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A rapid, targeted, neuron-selective, *in vivo* knockdown following a single intracerebroventricular injection of a novel chemically modified siRNA in the adult rat brain

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ABSTRACT

There has been a dramatic expansion of the literature on RNA interference and with it, increasing interest in the potential clinical utility of targeted inhibition of gene expression and associated protein knockdown. However, a critical factor limiting the experimental and therapeutic application of RNA interference is the ability to deliver small interfering RNAs (siRNAs), particularly in the central nervous system, without complications such as toxicity and inflammation. Here we show that a single intracerebroventricular injection of Accell siRNA, a new type of naked siRNA that has been modified chemically to allow for delivery in the absence of transfection reagents, even into differentiated cells such mature neurons, leads to neuron-specific protein knockdown in the adult rat brain. Following *in vivo* delivery, targeted Accell siRNAs were incorporated successfully into various types of mature neurons, but not glia, for 1 week in diverse brain regions (cortex, striatum, hippocampus, midbrain, and cerebellum) with an efficacy of delivery of approximately 97%. Immunohistochemical and Western blotting analyses revealed widespread, targeted inhibition of the expression of two well-known reference proteins, cyclophilin-B (38–68% knockdown) and glyceraldehyde 3-phosphate dehydrogenase (23–34% knockdown). These findings suggest that this novel procedure is likely to be useful in experimental investigations of neuropathophysiological mechanisms.

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1. Introduction

Gene silencing by RNA interference has emerged as a promising new method of inhibiting the expression of targeted genes and inducing knockdown of associated proteins both *in vitro* and *in vivo* (Akhtar and Benter, 2007; de Fougerolles et al., 2007; Elbashir et al., 2001; Shim and Kwon, 2010). RNA interference has been applied in experimental investigations of physiological and pathophysiological mechanisms in animal models, and also has been considered as a potential clinical tool in the treatment of intractable illnesses including cancer, infectious diseases, and neurodegenerative and neuropsychiatric disorders (Akhtar and Benter, 2007; Chen and Zhaori, 2011). Thus, findings from studies involving *in vivo* application of small interfering RNAs (siRNAs) in animal models may be useful in inspiring clinical studies and predicting unintended off-target effects and adverse reactions such as recruitment of immune responses (Akhtar and Benter, 2007; Chen and Zhaori, 2011).

Unfortunately, naked siRNAs generally are not stable enough to exert their expected effects *in vivo* because they are degraded rapidly by endo- and exonucleases (Shim and Kwon, 2010). Various delivery systems have been developed to circumvent this problem, including liposomes (Zimmermann et al., 2006), cationic polymers (Pulford et al., 2010), viruses (Dreyer, 2011), chemical modifications (Walton et al., 2010), short peptide-conjugations (Kumar et al., 2007), electroporation (Zhao et al., 2005), and exosomes (Alvarez-Erviti et al., 2011). In the central nervous system (CNS), topical injection of naked siRNAs has been used successfully to induce gene silencing, although the efficacy of siRNA delivery is adequate only immediately surrounding the injection site and not

Abbreviations: siRNA, small interfering RNA; i.c.v., intracerebraventricular or intracerebraventricularly; CNS, central nervous system; FAM, carboxyfluorescene; DAPI, 4′,6-diamidino-2-phenylindole; DAB, 3,3-diaminobenzidine; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule-1; CNP, 2′3′ cyclic nucleotide 3′ phosphodiesterase antibody; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IF, immunofluorescence.

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at more remote locations (Jean et al., 2007; Lingor et al., 2005; Makimura et al., 2002; Manrique et al., 2009). Intracerebroventricular (i.c.v.) injection of siRNAs conjugated to a lipid-based reagent (e.g., Lipofectamine 2000) has also shown promise, but this approach may possibly raise concerns about toxicity and recruitment of immune responses induced by the lipid-based reagent (Chen et al., 2009; Hu et al., 2009; Thakker et al., 2004, 2005).

Thermo Scientific Dharmacon Accell siRNA is a new type of naked siRNA that has been modified chemically to allow for delivery without requiring transfection reagents, resulting in robust silencing of selected genes and knockdown of associated proteins. Accell siRNA is designed to minimize off-target effects, toxicity, and recruitment of immune responses (Baskin et al., 2008). To date, there have been several studies in which Accell siRNA has been used successfully to induce robust gene silencing and knockdown of targeted genes and proteins in neurons, but these have been exclusively *in vitro* cell culture studies (Dolga et al., 2008; Dreses-Werringloer et al., 2008; Sebeo et al., 2009; Suzuki et al., 2010). A few reports have appeared in which Accell siRNA was delivered *in vivo* via intravenous and nasal routes (Bonifazi et al., 2010; Difeo et al., 2009), but none of these studies involved delivery into the adult brain.

In the present study we demonstrate that rapid, targeted, neuron-selective, *in vivo* knockdown can be achieved in the adult rat brain following a single i.c.v. injection of Accell siRNA. This novel methodology has considerable potential utility as an experimental tool.

2. Materials and methods

2.1. Materials

Chemicals: Unless otherwise noted, reagents were of analytical grade.

Accell siRNA: The sequences of the siRNAs were as follows:

Accell carboxyfluroescence (FAM)-labeled control siRNA and Accell control siRNA, 5′-UGGUUUACAUGUCGACUAA-3′; Accell rat cyclophilin-B siRNA, 5′-CCUUUGGACUCUUUGGAAA-3′; Accell rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA, 5′-UCUACAUGUUCCAGUAUGA-3′.

2.2. A single i.c.v. injection of Accell siRNA

The experiments were performed in accordance with the guidelines of the Animal Ethical Committee of Osaka Prefecture University. Male Wistar rats (SLC Japan, Shizuoka, Japan) weighing 250–350 g were anesthetized with pentobarbital (50 mg/kg, i.p., Abbott Japan, Tokyo, Japan) and placed in a stereotaxic instrument (Narishige, Tokyo, Japan). A single 28 gauge stainless steel injection cannula (Eicom, Kyoto, Japan) was lowered into the right lateral ventricle (coordinates: −0.8 mm posterior to bregma, −1.5 mm lateral to midline, and −4.6 mm ventral to the skull surface) (Paxinos et al., 1985). The rats then received an acute i.c.v. injection of Accell siRNA (5 μg/rat) in 5 μL of Accell siRNA delivery media (Accell Control siRNA kit, Green, for rat; Thermo Scientific Dharmacon, Rockford, IL) at a rate of 0.5 μL/min using a microinfusion pump (type ESP-32, Eicom Corporation, Kyoto, Japan) and a 10 μL microsyringe (Hamilton Company, Tokyo, Japan). After the infusion was complete, the cannula was left in place for 5 min, then removed at a rate of 1 mm/min.

2.3. Histological analysis

Rats were deeply anesthetized with pentobarbital (70 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) on day 2, 4, or 7 post-siRNA injection. The brains were removed and postfixed in 4% paraformaldehyde in PBS at 4 °C overnight, stored in 30% sucrose in PBS at 4 °C for 2–3 days, and frozen at −80 °C until sectioning. The frozen tissue was cut on a cryostat into 15-μM coronal sections. Sections were taken through the following brain regions, relative to bregma (Paxinos et al., 1985): cortex and striatum (+2.16 to 0 mm), hippocampus (−2.28 to −3.96 mm), midbrain (−4.92 to −6.24 mm), and cerebellum (−9.24 to −9.96 mm).

2.3.1. Semi-quantification of FAM-labeled cells

To count cells incorporating FAM-labeled siRNA, brain sections were incubated for 10 min with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/mL; Dojindo, Kumamoto, Japan) to visualize all cells in the area of interest. Square images (5 × 5 mm) were captured by confocal microscopy (C1si-TE2000-E; Nikon, Tokyo, Japan). The raw images first were grayed, then were made binary using the Scion image software package (version 4.0.3., Scion cooperation, Fredrick, MD). The images were analyzed in two ways to detect the efficiency of siRNA incorporation into cells. First, the number of FAM-labeled cells among at least 500 total cells in the square image were counted manually. Second, the number of binary FAM-derived pixels in the square images were measured automatically. For each approach, three different sections were evaluated in each experimental group.

2.3.2. Identification of the types of cells labeled by FAM

Brain sections were treated with 0.3% Triton-X 100 in PBS three times for 5 min each, then were incubated with 10% goat serum in PBS for 30 min at room temperature to block nonspecific binding, and finally were incubated overnight at 4 °C with one of the following antibodies: mouse monoclonal anti-NeuN (1:1000; Millipore Japan, Tokyo, Japan), mouse monoclonal anti-glial fibrillary acidic protein (GFAP; 1:500; DAKO, Glostrup, Denmark), rabbit polyclonal anti-ionized calcium binding adaptor molecule-1 (Iba1; 1:500; WAKO pure chemicals, Osaka, Japan), or Fluoro 555-conjugated mouse monoclonal anti-2′3′ cyclic nucleotide 3′ phosphodiesterase (CNP; 1:200; Sigma–Aldrich, St. Louis, MO). The sections then were washed three times in PBS before being treated with Alexa 568-conjugated anti-mouse (for NeuN and GFAP) or anti-rabbit (for Iba1) IgG antibody (1:1000; Invitrogen, Carlsbad, CA) for 1 h. Cells double-labeled with FAM and a marker protein (NeuN, GFAP, Iba1, or CNP) were counted manually. Three different sections were evaluated in each experimental group.

2.3.3. Immunohistochemistry for cyclophilin-B and GAPDH

Brain sections were incubated with 10% goat serum in PBS for 30 min at room temperature to block nonspecific binding. For cyclophilin-B staining, the sections were incubated overnight at 4% with rabbit polyclonal anti-cyclophilin-B (1:1000; Abcam, Cambridge, UK). After three PBS washes, the sections were incubated in 0.3% hydrogen peroxide for 30 min, then were treated with a peroxidase-conjugated anti-rabbit IgG antibody (Histofine Simplestain MAX PO, Nichirei, Tokyo, Japan) for 1 h. The signal was visualized using a 3,3-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA). For staining of GAPDH, brain sections were pretreated in a microwave in 10 mM citrate buffer (pH 6.0) for 10 min, and incubated with 0.3% Triton-X 100 in PBS three times for 5 min each. The sections were incubated with 10% goat serum in PBS for 30 min at room temperature to block nonspecific binding, and finally were incubated with mouse monoclonal anti-GAPDH antibody (1:500; Millipore Japan) for 3 days at room temperature. After three PBS washes, the sections were incubated with Alexa 488-conjugated anti-mouse IgG antibody