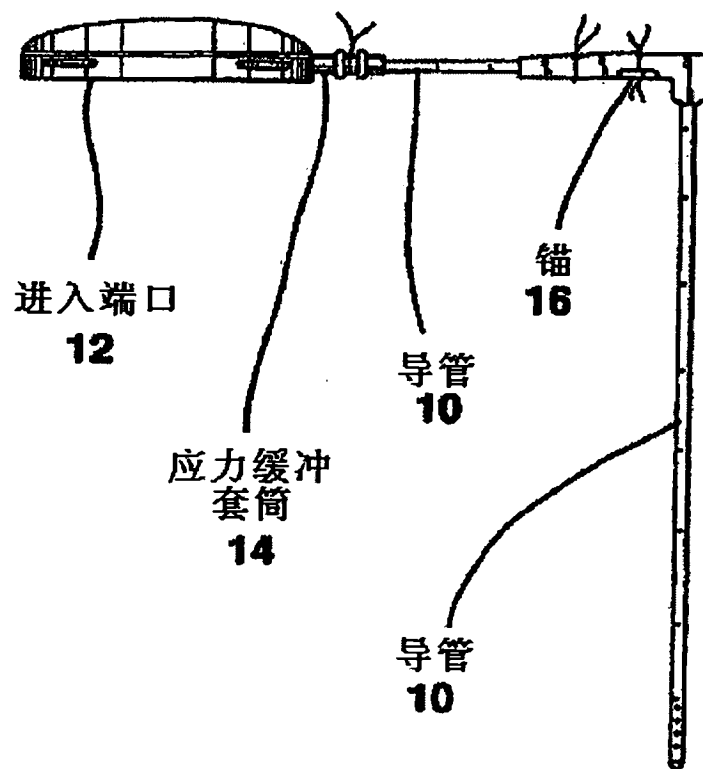


图 4

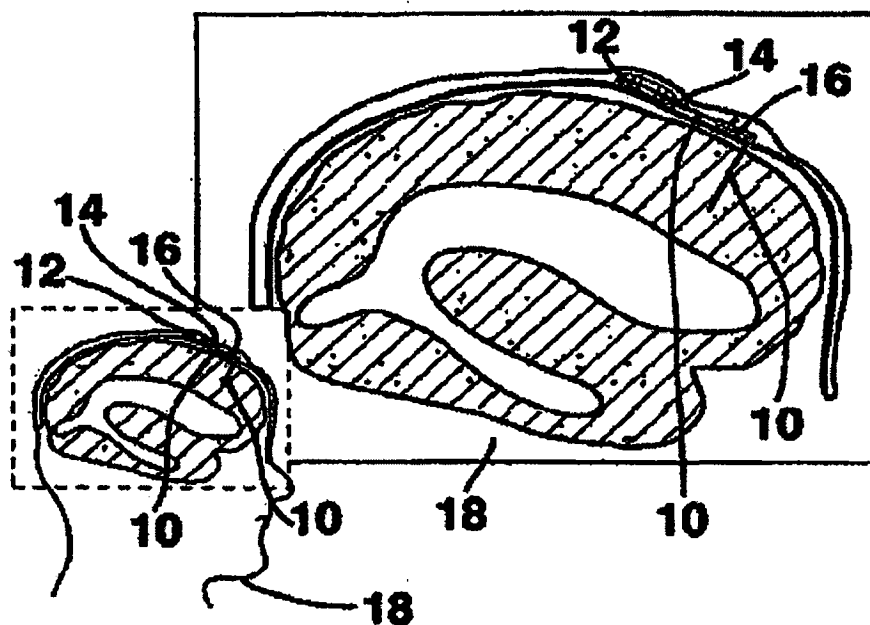


图 5

神经退行性疾病中的小干扰 RNA 治疗

疾病	部位	基因产物
帕金森氏病	黑质	α -突触核蛋白
阿尔兹海默氏病	梅纳特(Meynart)基底核 大脑皮质	1 型 β -淀粉样前体蛋白裂解酶(BACE1 (包括其变异体, 例如 A、B、C 和 D 型变异体))
亨廷顿氏舞蹈症	纹状体 尾状核 壳核	亨廷顿蛋白 IT15
脊髓小脑性共济失调 1 型 2 型 3 型 (马查多-约瑟夫病)	小脑深部核 齿状核 栓状核 苍白核 小脑顶核 大脑皮质	1 型共济失调蛋白 2 型共济失调蛋白 3 型共济失调蛋白
齿状核-红核-苍白球-路易氏体萎缩	红核 苍白球	1 型萎缩蛋白

图 6