Current Topics

Recent Progress in Drug Delivery System for Cancer Therapy

Review

Recent Strategies for Targeted Brain Drug Delivery

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Because the brain is the most important human organ, many brain disorders can cause severe symptoms. For example, glioma, one type of brain tumor, is progressive and lethal, while neurodegenerative diseases cause severe disability. Nevertheless, medical treatment for brain diseases remains unsatisfactory, and therefore innovative therapies are desired. However, the development of therapies to treat some cerebral diseases is difficult because the blood–brain barrier (BBB) or blood–brain tumor barrier prevents drugs from entering the brain. Hence, drug delivery system (DDS) strategies are required to deliver therapeutic agents to the brain. Recently, brain-targeted DDS have been developed, which increases the quality of therapy for cerebral disorders. This review gives an overview of recent brain-targeting DDS strategies. First, it describes strategies to cross the BBB. This includes BBB-crossing ligand modification or temporal BBB permeabilization. Strategies to avoid the BBB using local administration are also summarized. Intrabrain drug distribution is a crucial factor that directly determines the therapeutic effect, and thus it is important to evaluate drug distribution using optimal methods. We introduce some methods for evaluating drug distribution in the brain. Finally, applications of brain-targeted DDS for the treatment of brain tumors, Alzheimer's disease, Parkinson's disease, and stroke are explained.

Key words blood–brain barrier; brain targeting; glioma; ultrasound; multicolor deep imaging

1. Introduction

Because the brain is the most important human organ, many brain disorders can cause death or disability. For example, glioblastoma, one type of brain tumor, is extremely lethal, with a 5-year survival rate of only 5%. Neurodegenerative disorders such as Alzheimer's disease (AD) or Parkinson's disease (PD) cause severe disability associated with motor or cognitive impairment, which reduces patients' QOL. With an increasingly aging population, the number of patients with brain disorders tends to increase. As highly potent drugs to treat brain diseases are currently available, the symptoms of many diseases can be ameliorated. Nevertheless, satisfaction with medications for the treatment of brain diseases remains low compared with those to treat other diseases, and therefore innovative therapies are desirable.

Recently, new types of therapies such as gene vectors[1)] and oligonucleotides[2)] have been developed and approved. These can directly act on upstream biological targets, *e.g.*, DNA or RNA, that were not targeted by conventional drugs. Thus, these therapies are expected to be more effective than conventional drugs and offer cure of refractory brain diseases.

The blood–brain barrier (BBB) is the barrier between the blood and brain parenchyma, which prevents drugs from entering the parenchyma. Drugs with large molecular weights or high polarity cannot pass the BBB; therefore only a few small-molecule drugs can enter the brain parenchyma from the bloodstream.[3)] In the development of drugs for the treatment of central nervous system disorders, potent pharmacological activity alone is not sufficient due to the existence of the BBB. For these reasons, brain-targeting drug delivery systems (DDS) are now the subject of intensive development efforts to overcome the BBB and efficiently deliver therapeutic agents to the brain (Fig. 1).

The evaluation of DDS is important in the clinical phase. The brain has a complex structure and contains many cell populations. Moreover, each brain region is assigned a unique role, unlike other organs such as the liver which have homogeneous functions. Thus, information on drug distribution in the brain is necessary for evaluating the feasibility and utility of DDS. In addition, such information obtained in the evaluation stage can be useful for further development of DDS by feeding back the results to researchers.

This review first discusses recent progress in brain-targeting DDS, followed by methods to evaluate DDS. Finally, applications of brain DDS for the treatment of tumors, AD, PD, and stroke as representative brain disorders are described.

2. Approaches for Drug Delivery to the Brain

2.1. Systemic Route of Administration Brain-targeting DDS *via* the systemic route need a strategy to cross the two barriers between brain parenchyma and brain blood vessels: the BBB and blood–cerebrospinal fluid barrier (BCSFB). The BBB is a multiple layer composed of brain microvascular endothelial cells connected by tight junctions (TJs) and covered by pericytes and glial cells. Because of the BBB, brain microvascular endothelial cells do not have a fenestration to

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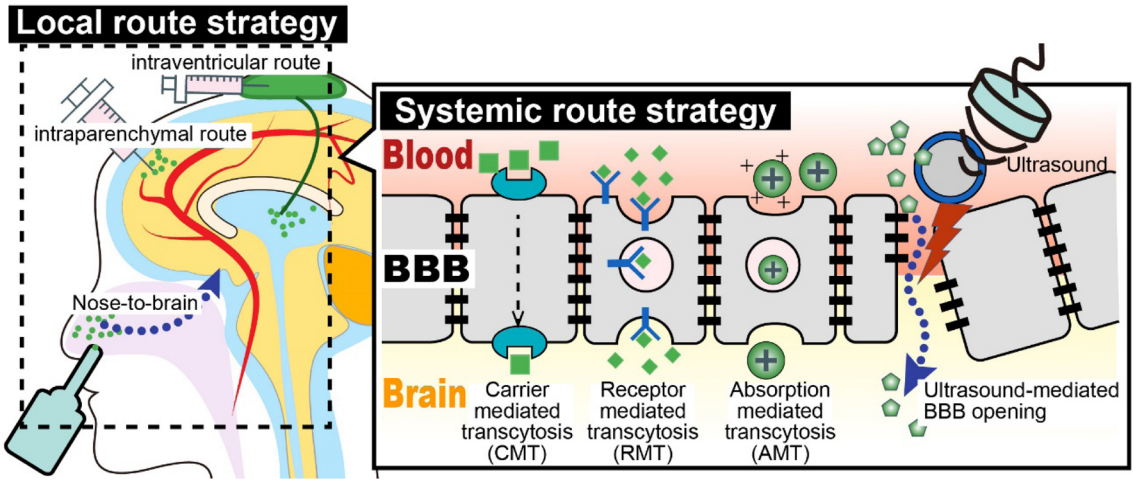

Fig. 1. Overview of Brain-Targeting Drug Delivery Systems

(Color figure can be accessed in the online version.)

Table 1. Efficiency of Brain-Targeting Drug Conjugates and Nanocarriers

Formulation	Ligand (mechanism)	% ID	Model animal	Ref.
Drug conjugates				
^{125}I-labeled 8D3	Anti-mouse TfR mAb (RMT, TfR)	3.1% ID/g brain	Normal mice	171
^{125}I-labeled RI7217	Anti-mouse TfR mAb (RMT, TfR)	1.6% ID/g brain		
^{125}I-labeled OX26	Anti-rat TfR mAb (RMT, TfR)	0.06% ID/g brain		
α-L-Iduronidase fused antibody	Anti-human IR mAb (RMT, IR)	1% ID/brain	Normal rhesus monkey	25
Iduronate 2-sulfatase fused antibody	Anti-human IR mAb (RMT, IR)	1% ID/brain	Normal rhesus monkey	27
Doxorubicin conjugates	Angiopep-2 (RMT, LRP1)	0.2% ID/g brain* 1.0% ID/g brain*	Normal mice Orthotopic U87 glioma-bearing mice	31
Etoposide conjugates	Angiopep-2 (RMT, LRP1)	0.3% ID/g brain* 1.2% ID/g brain*	Normal mice Orthotopic U87 glioma-bearing mice	
NIP228 conjugates	MTf-derived peptide (RMT, LRP1)	4% ID/g brain	Normal mice	34
Nanocarriers				
PEGylated liposomal doxorubicin	Glutathione (CMT)	0.08% ID/g brain*	Normal mice	56
PIC micelles	Glucose (CMT, GLUT1)	6% ID/g brain	24-h fasted mice (30 min after i.v. injection, i.p. glucose injection)	16
Liposomes	Anti-mouse TfR mAb (RMT, TfR)	0.22% ID/brain	Normal mice (1 mol% modification of each ligand)	58
Liposomes	Holo-Tf (RMT, TfR)	0.11% ID/brain*		
Liposomes	Angiopep-2 (RMT, LRP1)	0.07% ID/brain*		
Liposomes	ApoE mimetic peptide (RMT, LDLR)	0.09% ID/brain*		
Liposomes	DT mutated protein (RMT, DT receptor)	0.08% ID/brain*		

PIC, polyion complex; TfR, transferrin receptor; mAb, monoclonal antibody; RMT, receptor-mediated transcytosis; IR, insulin receptor; LRP1, low-density lipoprotein receptor-related protein-1; CMT, carrier-mediated transcytosis; LDLR, low-density lipoprotein receptor; DT, diphtheria toxin; % ID, % of injected dose; i.v., intravenous injection; i.p., intraperitoneal injection; *approximate value.

cross to the brain parenchyma except for the choroid plexus. On the other hand, the choroid plexus also has the BCSFB, which is tightly connected with the choroid plexus epithelial cells. These barriers play a crucial role in maintaining homeostasis in the brain and restricting 98% of low molecular-weight drugs and almost 100% of large molecules such as proteins, antibodies, nanoparticles, and nucleic acids.[3] Therefore, it is essential to develop brain-targeting DDS regardless of molecular size. In particular, we need to consider BBB-crossing strategies because its surface area is 5000-fold larger than that of the BCSFB.[4]

The three main pathways of drugs across the BBB are passive diffusion, transcellular pathway, and paracellular pathway. Passive diffusion of drugs is limited to molecules with the following characteristics: less than 400–500 Da; and high lipophilicity forming fewer than 8 hydrogen bonds with water solvent.[5] In the transcellular pathway, drugs are uptaken through transporters, receptors, and surface proteins expressed on the brain microvascular endothelial cells. Many researchers identified and analyzed these targets[6] and then developed ligand-grafted brain-targeting DDS.[7–9] The paracellular pathway passes through the junctions between endothelial cells, and DDS strategies like ultrasound-mediated temporal opening and modulation of junction expression levels have been proposed.[10,11] Drug delivery approaches through the transcellular and paracellular pathways are discussed in detail below.

2.1.1. Ligand Modification Approaches Across the BBB

For the purpose of targeting the transcellular pathway, many ligands for target transporters or receptors have been reported such as antibodies[12] or peptides.[13] These ligands are categorized into three transcytosis pathway types: carrier-mediated transcytosis (CMT); receptor-mediated transcytosis (RMT); and adsorptive-mediated transcytosis (AMT).

The CMT pathway is mediated by transporters to the brain such as glucose, amino acids, and metal ions. Typical transporters are glucose transporter 1 (GLUT1) and large neutral amino acid transporter (LAT1). The RMT pathway is the common approach to target receptors such as the transferrin receptor (TfR), insulin receptor (IR), low-density lipoprotein receptor (LDLR) and related protein-1 (LRP1), and nicotinic acetylcholine receptor (nAChR).[14] In the AMT pathway, cationic substance-conjugated drugs interact electronically with anionic proteoglycan on the cell membrane.[15]

The CMT or RMT pathway is expected to transport drugs with high selectivity because they target transporters or receptors that are highly expressed on the BBB. However, expression levels of these molecules change in response to nutrients[16] or circadian rhythm.[17] Transporters or receptors can also compete with endogenous compounds, and transcytosis efficiency tends to become saturated.[18] In contrast, saturation of the AMT pathway tends to be difficult because drugs interact not with transporters or receptors but directly with the cell membrane. The AMT pathway has greater ability to bind to membrane proteins (about several nmol/g) than the RMT pathway (a maximum of about 1 pmol/g).[19] Nevertheless, cytotoxicity and nonspecific uptake by other organs are concerns. Because each pathway has advantages and disadvantages, we need to determine which mechanism is the best brain-targeting system.

Based on ligand-modified drug delivery, these systems can be classified into direct ligand conjugation and encapsulation in ligand-modified nanocarriers. According to a summary by Kozlovskaya and Stepensky, brain accumulation efficiency was an average 1% of the injected dose (ID) of various ligand-modified conjugates and nanocarriers.[20] However, more recently, brain accumulation efficiency has increased (Table 1), along with the number of brain-targeting DDS in the clinical trial phase.

Drug conjugates and encapsulated nanoparticles are discussed separately below in the basic study and clinical study phases. In addition, we introduce exosomes, which have been developed as endogenous drug-delivery carriers.

2.1.1.1. Drug Conjugates

Drug conjugates have the potential to dramatically improve pharmacokinetics compared with the original drugs. Recently, various brain-targeting antibody–drug conjugates (ADCs) and peptide–drug conjugates (PDCs) have been developed, and clinical applications are expected in the near future. Three receptors have been widely used as drug conjugate targets: TfR and IR for ADC targets; and LRP1 for PDC targets.

The TfR is the most common target because it has the ability to transport transferrin (Tf), which is a very large (80000-Da) endogenous protein, among substances transported to the brain. Mucopolysaccharidoses II (MPS II) is an inherited genetic disorder caused by a lack or malfunctioning of iduronate 2-sulfatase (IDS). Sonoda *et al.* reported that enzyme replacement using anti-TfR antibody fused with IDS (JR-141) reduced glycosaminoglycans in MPS II model mice.[21] JR-141 is in phase III clinical trials for treating Hunter syndrome (NCT03568175)[21,22] and expected to become a new enzyme replacement therapy.

Niewoehner *et al.* demonstrated that anti-amyloid-β (Aβ) monoclonal antibody (mAb) conjugated with monovalent and bivalent Fab fragments of anti-TfR antibody decreased Aβ plaques in the cortex and hippocampus of AD model mice.[23] Interestingly, monovalent anti-TfR antibody conjugates transferred mAb to the brain and eliminated Aβ more efficiently rather than the bivalent form, although monovalent conjugates have lower binding affinity to TfR than bivalent ones. Those authors hypothesized that bivalent conjugates may induce dimerization of TfR, followed by internalization, causing TfR arrest at lysosomes.[23] On the other hand, TfR tends to become saturated because endogenous iron-bound Tf has a high affinity for TfR.[18,23] Therefore, it is important to design an anti-TfR antibody with the optimal binding site and binding affinity.

Insulin is involved in the synthesis of glycogen and sustaining cognitive function and neurogenesis in the brain. Anti-human IR antibody can cross the primate BBB 10-fold faster than anti-TfR antibody,[24] which could be a superior approach. Boado *et al.* indicated that anti-human IR antibody fused with α-iduronidase (AGT-181) accumulated in the brain of rhesus monkeys and decreased glycosaminoglycans by 70%.[25] Open-level phase 1/2 clinical trials of AGT-181 for treating Hurler syndrome (NCT03053089) were completed.[26] This system was also examined as a replacement for IDS in treating Hunter syndrome (AGT-182, finished in phase I, NCT02262338).[27]

Angiopep-2, which is a 19-amino acid sequence (TFFYGGSRGKRNNFKTEEY), targets LRP1 and has greater potential to accumulate in the brain parenchyma than Tf or lactoferrin.[28,29] For clinical use, Angiopep-2 has been applied for PDC with three drug moieties bound by cleavable ester bonds.[30,31] In particular, Angiopep-2–paclitaxel conjugates (ANG1005, in phase III preparation, NCT03613181) bypassed P-glycoprotein and increased the efficacy of treatment in brain tumor model mice.[30] Angiopep-2-conjugated anti-HER2 mAb (ANG4043) had greater brain accumulation and resulted in longer survival times than anti-HER2 mAb in BT-474 intracranial tumor model mice.[32]

Human melanotransferrin (MTf) is a protein with 37–39% homology to human serum Tf. BBB crossing by recombinant MTf was 14-fold greater *in vitro* and 5.7-fold greater *in vivo* compared with crossing by Tf.[33] Thom *et al.* investigated an MTf-derived peptide (MTfpep) composed of a 12-amino acid sequence (DSSHAFTLDELR). MTfpep-conjugated immunoglobulin G (IgG) mAb showed similar retention in the blood to control IgG mAb, but MTf-fused IgG mAb was quickly eliminated. In addition, fusion to an interleukin (IL)-1 receptor antagonist allowed it to cross the BBB and suppress neuropathic pain.[34]

So far, nucleic acid has mainly been encapsulated into nanocarriers because of low serum stability. However, advances in nucleic acid technology have enhanced stability. In particular, gapmer-type antisense oligonucleotide (ASO) is formed with phosphorothioated DNA flanked by locked nucleic acid to achieve nuclease resistance. Yokota's group developed a DNA/RNA heteroduplex oligonucleotide (HDO), which is complementarily formed with gapmer-type ASO and

its RNA. HDO mechanisms have two steps: 1) cleavage of complementary RNA by intracellular ribonuclease (Rnase) H; and 2) gene silencing by binding ASO and target RNA. They prepared HDO modified with α-tocopherol as a ligand, based on the result that α-tocopherol-conjugated small interfering RNA (siRNA) increased liver and brain accumulation.[35,36] α-Tocopherol-modified HDO has much higher apolipoprotein B (ApoB) mRNA gene silencing effects than ASO *in vivo*.[37] Moreover, in the case of targeting organic anion transporter (OAT3) expressed on the BBB, α-tocopherol-modified HDO suppressed OAT3 mRNA levels by 50% with a single dose *via* intravenous injection and by 76% after four doses.[38]

2.1.1.2. Nanocarriers

Nanocarriers can selectively deliver various therapeutic agents such as low molecular-weight drugs and nucleic acids to target organs. Therefore, brain-targeting nanocarriers are desirable to fulfill the unmet medical needs of patients with brain diseases.

The RMT pathway has been widely investigated as the most common approach. The LDLR family is often chosen as the target receptor for nanoparticle delivery. Tröster *et al.* first reported the pharmacokinetics of poly(methyl methacrylate) (PMMA) nanoparticles coated with various surfactants in order to identify compounds possessing superior targeting ability *in vivo*.[39] They found that polysorbate-80 led to much higher brain concentrations.[39] Kreuter *et al.* also demonstrated that dalargin-bound poly(butyl cyanoacrylate) (PBCA) nanoparticles coated with polysorbate-80 showed better analgesic effects compared with polysorbate-20, -40, and -60, and that the nanoparticles were taken up by the phagocytic pathway into the brain.[40,41] Those authors suggested that ApoB or ApoE was adsorped on the surface of PBCA nanoparticles coated with polysorbate-80.[42] However, that approach is not optimal because high-dose (3–30 mg/kg) polysorbate-80 injection induced BBB disruption due to osmotic effects in mice.[43] Therefore, the ApoE protein or ApoE-derived peptide modification approach targeting LDLR was developed.[44–46] LDLR family-targeting peptides, such as Angiopep-2[47–49] or Peptide-22[50,51] were also developed. Chen *et al.* evaluated the BBB-targeting ability of liposomes modified with various BBB-targeting (Angiopep-2, T7, Peptide-22) and glioma-targeting (c(RGDfK), D-SP5, peptide-1) ligands.[51] According to those results, Peptide-22 and c(RGDfK) dual-modified liposomes showed the best targeting abilities *in vitro* and *in vivo* against human glioblastoma cells.[51]

More recently, good results have been reported with the administration of glutathione transporter- and GLUT1-targeting nanoparticles *via* the CMT pathway. Kannan *et al.* first suggested that glutathione crosses the rat BBB and enters the brain directly.[52] Since then, glutathione has been utilized as a nanocarrier ligand.[53–56] For example, glutathione-modified PEGylated liposomes encapsulated 6.5-fold more methylprednisolone (2B3-201) accumulated in the brain than did free methylprednisolone. 2B3-201 decreased the clinical score to $42 \pm 6.4\%$ in the silane-alone group in experimental autoimmune encephalomyelitis rats.[54] In addition, glutathione-modified PEGylated liposome-encapsulated doxorubicin (2B3-101) prolonged survival time by 16.1% compared with the unmodified form. This formulation proceeded to a phase I/IIa trial (NCT01386580).[55,56] Xie *et al.* evaluated PEGylated liposomes with glucose-derived cholesterol composed of different PEG chain lengths (MW: 200, 400, 1000, and 2000). As a result, glucose-PEG_{1000}-modified liposomes showed the highest accumulation in the brain, which was 3.93-fold higher than that of unmodified liposomes.[57] Anraku *et al.* reported that glucose-modified self-assembly polyion complex (PIC) micelles had the potential to cross the BBB corresponding to glucose concentration.[16] Thirty minutes after PIC micelles were intravenously injected, glucose solution was intraperitoneally injected in 24-h fasted mice. Subsequently, PIC micelle accumulation in the brain was up to 6% of the dose per gram of organ, which was 56-fold higher than the rate observed in free-feeding mice.[16]

In efforts to design brain-targeting nanoparticles, many brain-targeting ligands have been identified using phage display and analysis of the structure of viruses or toxins.[13] Therefore, it is important to determine the appropriate ligand or pathway. Van Rooy *et al.* compared the brain accumulation of five different brain-targeting ligand (RI7217, transferrin, Angiopep-2, COG133, CRM197)-modified liposomes.[58] When each liposome was modified with 1% ligand, only RI7217 was able to significantly enhance brain uptake.[58] This result indicated the need to consider the type of ligand, optimal ligand density, and physicochemical characteristics when designing functionalized formulations.

2.1.1.3. Exosomes

Exosomes are endogenous vesicles of 30–100 nm in size secreted from various cells containing cancer cells, which mainly communicate between cells and sustain functionality. The characteristics of the types of receptors expressed on the exosomal surface or encapsulated mRNA/miRNA corresponding to secreted cells differ among exosomes. Therefore, analysis of the functionality of exosomes is expected to lead to the development of methods for clinical diagnosis and therapy.

Several studies demonstrated that mesenchymal stem cell (MSC)-derived exosomes have the potential to act as novel brain-targeting carriers. Xin *et al.* showed that MSC-derived exosomes promoted neurite remodeling and functional recovery from stroke through the delivery of miR-133b to astrocytes or neurons.[59] Katsuda *et al.* demonstrated that human adipose tissue-derived MSCs secreted neprilysin (NEP)-containing exosomes, which suppressed the accumulation of Aβ in AD model mice.[60] In addition, MSC-derived exosomes modified with rabies viral glycoprotein-derived peptide (RVG peptide) suppressed the inflammatory response to a greater degree than unmodified ones in AD model mice.[61]

On the other hand, exosomes are difficult to isolate in sufficient amounts to act as DDS carriers. To solve this problem, Qu *et al.* focused on blood-derived exosomes (blood-exos), which are abundantly released by reticulocytes.[62] They indicated that unmodified blood-exos accumulated in the brain and were able to interact with Tf dimers in the blood and two TfRs, one on the blood-exos and the other on brain microvascular endothelial cells. Moreover, dopamine-loaded blood-exos recovered dopaminergic neuron activity in PD model mice.[62] Kojima *et al.* reported on synthetic biology-inspired control devices called EXOsomal Transfer into Cells (EXOtic) devices.[63] The exosomal production system focused on genetic engineering for efficient production, mRNA packaging, and delivery into the cytosol of target cells. First, they utilizeded STEAP3, SDC4, and NadB fragments to boost exosomal production and transfection into the cells. Second, they packed

catalase mRNA by mediating C/D_{box} and L7Ae interaction to treat PD. Finally, they modified RVG peptides and Cx43, which can be delivered into the cytosol by targeting nAChR and gap junctions. HEK-293T cell-produced exosomes using EXOtic devices suppressed attenuated neurotoxicity and neuroinflammation in PD model mice.[63]

Research on brain-targeting exosomes then proceeded to improve targeting abilities or manufacturing approaches. For clinical applications, work on the normalization of various exosomes should consider the use of endogenous substances or improved manufacturing methods because detailed information remains unknown.

2.1.2. Permeabilization of the BBB

2.1.2.1. Micro/Nanobubbles Plus Ultrasound

Ultrasound is widely used in the field of diagnostic imaging and as a DDS tool in combination with bubble formation. Ultrasound-mediated DDS are based on the cavitation or oscillation generated when bubble formation is irradiated with ultrasound waves. Many reports showed that the BBB can be temporally opened by cavitation, allowing drugs to be delivered to the brain parenchyma. A clinical study was also conducted on opening the BBB of patients with glioblastoma by ultrasound irradiation using an implantable transducer after injection of microbubbles.[64] Although that study demonstrated a clinical proof of concept for BBB opening by ultrasound, invasive surgery was needed to install ultrasound transducers into patients' brains. Magnetic resonance imaging (MRI)-guided focused ultrasound (MRgFUS) can precisely deliver ultrasound irradiation to the targeted region of the brain.[65,66] Noninvasive BBB opening and drug delivery to the brain using MRgFUS are expected to be clinically useful because surgery is not required. Temporal BBB opening by MRgFUS was successfully performed in patients with AD or amyotrophic lateral sclerosis (ALS).

Although the mechanism of ultrasound-mediated BBB opening is not fully understood, it appears that the cavitation or oscillation acts on the function of the BBB. Some reports showed that claudin, occludin, and ZO-1, which are components of TJs, were decreased after ultrasound irradiation.[67,68] P-glycoprotein (P-gp), which pumps drugs to the blood from the brain parenchyma, was also reported to be decreased.[69] Another report showed an increase in caveolin-1, which is involved in caveola-mediated transcytosis.[70] Those reports led us to hypothesize that loosening of TJs, enhancement of transcytosis, and suppression of drug excretion together result in increased drug delivery to the brain.

Ultrasound-mediated DDS can deliver a wide range of drugs to the brain. Table 2 shows the therapeutic agents delivered by ultrasound-mediated DDS in animal experiments, which include not only small-molecule drugs but also antibodies, plasmid DNA, viral vectors,[71,72] nanoparticles, and stem cells.[73] Reports on ultrasound-mediated gene transfection systems for the treatment of neurodegenerative diseases are increasing. We and other groups showed that high transgene expression was observed with intravenous injection of bubble formulations and plasmid DNA followed by ultrasound

Table 2. Therapeutic Delivery by Micro/nanobubbles Plus Ultrasound

Agent	MW/size	Result	Ref.
Small molecule drugs			
Doxorubicin	549 Da	7.1-Fold increase in brain tumor *vs.* nonirradiated controls	126
		Prolonged survival in glioma model	142
5-FU	130 Da	6-Fold increase in brain tissue conc. *vs.* nonirradiated controls	125
Temozolomide	190 Da	2.7-Fold increase in brain tissue conc. *vs.* nonirradiated controls	141
Oligonucleotides			
Morpholino oligomers	6 kDa	Greater accumulation on *ex vivo* imaging	172
Htt siRNA	13 kDa	Decreased *Htt* mRNA in striatum	173
Antibodies			
Bevacizumab	149 kDa	3-Fold increase in brain tissue *vs.* nonirradiated controls Increased survival time of glioma-bearing mice	174
Trastuzumab	185 kDa	Decreased tumor volume and prolonged survival *vs.* nonirradiated BT464-bearing mice	175
Tau antibody	156 kDa	19-Fold increase in tau antibody in brain *vs.* nonirradiated controls	176
Protein drugs			
IL-12	75 kDa	1.5-Fold increase in IL-12 conc. *vs.* nonirradiated controls Suppressed tumor progression in C-6 glioma-bearing rats	177
BDNF	14 kDa	1.8-Fold increase in BDNF conc. *vs.* nonirradiated controls	169
Plasmid DNA			
pDNA coding Birc5 shRNA	Unknown	Suppressed tumor progression and prolonged survival in C-6 glioma-bearing rats	178
GDNF pDNA	Unknown	1.5-Fold increase in GDNF expression *vs.* nonirradiated controls and increased dopamine level	161
Viral vector			
scAAV9	22 nm	Site-selective gene expression in irradiated area	71
Nanoparticles			
Liposomes	55, 120, 200 nm	Correlation between ultrasound intensity and liposomal accumulation in brain.	179
Au nanoparticles	6.3, 9.5, 14.2 nm	Size-dependent delivery specific to irradiated brain region	180
Cells			
NSCs	10 μm	GFP-expressing stem cells observed across the BBB	73

MW, molecular weight; 5-FU, 5-fluorouracil; Htt, huntingtin; IL-12, interleukin-12; BDNF, brain-derived neurotrophic factor; pDNA, plasmid DNA; shRNA, short hairpin RNA; GDNF, glial cell line-derived neurotrophic factor; NSCs, neural stem cells; GFP, green fluorescent protein; BBB, blood–brain barrier.

irradiation to the brain.[74,75] Furthermore, we evaluated the spatial distribution of transgene expression using multicolor deep imaging, which is a 3D observation system using tissue clearing and biological staining.[75] The results showed that transgene expression occurred in both endothelial cells and the brain parenchyma.

Currently, five types of microbubbles, Levovist, Optison, Definity, Sonovue, and Sonazoid, are commercially available as ultrasound contrast agents. Recently, some researchers have attempted to add functions to bubble formulations. Fan *et al.* prepared carmustine-loaded microbubbles by incorporating carmustine into their lipid shells.[76] Wang *et al.* conjugated methotrexate-loaded liposomes to microbubbles *via* the biotin–avidin interaction.[77] We and other groups developed complexes of bubbles and plasmid DNA *via* electrostatic interaction.[75,78] The drug-conjugated bubbles are expected to enhance drug delivery efficiency and decrease side effects. On the other hand, microbubbles with targeting ability are also being studied to increase their accumulation in target regions. For glioma targeting, vascular endothelial growth factor (VEGF) receptor-2 mAb-conjugated microbubbles[79] or folate-conjugated microbubbles[80] have been developed. These ultrasound-responsive formulations may be utilized as diagnostic as well as therapeutic agents, referred to as "theranostics."

Ultrasound-mediated DDS can efficiently deliver therapeutic agents to targeted brain regions. However, there are risks of adverse effects including severe cerebral hemorrhage or inflammation because the BBB is forcibly opened and blood components can enter the brain parenchyma; therefore, safety research is necessary for clinical application. Several reports showed that there is a positive correlation between the intensity or duration of ultrasound and the extent of BBB opening.[67,81,82] At the same time, Song *et al.* found that the amount of injected echo gas volume also determines the extent of BBB opening with the administration of various sizes of microbubbles.[83] Further study is needed to establish the appropriate conditions for ultrasound-mediated DDS platforms with minimal damage to the BBB.

2.1.2.2 Tight Junction Modulators

Paracellular drug transport to brain is prevented by TJs and adherence junctions (AJs). The BBB has three types of physical barriers: bicellular tight junctions (bTJs); tricellular tight junctions (tTJs); and AJs. bTJs are formed between adherent cells like claudin-5 and occludin. tTJs are the proteins located at the meeting points of three cells such as tricellulin and angulin-1. AJs are composed of actin filaments bound *via* linked proteins, mainly vascular endothelial (VE)-cadherin. To break the junctions physiologically, Rapoport reported that brain microvascular endothelial cells were dehydrated and formed about 40-nm cavities after the administration of mannitol solution *via* the carotid artery.[84] However, because this method might allow the influx of toxic proteins to the brain, many researchers have tried to suppress the expression of these junctions to open them temporarily.

The barrier function of bTJs may depend on the expression level of the claudin family.[85] Nitta *et al.* reported that the BBB in claudin-5-deficient mice was affected by the primary amine-reactive biotinylation reagent (443 Da) and Gd-DTPA (742 Da) but not by microperoxidase (approx. 1900 Da).[86] On the other hand, the role of tTJs is still largely unknown because they were identified as tricellulin in 2005 and as members of the angulin family in 2011.[87,88] Among them, angulin-1 plays a role in recruiting tricellulin and forming a strong barrier.[87] Angulin-1-deficient E14.5 mice were found to leak Sulfo-NHS-biotin (446 Da) from the BBB. However, there was less leakage of fibrinogen (52000 Da), albumin (69000 Da), and antibodies (160000 Da).[89] Moreover, more 16-mer ASO entered the BBB after pretreatment with angubindin-1 to inhibit angulin-1.[90]

AJs are formed by mediating the intracellular signaling pathway of the G protein-coupled receptor (GPCR). Therefore, modulation of AJs or knockout of endothelial GPCR expression is expected to increase BBB permeability. Sphingosin-1-phosphate receptor-1 ($S1P_1$) is associated with AJs through Gi-dependent Rac activation. BBBs of endothelial $S1P_1$-deficient mice were permeable to cadaverine (1000 Da) and dextran (3000 Da) but not to dextran 10000 Da or 70000 Da. BBB opening was reversible by the injection of the $S1P_1$ modulator FTY720 (fingolimod).[91] High mRNA expression levels of Gpr116, a GPCR, are seen in brain blood vessels. Gpr116-knockout mice accumulated Alexa Fluor 555-cadaverine (1000 Da) in the cerebral vessels, but tetramethylrhodamine-conjugated dextran (70000 Da) did not accumulate.[92]

Thus, junction modulators might be useful to cross the BBB depending on their molecular weight. Because it is unclear whether BBB opening is temporary and reversible, further studies are needed before clinical use.

2.2. Local Route of Administration Systemic administration such as the intravenous route is commonly used in clinical practice because it is less invasive and makes it easy to administer drugs. However, high doses of drugs must be administered *via* the systemic route to obtain sufficient therapeutic effects when treating cerebral diseases because of the low percentage of the ID accumulated in the brain. Moreover, drugs can be distributed in unintended organs, leading to systemic adverse side effects. In contrast to the systemic route, local administration delivers drugs selectively to the brain without nonspecific distribution to untargeted organs. Conventionally, only "naked" drug molecules were administered locally, and the DDS technique was not utilized. Recently, DDS for local administration have been studied based on reports that they may enhance the therapeutic effects.

2.2.1. Intraparenchymal Administration

The intraparenchymal route is commonly used for the local administration of antitumor drugs or gene vectors, both clinically and experimentally. Drugs are injected into the targeted brain region after craniotomy, with little nonspecific drug distribution. One problem is that the diffusion of drugs in the brain does not cover the entire lesion site. In mouse or rat models, cerebral diseases can be treated with a single injection because the brain is small (about 0.45 mL in mice). However, the volume of the human brain is about 1.3 L, and wider drug distribution is needed. Thus, in clinical practice, multiple injections are administered to increase drug distribution in the brain. For example, a viral vector was injected into 4 sites for PD gene therapy.[93] However, multiple-injection procedures place burdens on patients and medical staff, are complicated, and are time consuming.

Convection enhanced delivery (CED) to the brain parenchyma applies sustained pressure by a syringe pump to generate bulk drug flow, resulting in wider drug distribution in the brain parenchyma than with simple parenchymal injection.[94]

Clinically, antitumor drugs, antibodies, or oligonucleotides have been injected using CED to treat glioma.[95] Recently, drug carriers have been developed to enhance the therapeutic effects of CED, although they have not yet been approved for clinical use. The pharmacokinetics of drug CED are affected by the physicochemical properties of carriers, especially the surface potential.[96] According to some reports, CED drug carriers should have an anionic surface charge[97,98] because it results in fewer interactions with cell surface moieties such as proteoglycans which contribute to wide drug dispersion. Intratumoral injection of carboplatin encapsulated in anionic liposomes showed greater therapeutic effects against glioma than free carboplatin, while carboplatin encapsulated in cationic liposomes was less effective than free carboplatin.[97] This was also confirmed in a transfection study, since an anionic complex of liposomes and plasmid DNA transferred genes to wider brain regions than a cationic one.[98]

Clinical applications of CED are restricted to severe brain diseases because it requires surgery with the concomitant risk of infection. However, CED is expected to be a useful therapeutic tool to deliver high concentrations of drugs to lesions using sophisticated DDS carriers and medical devices.

2.2.2. Intraventricular Administration

Intraventricular (ICV) injections deliver drugs locally to the cerebral ventricle. The barrier between the ventricle and parenchyma is so loose that it allows simple diffusion into the brain,[99] and it is not necessary to overcome the BBB or BCSFB when administering ICV drugs. In experiments using mice and rats, ICV injections can be administered without special equipment. For this reason, the ICV route is commonly used in pharmacological research to evaluate the therapeutic effects of CNS drugs. In clinical practice, a drug reservoir such as the Ommaya reservoir is surgically implanted before drug administration, enabling repeated administration. Anticancer or antibacterial drugs are commonly injected by the ICV route to treat meningitis.[100,101] In ICV administration, drugs are not directly injected into the parenchyma containing neurons and glial cells and therefore it is considered safer than the intraparenchymal route.

Regenerative medicine using endogenous neural stem cells (NSCs) is being intensively studied to treat neurodegenerative diseases. The subventricular zone (SVZ) contains many tightly packed NSCs that contribute to neurogenesis.[102] NSCs in the SVZ are activated by the administration of certain therapeutic proteins such as growth factor or neurotrophic factor.[103] Ochi *et al.* demonstrated that the number of NSCs increased after 7-d ICV infusion of fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF).[103] However, protein should be continuously supplied to the cerebrospinal fluid to activate NSCs because it is quickly degraded.

Gene transfection to the region around cerebral ventricles and subsequent secretion of therapeutic proteins is one approach to obtain a sustainable supply. An electrostatic-based complex of plasmid DNA and polyethyleneimine (PEI) is conventionally used as a transfection reagent for ICV injection.[104] Although plasmid DNA/PEI complexes can deliver transfected genes to the SVZ efficiently, neurotoxicity due to their cationic surface charge is a concern.[105] However, Tamaru *et al.* developed plasmid DNA-encapsulated liposomal nanoparticles with a neutral charge and they succeeded in efficient transfection in the SVZ.[46]

Recently, external stimuli have been used to deliver transfected genes *via* ICV administration. Bugeon *et al.* genetically manipulated NSCs and neuronal cells in the SVZ by ICV injection of mRNA followed by electroporation.[106] Tan *et al.* first reported ultrasound-mediated ICV gene transfection.[107] They showed that transgene expression by polyplexes can be enhanced by microbubble administration and subsequent ultrasound irradiation. Furthermore, we investigated ultrasound and ICV-mediated gene transfection from the viewpoint of distribution of exogenous gene expression.[108] While gene expression by the plasmid DNA/PEI complex, the conventional gene carrier for ICV, was observed at the ventricular wall, ultrasound and nanobubble-mediated gene expression was observed at both the ventricular wall and choroid plexus. This might be due to the difference in the transfection mechanism. Although only a few reports are available on external stimuli-mediated transfection after local administration in the brain, the characteristics of transgene expression may be unique depending on the type of stimuli.

2.2.3. Nose-to-Brain Drug Delivery

Although the intraparenchymal/intraventricular route delivers drugs efficiently and specifically to the brain, it requires surgery and thus burdens patients. Intranasal drug delivery, known as the nose-to-brain (N2B) route, is a noninvasive method to deliver agents of various sizes, including small-molecule drugs,[109,110] proteins,[111,112] oligonucleotides,[113–115] or gene vectors.[116,117] N2B delivery of insulin was shown to improve memory impairment in AD patients.[118]

Intranasally administered drugs reach the brain through two pathways, the olfactory pathway and trigeminal pathway. In the first, drugs are distributed in the olfactory bulb through axonal transport by the olfactory nerve or passage through the nasal epithelium. In the trigeminal pathway, drugs are distributed in the hypothalamus and brainstem, where trigeminal nerve ganglia are located. Intranasally administered ^{125}I-labeled IgG was distributed in the olfactory bulb, hypothalamus, and brainstem.[111] Major obstacles to N2B administration are the mucus layer and olfactory epithelium, which are responsible for eliminating exogeneous substances that invade the nasal cavity. Thus, carrier systems are being developed to overcome those barriers and enhance the efficiency of N2B delivery.

Chitosan is biocompatible cationic polysaccharide derived from deacetylated chitin. It has unique properties useful for DDS such as mucoadhesive or cell-permeable effects. Thus, chitosan-derived nanoparticles have been utilized as N2B nanocarriers. Rivastigmine, an inhibitor of acetylcholine esterase, is used to treat AD but only a limited amount reaches the brain with intravenous administration because of its hydrophilicity and low bioavailability. Some groups developed rivastigmine-loaded chitosan nanoparticles using the ionotropic gelation method for more efficient N2B delivery of rivastigmine. When nanoparticles were administered intranasally, the intrabrain concentration of rivastigmine was higher than when free rivastigmine was administered.[109] On the other hand, Van Woensel *et al.* developed siRNA-loaded chitosan nanoparticles targeting galactine-1, which is overexpressed in glioblastoma, and administered them to tumor-bearing mice.[113] Efficient downregulation of galectin-1 was achieved with the accumulation of siRNA in glioma lesions.

Some studies showed that cell-penetrating peptide (CPP) can increase the delivery of therapeutic agents to the brain

via the N2B route due to accelerated penetration of the epithelial layer. Kamei *et al.* delivered exendin-4, an analogue of glucagon-like peptide-1 (GLP-1) co-administered with L-penetratin, which improved cognitive dysfunction in AD model mice.[119] On the other hand, Kanazawa *et al.* developed an N2B delivery system using CPP-modified nanomicelles.[115] They confirmed that drug was more efficiently transferred to the olfactory bulb and brainstem by N2B delivery of Tat peptide-modified nanomicelles than the free drug.[120] They also prepared Tat-modified micelles loaded with camptothecin (CPT) and siRNA for the treatment of glioma and found that the micellar formulation suppressed tumor growth and increased survival times.[115]

Because of the large gene vector size, it was assumed that gene transfection through N2B delivery would be difficult. Belur *et al.* demonstrated that N2B delivery of AAV9 exerted efficient gene expression in the olfactory bulb.[116] Furthermore, Aly *et al.* succeeded in N2B gene delivery of plasmid DNA compacted by PEG-conjugated poly-L-lysine.[117] Transgene expression was observed in the perivascular region of the olfactory bulb and brainstem. After the transfection of GDNF plasmid DNA in PD model rats, dopaminergic neurons were restored in the SN. Those studies indicated that noninvasive, brain-specific gene therapy can be achieved by N2B delivery of viral vectors.

3. Methods to Evaluate Drug Distribution Characteristics

Generally, the development of DDS starts from experimental research using rodent models and then nonhuman primates. Clinical studies follow to evaluate efficacy and safety. For the smooth transition to clinical application, the evaluation of DDS is important at the animal research stage. Information on the amount and distribution of drugs or exogeneous genes is especially critical for drug or gene delivery systems targeting the brain to determine the therapeutic strategy. From another perspective, those results are useful for researchers to develop superior DDS by feeding back the evaluation results to the development stage.

With progress in bioimaging technology, *in vivo* imaging systems now allow drug distribution to be observed in living animals. Although *in vivo* imaging is a powerful tool for evaluating drug distribution, its resolution is not sufficiently high to observe tissues at the single-cell level. Table 3 lists methods used to evaluate brain-targeting DDS. Each method has advantages and disadvantages, and therefore it is important to combine several depending on the purpose of evaluation.

3.1. Systemic Imaging Methods Systemic *in vivo* imaging can monitor drug distribution in living animals after the administration of labeled drugs. The current modalities include MRI, positron-emission tomography (PET), and optical imaging (OI).

PET detects the annihilation radiation generated by the interaction of positrons and electrons in the body. To enable detection by PET, therapeutic agents are labeled with positron-emitting isotopes. The advantage of PET is high sensitivity, so that systemic pharmacokinetics can be analyzed at low drug doses. However, long-term evaluation is not possible because the radioactive half-life is short.

Small-molecule drugs such as anticancer agents can be labeled with the positron-emitting isotopes ^{11}C or ^{18}F. Tosi *et al.* synthesized ^{18}F-labeled dasatinib and evaluated the pharmacokinetics using PET after intraparenchymal administration.[96] Mukai *et al.* developed a method to synthesize radioactive oligonucleotides by incorporating ^{18}F.[121] Therapeutic agents composed of oligonucleotides are promising and some, *e.g.*, nusinersen and viltolarsen, are approved for the treatment of neurological diseases. This labeling technique makes it possible to elucidate the pharmacokinetics of oligonucleotide-based drugs with high sensitivity in the laboratory and clinically.

Nanocarriers such as liposomes or micelles can be labeled by incorporating positron emitters. Oku *et al.* evaluated the distribution of ^{18}F-labeled liposomes in glioma-bearing mice.[122] Because the half-life of ^{18}F is only 109 min, time-course evaluation over several hours is not possible. Hence, studies in which DDS nanocarriers are labeled with radiometals have recently increased.[123] Seo *et al.* prepared ^{64}Cu-labeled liposomes and micelles and evaluated their accumulation in brain tumors after intravenous administration to glioma-bearing rats.[124] Those reports suggested that radioactive nanocarriers may be useful in the diagnosis of cerebral diseases.

OI can trace the dynamics of fluorescent-labeled drug molecules or formulations without the need for radioactive substances. Because OI is a simple method, it can be used to evaluate the pharmacodynamics of nanoparticle formulations such as liposomes, micelles, or exosomes after fluorescence dye loading. In the field of gene delivery, the luciferase gene is transfected as a reporter gene, and then bioluminescence emitted by the reaction with luciferin is detected. Fluorescent proteins excited with long-wavelength labels such as mCherry

Table 3. Methods for Evaluating Drug Distribution in the Laboratory

	Maximum resolution	Object	Advantage	Limitation
Systemic imaging methods				
PET	700 μm	Drug labeled with positron emitter (^{11}C, ^{18}F, ^{64}Cu)	High sensitivity; many probes available	Short-lived signals
MRI	10 μm	Paramagnetic chelator (Gd-DTPA)	Visualization of anatomic structure; non-RI	Low sensitivity
OI	Dependent on depth and wavelength	Fluorescent dye/protein, bioluminescence	High throughput; non-RI	Attenuated signal intensity of deep region
Microdialysis method	—	Drug itself	Highly quantitative	Limited to hydrophilic and small-molecule drugs
Deep imaging of tissue method				
Tissue clearing + microscopic analysis	0.2 μm	Fluorescent dye/protein	High spatial resolution of 3D images	Only *ex vivo*

PET, positron emission tomography; MRI, magnetic resonance imaging; OI, optical imaging; DTPA, diethylenetriaminepentaacetic acid; RI, radioisotope.

or E2Crimson are also used as reporter genes because longer wavelengths have higher transmission rates. Although OI is a simple, convenient imaging modality, it is quantitativity inferior because fluorescence from deep regions tends to be attenuated. Hence OI may be suitable only for approximate evaluations of drug or gene distribution.

3.2. Brain Microdialysis Methods Brain microdialysis is a technique for recovering small molecules in the interstitial fluid in the target region by inserting a probe into the brain. When the results are combined with quantitative HPLC analysis, drug concentrations can be measured. This is conventionally used for the measurement of endogenous neurotransmitters such as dopamine, serotonin, or acetylcholine in the brain. Recently, brain microdialysis has also been used to evaluate brain-targeting DDS because it can clarify the time course of pharmacokinetics after drug administration. We and other researchers showed that brain microdialysis is useful to evaluate the pharmacokinetics of ultrasound-mediated drugs delivered.[125,126] We measured the concentration of 5-fluorouracil (5-FU) in the brain after intravenous injection of 5-FU and nanobubbles followed by ultrasound irradiation.[125] Lin *et al.* evaluated the pharmacokinetics of ultrasound-mediated doxorubicin delivery to glioma lesions.[126]

Brain microdialysis can measure the time course of drug concentrations in targeted regions with high quantitative accuracy because the microdialysis probe is inserted directly into the brain. However, measurable drugs are limited in terms of molecular weight and hydrophobicity. Drugs that are too large are difficult to recover, while those that are too hydrophobic can be adsorbed to the dialysis membrane. Recently, a microdialysis probe with a high molecular-weight cutoff membrane has become available,[127] which will make it possible to analyze large-molecule drugs such as cytokines, antibodies, or oligonucleotides. Hence, brain microdialysis is expected to provide fundamental information on the pharmacokinetics of a wide variety of drugs.

3.3. Deep-Tissue Imaging Methods The brain contains populations of cells such as neurons, astrocytes, microglia, and endothelial cells, and each has a unique role in maintaining brain function. It would therefore be ideal to evaluate drug distribution in individual cell populations. Immunohistochemical analysis of brain sections is performed by staining specific cells. Drug distribution in the tissue sections can be observed by labeling drugs, making it possible to perform detailed evaluation. However, comprehensive evaluation using brain tissue sectioning is difficult because the technique only provides 2D information.

Tissue clearing suppresses light scattering in biological specimens, making them appear transparent. Comprehensive evaluations in deep brain regions may become possible with tissue clearing and microscopic observation. Tissue-clearing reagents or protocols have been reported *e.g.*, CUBIC,[128] Sca*l*e,[129] SeeDB, Clear,[130] and CLARITY.[131] Some groups succeeded in evaluating biological events using tissue-clearing techniques. For example, Susaki *et al.* spatially visualized the neural circuits of transgenic mice that consistently express fluorescent protein by tissue clearing with CUBIC,[128] and Hama *et al.* evaluated the distribution of Aβ plaques in AD mice using Sca*l*e.[132]

Our group developed a new observation system using tissue clearing and confocal microscopy, called the "multicolor deep imaging" system, for 3D evaluation of DDS.[75,108,133–137] It allows evaluation of the spatial distribution of drugs or exogeneous gene expression while visualizing the biological structure with immunological staining or fluorescent probe labeling. We evaluated the spatial distribution of transgene expression in brains transfected by systemic administration of plasmid DNA and nanobubbles followed by ultrasound irradiation when blood capillaries were labeled with lipophilic carbocyanine dye.[75] We also evaluated ultrasound-facilitated transfection *via* the ICV route with ventricular wall staining.[108] Importantly, we found that the distribution of transgene expression changed after ultrasound irradiation. Multicolor deep imaging can lead to the precise observation of brain-targeting DDS.

4. Applications in Cerebral Diseases

4.1. Brain Tumors Brain tumors are divided into the primary and migrating or metastatic categories. Their malignancy is pathologically diagnosed from grades I to IV based on the affected sites or cell type. Glioma, which arises from glial cells, is progressive and difficult to distinguish from normal cells, and therefore complete surgical resection is difficult. In conventional glioma treatment, chemotherapy using anticancer agents (temozolomide) or anti-VEGF mAb (bevacizumab) is performed after surgery and radiotherapy. However, the 5-year survival rate is about 30% for grade III patients and 5% for grade IV glioma patients. Therefore, novel therapeutic agents and glioma-targeting DDS are needed to improve glioma treatment and patient outcomes.

When tumor occurs in or metastasizes to the brain, tumor partly damages the BBB and induces angiogenesis. This damaged BBB is called the blood–brain tumor barrier (BBTB). The BBTB has greater permeability than the BBB because of collapsed pericytes, lost astrocytic endfeet connections, and lower expression levels of TJs, GLUT1, and the sodium-dependent lysophosphatidylcholine symporter (NLS1).[138] Angiopep-2-conjugated drugs showed 4–5-fold greater accumulation in the brain of orthotopic U87 glioma-inoculated mice than in normal mice.[31] However, the efficiency of nanocarrier delivery was still extremely low, at about 0.8% of the ID in brain tumors.[139] To resolve this problem, many researchers developed multiligand-grafted nanocarriers conjugated with BBB-crossing and tumor-targeting ligands (Table 4). For example, Zong *et al.* demonstrated that Tat- and T7-modified doxorubicin-encapsulated liposomes prolonged the median survival time of C6 glioma-bearing mice by 1.65-fold compared with unmodified ones.[140] The survival time of mice treated with dual ligand-modified liposomes is 1.54-fold longer than in those treated with Tat-modified ones and 1.23-fold longer than in those receiving T7-modified ones. Yang *et al.* reported that Angiopep-2 and tLyp-1 multiligand-modified cationic liposomes showed greater cellular association than monoligand-modified cationic liposomes in murine brain microvascular endothelial cells and three types of glioma cells.[49] Those findings suggest that multiligand-modified nanocarriers exert synergistic therapeutic effects against glioma.

Ultrasound-mediated drug delivery has been intensively studied for the treatment of glioma. MRgFUS can irradiate glioma cells selectively with MRI visualization. Therefore, selective drug delivery to glioma is expected. The concentration of temozolomide[141] or doxorubicin[142] in the brain was

Table 4. Brain Tumor-Targeting DDS Carriers and Ligand Characteristics

Formulation	Targeting BBB	Targeting tumor	Loading drug	Ref.
Drug conjugates				
PDC	Angiopep-2 peptide (RMT, LRP1)		Paclitaxel	30
			Doxorubicin	31
Antibody-peptide conjugates	Angiopep-2 peptide (RMT, LRP1)		Anti-HER2 mAb	32
PDC	Angiopep-2 peptide (RMT, LRP1) Tat peptide (AMT)		Paclitaxel	181
Nanocarriers				
Liposomes	Tat peptide (AMT) T7 peptide (RMT, TfR)	T7 peptide (TfR)	Doxorubicin	140
Liposomes	Glucose (CMT, GLUT1)	c(RGDfK) peptide (integrin receptor)	Epirubicin	182
Liposomes	$_{D}$(CDX) peptide (RMT, nAChR)	c(RGDyK) peptide (integrin receptor)	Doxorubicin	183
Liposomes	c(CMPRLRGC) peptide (RMT, LDLR)	c(RGDfK) peptide (integrin receptor)	Doxorubicin	51
Liposomes (tandem peptide)	R8 peptide (AMT)	c(RGDfK) peptide (integrin receptor)	Paclitaxel	184
Liposomes	Glutathione (CMT)		Doxorubicin	56
Liposomes		RGD peptide (integrin receptor) IL-13 peptide (IL13Rα2)		185
Liposomes		VTW peptide (gp130)		186
Cationic liposomes	Angiopep-2 peptide (RMT, LRP1)	tLyP-1 peptide (neuropilin-1 receptor)	VEGF-siRNA and docetaxel	49
PEI/pDNA complex		$_{D}$(RPPREGR) peptide (neuropilin-1 receptor)	pORF-hTRAIL	146
DGL	T7 peptide (RMT, TfR)	T7 peptide (TfR)	Doxorubicin and pORF-hTRAIL	145
PBCA nanoparticles	Poloxamer 188 (RMT, SR-B1)		Doxorubicin	187
PLGA nanoparticles	Angiopep-2 peptide (RMT, LRP1)		GOLPH3 siRNA Gefitinib	47

PDC, peptide drug conjugate; DGL, dendrigraft poly-L-lysine; PBCA, poly(butyl cyanoacrylate); PLGA, poly(lactic-*co*-glycolic acid); BBB, blood–brain barrier; RMT, receptor-mediated transcytosis; LRP1, low-density lipoprotein receptor-related protein-1; AMT, adsorptive-mediated transcytosis; CMT, carrier-mediated transcytosis; GLUT1, glucose transporter-1; nAChR, nicotinic acetylcholine receptor; LDLR, low-density lipoprotein receptor; TfR, transferrin receptor; SR-B1, scavenger receptor class B member 1; IL-13, interleukin-13; IL13Rα2, interleukin-13 receptor α2; VEGF, vascular endothelial growth factor; ORF, open reading frame; hTRAIL, human tumor necrosis factor-related apoptosis-inducing ligand; GOLPH3, Golgi phosphoprotein 3; $_{D}$( ) peptide, retro-inverso peptide; $_{C}$( ) peptide, cyclic peptide.

increased by microbubble injection and ultrasound irradiation, with enhanced antiglioma effects. Currently, some mAbs, *e.g.*, bevacizumab, trastuzumab, cetuximab, and nivolumab, are available to treat some solid cancers. However, only bevacizumab can be used for the treatment of glioma because of the impermeability of glioma tissue. Some reports showed that mAbs can be efficiently delivered in glioma-bearing animals (Table 2), suggesting the possibility of mAb-based therapy. In addition, it was reported that ultrasound itself has anticancer effects arising from its thermal effect or immune activation.[143,144] These findings suggest that glioma therapy can be improved by combining ultrasound-mediated DDS with chemotherapy.

Patisiran, a form of siRNA-encapsulated nanoparticles, was approved in some countries. That paves the way for nucleic acid to be used as a novel therapeutic agent in combination with DDS. Several studies showed that plasmid DNA-mediated transfection of human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) may have antiglioma efficacy *in vivo*.[145,146] Liu *et al.* demonstrated that co-delivery of the TRAIL gene and doxorubicin through a dendrigraft poly-L-lysine (DGL) derivative modified with T7 peptide (DGDP/pORF-hTRAIL) prolonged the survival of glioma-bearing mice dramatically to 57 d, while the survival time in those that received unmodified DGDP/pORF-hTRAIL was 27 d.[145] In another approach, epidermal growth factor receptor (EGFR) mutation is related to the proliferation and invasion of glioma. Golgi phosphoprotein 3 (GOLPH3) also affects the progression of glioma and could downregulate the expression of EGFR. Ye *et al.* indicated that Angiopep-2-modified PLGA nanoparticles encapsulating GOLPH3 siRNA and gefitinib efficiently silenced GOLPH3 mRNA expression levels and promoted antiglioma effects.[47]

Because metastatic intracranial tumors such as from breast cancer also occur, the migration mechanism and heterogenous intratumoral environment must be analyzed when determining the treatment strategy. Tominaga *et al.* reported that brain metastatic cancer cells released exosomes encapsulating microRNA-181c and destroyed the BBB.[147] This could be because the suppression of cofilin phosphorylation by microRNA-181c upregulates cofillin and depolymerizes actin.[147] Moreover, we utilized various fluorescence probes to analyze the heterogenous intratumoral environment to visualize the distribution of nanocarriers and tumor blood vessels in a colon cancer model using the tissue-clearing method.[136] This information could lead to elucidation of the mechanisms and therapeutic efficacy of DDS in the heterogenous glioma environment.

4.2. Alzheimer's Disease AD is the most common neurodegenerative disease causing dementia. The major symptom of AD is memory loss, but patients also exhibit neurological manifestations such as depression, social withdrawal, and delusion. Because patients need increasing levels of care as the stage of AD advances, the socioeconomic burden is consider-

able. The number of AD patients increases with population aging, and therefore AD treatment is an urgent need. So far, only cholinesterase inhibitors, *e.g.*, donepezil, rivastigmine, and galantamine, and *N*-methyl-D-aspartic acid (NMDA) glutamate receptor antagonists, *e.g.*, memantine, have been approved to treat the symptoms of AD.

AD is caused by brain atrophy, mainly of the hippocampus and cortex. Aggregation of insoluble Aβ and subsequent accumulation of tau protein is the proposed molecular pathology, and research into AD treatment is focused on their reduction. β-site-cleaving enzyme 1 (BACE1) is an enzyme that produces Aβ by processing the N-terminal of amyloid precursor protein (APP). Several reports showed that RNA interference with BACE1 in combination with DDS potently reduced Aβ plaque in animal AD models. Intraparenchymal injection of BACE1 siRNA nanocomplex conjugated with peptide ligand effectively reduced BACE1 expression.[148] Shyam *et al.* showed that efficient BACE1 knockdown was achieved by ICV administration of micelles composed of linear PEI–PEG copolymer-loaded BACE1 siRNA.[149] Carriers for systemic administration have been also developed. Liu *et al.* prepared a complex of BACE1 shRNA coding plasmid DNA and DGLs conjugated with the BBB-crossing peptide RVG29.[150] After intravenous administration of the complex to AD model mice, they confirmed that Aβ in the hippocampus was reduced and memory and learning ability was recovered. In addition, Wang *et al.* developed an siRNA nanocomplex composed of PEG-derived block copolymer conjugated with Tet1 peptide to treat AD with intravenous siRNA delivery.[151]

NEP, a peptidase involved in Aβ catabolism, may be a target for the treatment of AD. Intraparenchymal administration of NEP using CED resulted in widespread NEP distribution in the brain and significant reduction of endogenous Aβ,[152] suggesting the NEP enzyme as a therapeutic candidate. Because NEP protein disappears rapidly, a gene transfer approach has been developed by some groups. Iwata *et al.* demonstrated an increase in NEP expression and decrease in the hippocampal Aβ level when NEP coding AAV that can penetrate the BBB was administered intravenously.[153] Lin *et al.* developed an mRNA-based NEP gene transfer system using polyplex micelles *via* ICV.[154] mRNA is a promising gene vector because it has no risk of integration into the genome, which may occur when using a DNA vector. Although the physiological function of NEP has been partially clarified, there are only a few reports of therapeutic strategies targeting NEP and therefore further studies are needed.

4.3. Parkinson's Disease PD causes motor symptoms including tremor, bradykinesia, and rigidity. The cause of PD is thought to be the loss of nigrostriatal dopaminergic neurons and subsequent loss of dopamine. The current main treatment of PD is dopamine replacement therapy, in which dopamine agonists, monoamine oxidase (MAO) inhibitors, or dopamine analogues are administered orally. Although temporary symptom relief can be obtained with dopamine replacement, long-term administration can reduce the duration of action or cause side effects such as dyskinesia. Dopamine encapsulated in poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles 100 nm in diameter can sustain the release of dopamine, resulting in greater therapeutic effects than free dopamine when administered to PD model rats.[155] However, dopaminergic replacement therapy merely suppresses the symptoms of PD, meaning that a strategy for radical therapy by modifying the pathology of PD is required.

The accumulation of aggregated Lewy bodies mainly composed of α-syn has been proposed as the pathologic mechanism of PD. The mutation of the gene coding α-syn (*SNCA*) is reported to be associated with familial and even sporadic PD. Recently, therapeutic agents that reduce α-syn expression have been proposed as a radical treatment for PD. As therapeutic molecules to reduce α-syn, antibodies, siRNA, ASO, and shRNA-expressing plasmid DNA were proposed. Delivery of siRNA by PEI complex *via* the ICV route reduced the expression of α-syn in the whole brain region in PD transgenic mice overexpressing *SNCA*.[156] N2B delivery of α-syn-targeted siRNA or ASO reduced α-syn expression in the SN and increased the dopamine concentration in the forebrain.[157] Because siRNA has only transient effects, shRNA-coded minicircle DNA that mimics plasmid DNA was proposed as an alternative.[158] Izco *et al.* prepared RVG ligand-modified exosomes isolated with dendritic cells containing minicircles that code shRNA against α-syn and showed therapeutic effects after systemic administration.[159]

Neurogenerative approaches by delivering therapeutic proteins such as neurotrophic factors or growth factors could also become radical PD treatments. Because protein is quickly degraded, a sustained supply of therapeutic protein is required to achieve neurogenesis and therapeutic effects against PD. Glial cell line-derived neurotrophic factor (GDNF)-loaded PLGA particles have the ability to release GDNF over at least 40 d and increased the number of dopaminergic neurons in the SN in a PD model.[160]

Exogeneous gene transfection and subsequent production of therapeutic protein could also recover lost dopaminergic neurons. Several reports showed that ultrasound-mediated delivery of plasmid DNA to the SN *via* transient BBB opening restored dopaminergic neurons in an animal PD model. Lin *et al.* developed GDNF plasmid DNA-loaded microbubbles as an ultrasound-responsive GDNF gene carrier.[161] Intravenous injection of the formulation and focused ultrasound irradiation to the SN increased the number of dopaminergic neurons and restored motor function in mice in which PD was induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. On the other hand, Aly *et al.* showed that N2B delivery of GDNF plasmid DNA nanoparticles induced GDNF expression in the rostral midbrain and ameliorated the loss of dopaminergic neurons in the SN,[162] suggesting that noninvasive gene therapy for PD is possible.

4.4. Ischemic Stroke Ischemic stroke is a neurological condition in which cerebral blood flow is decreased due to the transient infarction of blood capillaries. In regions where ischemic stroke occurred, neurons and glial cells die due to the limited supply of oxygen, leading to death or disability. Although thrombolysis using recombinant tissue-plasminogen activator (rt-PA) is the treatment with the most evidence accumulated to date, the percentage of patients administered rt-PA is limited because it must be administered within 4.5 h after stroke onset due to the risk of severe hemorrhage. After thrombolysis with rt-PA, the production of reactive oxygen species or excessive inflammatory cytokines can be induced by ischemic/reperfusion (I/R) injury. The ischemic region is divided into the infarct core and penumbra. The infarct core is the center of ischemia and subject to severe irreversible

damage. The penumbra is the region adjacent to the infarct core which is subject to functional but reversible impairment. There are two types of DDS approaches to the treatment of ischemic stroke. One is to protect the penumbra from neuronal loss by administering neuroprotectants as soon as possible after stroke occurrence, and the other is to regenerate lost cells by transplanting exogeneous stem cells or activating endogenous ones.

BBB dysfunction with increased permeability is observed after infarction and reperfusion. Before the loss of neurological function, increased BBB permeability is observed within 30 min after infarction. Utilizing this pathology of ischemic stroke, enhanced permeability and retention-based neuroprotectant delivery systems have been developed. Ishii *et al.* developed asialo-erythropoietin-modified PEGylated liposomes and evaluated their distribution and neuroprotective effects in a middle cerebral artery occlusion model.[163] Therapeutic liposomes injected after I/R injury accumulated in the ischemic hemisphere and ameliorated I/R-mediated neuronal loss and motor dysfunction. Moreover, the administration of fasudil-loaded liposomes under occlusion ameliorated the injury associated with reperfusion by rt-PA.[164] Xu *et al.* developed a multifunctional formulation called "nanoplatelets" for sequential selective delivery of rt-PA and neuroprotectant to ischemic sites.[165] Those neuroprotectant-loaded nanoparticles with thrombin-cleavable Tat peptide-coupled rt-PA can deliver rt-PA to thrombi and enhance migration to ischemic sites.

Regenerative medicine approaches may also be useful in the treatment of ischemic stroke, involving: 1) transplantation of exogenous stem cells; and 2) promoting neurogenesis by activating endogenous NSCs. The current status and details of exogenous stem cell transplantation are summarized in other reviews.[166,167] Endogenous NSC activation is receiving increased attention because it does not require the processing of living cells. Some therapeutic systems have been developed in combination with DDS techniques. Nakaguchi *et al.* investigated a controlled-release carrier of hepatocyte growth factor (HGF) composed of gelatin hydrogel microspheres.[168] The sustained release of HGF increased the number of new neurons migrating from the SVZ toward the injured striatum in a mouse stroke model after HGF-loaded microspheres were injected into the striatum.[168] Furthermore, Rodríguez-Frutos *et al.* demonstrated that ultrasound-enhanced BDNF delivery improved motor function in a rat stroke model.[169] Importantly, no adverse effects associated with hemorrhage induced by ultrasound were seen. On the other hand, Yang *et al.* prepared RVG-modified MSC-derived exosomes carrying miR-124 which have neuroprotective and neurorestorative potential.[170] miR-124-loaded exosomes significantly promoted neurogenesis in ischemic regions. These recent findings may offer hope for improved prognosis in stroke patients.

5. Conclusion and Future Prospects

Brain-targeting DDS can potentially increase treatment efficacy in patients with cerebral disorders. As described in this review, the safest, least-invasive approach to crossing the BBB appears to be transcytosis by presenting ligands. Many DDS for this have been proposed and shown therapeutic effects. Recent studies have found that accumulation in the brain reached around 5% ID,[16,34] and future targeting systems are expected to improve that rate. Some brain-targeting DDS formulations have complicated structures because they are functionalized by modification with ligand molecules. For clinical applications, it is necessary to establish production systems for high-quality formulations with good reproducibility under Good Manufacturing Practices standards. Research on the design and production of those formulations should be conducted in combination with developments in the laboratory.

Targeted region-specific drug delivery is required for safe, effective treatment of disorders characterized by local lesions. BBB-crossing systems using ligand-modified carriers remain far from satisfactory because most deliver drugs to large regions of the brain. With progress in medical technologies such as MRI or focused ultrasound, precise brain-targeting DDS have become feasible with pinpoint delivery to tumors, ischemic sites, or the SN. However, further confirmation of safety is required before widespread clinical application. Research on regulating the extent of BBB opening is ongoing.

Intrabrain and intracellular drug distribution directly determines the therapeutic effect. New oligonucleotide or gene vectors exert activity after reaching specific cell regions such as the cytosol or nucleus. Therefore, precise evaluation is required. This review introduced evaluation methods including *in vivo* imaging and histological techniques like multicolor deep tissue imaging. However, these have both advantages and disadvantages. As yet, no single system meets all the requirements for DDS evaluation. Consequently, it will be important to combine several methods depending on the purpose of evaluation.

Conflict of Interest Shigeru Kawakami received scholarship support from Towa Pharmaceutical Co., Ltd., Japan, to study the incompatibility of vancomycin with other drugs used clinically. The other authors declare no conflict of interest.

References

1) Shahryari A., Saghaeian Jazi M., Mohammadi S., Razavi Nikoo H., Nazari Z., Hosseini E. S., Burtscher I., Mowla S. J., Lickert H., *Front. Genet.*, **10**, 868 (2019).
2) Rinaldi C., Wood M. J. A., *Nat. Rev. Neurol.*, **14**, 9–21 (2018).
3) Pardridge W. M., *Mol. Interv.*, **3**, 90–105, 151 (2003).
4) Reichel A., *Pharmacokinet. CNS Penetration*, **1**, 5–41 (2015).
5) Pardridge W. M., *NeuroRx*, **2**, 3–14 (2005).
6) Ohtsuki S., Terasaki T., *Pharm. Res.*, **24**, 1745–1758 (2007).
7) Sharma G., Sharma A. R., Lee S. S., Bhattacharya M., Nam J. S., Chakraborty C., *Int. J. Pharm.*, **559**, 360–372 (2019).
8) Xie J., Shen Z., Anraku Y., Kataoka K., Chen X., *Biomaterials*, **224**, 119491 (2019).
9) Elezaby R. S., Gad H. A., Metwally A. A., Geneidi A. S., Awad G. A., *J. Control. Release*, **261**, 43–61 (2017).
10) Liu W. Y., Wang Z. B., Zhang L. C., Wei X., Li L., *CNS Neurosci. Ther.*, **18**, 609–615 (2012).
11) Greene C., Campbell M., *Tissue Barriers*, **4**, e1138017 (2016).
12) Neves V., Aires-da-Silva F., Corte-Real S., Castanho M. A. R. B., *Trends Biotechnol.*, **34**, 36–48 (2016).
13) Oller-Salvia B., Sanchez-Navarro M., Giralt E., Teixido M., *Chem. Soc. Rev.*, **45**, 4690–4707 (2016).
14) Kirin S. C., Yanagisawa T., Oshino S., Edakawa K., Tanaka M., Kishima H., Nishimura Y., *Front. Neurosci.*, **13**, 1019 (2019).
15) Hervé F., Ghinea N., Scherrmann J. M., *AAPS J.*, **10**, 455–472 (2008).
16) Anraku Y., Kuwahara H., Fukusato Y., Mizoguchi A., Ishii T., Nitta K., Matsumoto Y., Toh K., Miyata K., Uchida S., Nishina K., Osada K., Itaka K., Nishiyama N., Mizusawa H., Yamasoba T.,

Yokota T., Kataoka K., *Nat. Commun.*, **8**, 1001 (2017).
17) Cuddapah V. A., Zhang S. L., Sehgal A., *Trends Neurosci.*, **42**, 500–510 (2019).
18) Qian Z. M., Li H., Sun H., Ho K., *Pharmacol. Rev.*, **54**, 561–587 (2002).
19) Bickel U., Yoshikawa T., Pardridge W. M., *Adv. Drug Deliv. Rev.*, **46**, 247–279 (2001).
20) Kozlovskaya L., Stepensky D., *J. Control. Release*, **171**, 17–23 (2013).
21) Sonoda H., Morimoto H., Yoden E., Koshimura Y., Kinoshita M., Golovina G., Takagi H., Yamamoto R., Minami K., Mizoguchi A., Tachibana K., Hirato T., Takahashi K., *Mol. Ther.*, **26**, 1366–1374 (2018).
22) Okuyama T., Eto Y., Sakai N., Minami K., Yamamoto T., Sonoda H., Yamaoka M., Tachibana K., Hirato T., Sato Y., *Mol. Ther.*, **27**, 456–464 (2019).
23) Niewoehner J., Bohrmann B., Collin L., Urich E., Sade H., Maier P., Rueger P., Stracke J., Lau W., Tissot A., Loetscher H., Ghosh A., Freskgård P. O., *Neuron*, **81**, 49–60 (2014).
24) Pardridge W. M., "Brain Drug Targeting: The Future of Brain Drug Development," Cambridge University Press, Cambridge, U.K., 2001, pp. 1–370.
25) Boado R. J., Zhang Y., Zhang Y., Xia C. F., Wang Y., Pardridge W. M., *Biotechnol. Bioeng.*, **99**, 475–484 (2008).
26) Giugliani R., Giugliani L., de Oliveira Poswar F., Donis K. C., Corte A. D., Schmidt M., Boado R. J., Nestrasil I., Nguyen C., Chen S., Pardridge W. M., *Orphanet J. Rare Dis.*, **13**, 110 (2018).
27) Boado R. J., Hui E. K. W., Lu J. Z., Sumbria R. K., Pardridge W. M., *Bioconjug. Chem.*, **24**, 1741–1749 (2013).
28) Demeule M., Regina A., Ché C., Poirier J., Nguyen T., Gabathuler R., Castaigne J. P., Béliveau R., *J. Pharmacol. Exp. Ther.*, **324**, 1064–1072 (2008).
29) Demeule M., Currie J. C., Bertrand Y., Ché C., Nguyen T., Régina A., Gabathuler R., Castaigne J. P., Béliveau R., *J. Neurochem.*, **106**, 1534–1544 (2008).
30) Régina A., Demeule M., Ché C., Lavallée I., Poirier J., Gabathuler R., Béliveau R., Castaigne J. P., *Br. J. Pharmacol.*, **155**, 185–197 (2008).
31) Ché C., Yang G., Thiot C., Lacoste M. C., Currie J. C., Demeule M., Régina A., Béliveau R., Castaigne J. P., *J. Med. Chem.*, **53**, 2814–2824 (2010).
32) Regina A., Demeule M., Tripathy S., Lord-Dufour S., Currie J. C., Iddir M., Annabi B., Castaigne J. P., Lachowicz J. E., *Mol. Cancer Ther.*, **14**, 129–140 (2015).
33) Demeule M., Poirier J., Jodoin J., Bertrand Y., Desrosiers R. R., Dagenais C., Nguyen T., Lanthier J., Gabathuler R., Kennard M., Jefferies W. A., Karkan D., Tsai S., Fenart L., Cecchelli R., Béliveau R., *J. Neurochem.*, **83**, 924–933 (2002).
34) Thom G., Tian M. M., Hatcher J. P., Rodrigo N., Burrell M., Gurrell I., Vitalis T. Z., Abraham T., Jefferies W. A., Webster C. I., Gabathuler R., *J. Cereb. Blood Flow Metab.*, **39**, 2074–2088 (2019).
35) Nishina K., Unno T., Uno Y., Kubodera T., Kanouchi T., Mizusawa H., Yokota T., *Mol. Ther.*, **16**, 734–740 (2008).
36) Uno Y., Piao W., Miyata K., Nishina K., Mizusawa H., Yokota T., *Hum. Gene Ther.*, **22**, 711–719 (2011).
37) Nishina K., Piao W., Yoshida-Tanaka K., *et al.*, *Nat. Commun.*, **6**, 7969 (2015).
38) Kuwahara H., Song J., Shimoura T., Yoshida-Tanaka K., Mizuno T., Mochizuki T., Zeniya S., Li F., Nishina K., Nagata T., Ito S., Kusuhara H., Yokota T., *Sci. Rep.*, **8**, 4377 (2018).
39) Tröster S. D., Müller U., Kreuter J., *Int. J. Pharm.*, **61**, 85–100 (1990).
40) Kreuter J., Petrov V. E., Kharkevich D. A., Alyautdin R. N., *J. Control. Release*, **49**, 81–87 (1997).
41) Kreuter J., Alyautdin R. N., Kharkevich D. A., Ivanov A. A., *Brain Res.*, **674**, 171–174 (1995).
42) Kreuter J., Shamenkov D., Petrov V., Ramge P., Cychutek K., Koch-Brandt C., Alyautdin R., *J. Drug Target.*, **10**, 317–325 (2002).
43) Azmin M. N., Stuart J. F. B., Florence A. T., *Cancer Chemother. Pharmacol.*, **14**, 238–242 (1985).
44) Re F., Cambianica I., Zona C., Sesana S., Gregori M., Rigolio R., La Ferla B., Nicotra F., Forloni G., Cagnotto A., Salmona M., Masserini M., Sancini G., *Nanomedicine*, **7**, 551–559 (2011).
45) Wang D., El-Amouri S. S., Dai M., Kuan C.-Y., Hui D. Y., Brady R. O., Pan D., *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 2999–3004 (2013).
46) Tamaru M., Akita H., Nakatani T., Kajimoto K., Sato Y., Hatakeyama H., Harashima H., *Int. J. Nanomedicine*, **9**, 4267–4276 (2014).
47) Ye C., Pan B., Xu H., Zhao Z., Shen J., Lu J., Yu R., Liu H., *J. Mol. Med.*, **97**, 1575–1588 (2019).
48) Tsai Y.-C., Vijayaraghavan P., Chiang W.-H., Chen H.-H., Liu T.-I., Shen M.-Y., Omoto A., Kamimura M., Soga K., Chiu H.-C., *Theranostics*, **8**, 1435–1448 (2018).
49) Yang Z. Z., Li J. Q., Wang Z. Z., Dong D. W., Qi X. R., *Biomaterials*, **35**, 5226–5239 (2014).
50) Malcor J.-D., Payrot N., David M., Faucon A., Abouzid K., Jacquot G., Floquet N., Debarbieux F., Rougon G., Martinez J., Khrestchatisky M., Vlieghe P., Lisowski V., *J. Med. Chem.*, **55**, 2227–2241 (2012).
51) Chen C., Duan Z., Yuan Y., Li R., Pang L., Liang J., Xu X., Wang J., *ACS Appl. Mater. Interfaces*, **9**, 5864–5873 (2017).
52) Kannan R., Kuhlenkamp J. F., Jeandidier E., Trinh H., Ookhtens M., Kaplowitz N., *J. Clin. Invest.*, **85**, 2009–2013 (1990).
53) Englert C., Trützschler A. K., Raasch M., Bus T., Borchers P., Mosig A. S., Traeger A., Schubert U. S., *J. Control. Release*, **241**, 1–14 (2016).
54) Gaillard P. J., Appeldoorn C. C. M., Rip J., Dorland R., Van Der Pol S. M. A., Kooij G., De Vries H. E., Reijerkerk A., *J. Control. Release*, **164**, 364–369 (2012).
55) Rip J., Chen L., Hartman R., van den Heuvel A., Reijerkerk A., van Kregten J., van der Boom B., Appeldoorn C., de Boer M., Maussang D., de Lange E. C., Gaillard P. J., *J. Drug Target.*, **22**, 460–467 (2014).
56) Gaillard P. J., Appeldoorn C. C. M., Dorland R., Van Kregten J., Manca F., Vugts D. J., Windhorst B., Van Dongen G. A. M. S., De Vries H. E., Maussang D., Van Tellingen O., *PLOS ONE*, **9**, e82331 (2014).
57) Xie F., Yao N., Qin Y., Zhang Q., Chen H., Yuan M., Tang J., Li X., Fan W., Zhang Q., Wu Y., Hai L., He Q., *Int. J. Nanomedicine*, **7**, 163–175 (2012).
58) Van Rooy I., Mastrobattista E., Storm G., Hennink W. E., Schiffelers R. M., *J. Control. Release*, **150**, 30–36 (2011).
59) Xin H., Li Y., Liu Z., Wang X., Shang X., Cui Y., Zhang Z. G., Chopp M., *Stem Cells*, **31**, 2737–2746 (2013).
60) Katsuda T., Tsuchiya R., Kosaka N., Yoshioka Y., Takagaki K., Oki K., Takeshita F., Sakai Y., Kuroda M., Ochiya T., *Sci. Rep.*, **3**, 1197 (2013).
61) Cui G. H., Guo H. D., Li H., Zhai Y., Gong Z. B., Wu J., Liu J. S., Dong Y. R., Hou S. X., Liu J. R., *Immun. Ageing*, **16**, 10 (2019).
62) Qu M., Lin Q., Huang L., Fu Y., Wang L., He S., Fu Y., Yang S., Zhang Z., Zhang L., Sun X., *J. Control. Release*, **287**, 156–166 (2018).
63) Kojima R., Bojar D., Rizzi G., Hamri G. C. E., El-Baba M. D., Saxena P., Ausländer S., Tan K. R., Fussenegger M., *Nat. Commun.*, **9**, 1305 (2018).
64) Carpentier A., Canney M., Vignot A., Reina V., Beccaria K., Horodyckid C., Karachi C., Leclercq D., Lafon C., Chapelon J. Y., Capelle L., Cornu P., Sanson M., Hoang-Xuan K., Delattre J. Y., Idbaih A., *Sci. Transl. Med.*, **8**, 343re342 (2016).
65) Lipsman N., Meng Y., Bethune A. J., Huang Y., Lam B., Masel-

lis M., Herrmann N., Heyn C., Aubert I., Boutet A., Smith G. S., Hynynen K., Black S. E., *Nat. Commun.*, **9**, 2336 (2018).
66) Abrahao A., Meng Y., Llinas M., Huang Y., Hamani C., Mainprize T., Aubert I., Heyn C., Black S. E., Hynynen K., Lipsman N., Zinman L., *Nat. Commun.*, **10**, 4373 (2019).
67) Zhao B., Chen Y., Liu J., Zhang L., Wang J., Yang Y., Lv Q., Xie M., *Oncotarget*, **9**, 4897–4914 (2018).
68) Tung Y. S., Vlachos F., Feshitan J. A., Borden M. A., Konofagou E. E., *J. Acoust. Soc. Am.*, **130**, 3059–3067 (2011).
69) Cho H., Lee H. Y., Han M., Choi J. R., Ahn S., Lee T., Chang Y., Park J., *Sci. Rep.*, **6**, 31201 (2016).
70) Deng J., Huang Q., Wang F., Liu Y., Wang Z., Wang Z., Zhang Q., Lei B., Cheng Y., *J. Mol. Neurosci.*, **46**, 677–687 (2012).
71) Thevenot E., Jordao J. F., O'Reilly M. A., Markham K., Weng Y. Q., Foust K. D., Kaspar B. K., Hynynen K., Aubert I., *Hum. Gene Ther.*, **23**, 1144–1155 (2012).
72) Alonso A., Reinz E., Leuchs B., Kleinschmidt J., Fatar M., Geers B., Lentacker I., Hennerici M. G., de Smedt S. C., Meairs S., *Mol. Ther. Nucleic Acids*, **2**, e73 (2013).
73) Burgess A., Ayala-Grosso C. A., Ganguly M., Jordao J. F., Aubert I., Hynynen K., *PLoS ONE*, **6**, e27877 (2011).
74) Mead B. P., Mastorakos P., Suk J. S., Klibanov A. L., Hanes J., Price R. J., *J. Control. Release*, **223**, 109–117 (2016).
75) Ogawa K., Fuchigami Y., Hagimori M., Fumoto S., Miura Y., Kawakami S., *Int. J. Nanomedicine*, **13**, 2309–2320 (2018).
76) Fan C. H., Ting C. Y., Chang Y. C., Wei K. C., Liu H. L., Yeh C. K., *Acta Biomater.*, **15**, 89–101 (2015).
77) Wang X., Liu P., Yang W., Li L., Li P., Liu Z., Zhuo Z., Gao Y., *Int. J. Nanomedicine*, **9**, 4899–4909 (2014).
78) Fan C. H., Ting C. Y., Lin C. Y., Chan H. L., Chang Y. C., Chen Y. Y., Liu H. L., Yeh C. K., *Sci. Rep.*, **6**, 19579 (2016).
79) Chang E. L., Ting C. Y., Hsu P. H., Lin Y. C., Liao E. C., Huang C. Y., Chang Y. C., Chan H. L., Chiang C. S., Liu H. L., Wei K. C., Fan C. H., Yeh C. K., *J. Control. Release*, **255**, 164–175 (2017).
80) Fan C. H., Chang E. L., Ting C. Y., Lin Y. C., Liao E. C., Huang C. Y., Chang Y. C., Chan H. L., Wei K. C., Yeh C. K., *Biomaterials*, **106**, 46–57 (2016).
81) Shin J., Kong C., Cho J. S., Lee J., Koh C. S., Yoon M. S., Na Y. C., Chang W. S., Chang J. W., *Neurosurg. Focus*, **44**, E15 (2018).
82) Fan C. H., Liu H. L., Ting C. Y., Lee Y. H., Huang C. Y., Ma Y. J., Wei K. C., Yen T. C., Yeh C. K., *PLOS ONE*, **9**, e96327 (2014).
83) Song K. H., Fan A. C., Hinkle J. J., Newman J., Borden M. A., Harvey B. K., *Theranostics*, **7**, 144–152 (2017).
84) Rapoport S. I., *Cell. Mol. Neurobiol.*, **20**, 217–230 (2000).
85) Angelow S., Ahlstrom R., Yu A. S. L., *Am. J. Physiol. Renal Physiol.*, **295**, F867–F876 (2008).
86) Nitta T., Hata M., Gotoh S., Seo Y., Sasaki H., Hashimoto N., Furuse M., Tsukita S., *J. Cell Biol.*, **161**, 653–660 (2003).
87) Higashi T., Tokuda S., Kitajiri S. I., Masuda S., Nakamura H., Oda Y., Furuse M., *J. Cell Sci.*, **126**, 966–977 (2013).
88) Masuda S., Oda Y., Sasaki H., Ikenouchi J., Higashi T., Akashi M., Nishi E., Furuse M., *J. Cell Sci.*, **124**, 548–555 (2011).
89) Sohet F., Lin C., Munji R. N., Lee S. Y., Ruderisch N., Soung A., Arnold T. D., Derugin N., Vexler Z. S., Yen F. T., Daneman R., *J. Cell Biol.*, **208**, 703–711 (2015).
90) Zeniya S., Kuwahara H., Daizo K., Watari A., Kondoh M., Yoshida-Tanaka K., Kaburagi H., Asada K., Nagata T., Nagahama M., Yagi K., Yokota T., *J. Control. Release*, **283**, 126–134 (2018).
91) Yanagida K., Liu C. H., Faraco G., Galvani S., Smith H. K., Burg N., Anrather J., Sanchez T., Iadecola C., Hla T., *Proc. Natl. Acad. Sci. U.S.A.*, **114**, 4531–4536 (2017).
92) Niaudet C., Hofmann J. J., Mäe M. A., *et al.*, *PLOS ONE*, **10**, e0137949 (2015).
93) Mittermeyer G., Christine C. W., Rosenbluth K. H., Baker S. L., Starr P., Larson P., Kaplan P. L., Forsayeth J., Aminoff M. J., Bankiewicz K. S., *Hum. Gene Ther.*, **23**, 377–381 (2012).
94) Mehta A. M., Sonabend A. M., Bruce J. N., *Neurotherapeutics*, **14**, 358–371 (2017).
95) Shi M., Sanche L., *J. Oncol.*, **2019**, 9342796 (2019).
96) Tosi U., Kommidi H., Bellat V., Marnell C. S., Guo H., Adeuyan O., Schweitzer M. E., Chen N., Su T., Zhang G., Maachani U. B., Pisapia D. J., Law B., Souweidane M. M., Ting R., *ACS Chem. Neurosci.*, **10**, 2287–2298 (2019).
97) Shi M., Anantha M., Wehbe M., Bally M. B., Fortin D., Roy L. O., Charest G., Richer M., Paquette B., Sanche L., *J. Nanobiotechnology*, **16**, 77 (2018).
98) Kenny G. D., Bienemann A. S., Tagalakis A. D., Pugh J. A., Welser K., Campbell F., Tabor A. B., Hailes H. C., Gill S. S., Lythgoe M. F., McLeod C. W., White E. A., Hart S. L., *Biomaterials*, **34**, 9190–9200 (2013).
99) Whish S., Dziegielewska K. M., Mollgard K., Noor N. M., Liddelow S. A., Habgood M. D., Richardson S. J., Saunders N. R., *Front. Neurosci.*, **9**, 16 (2015).
100) Cohen-Pfeffer J. L., Gururangan S., Lester T., Lim D. A., Shaywitz A. J., Westphal M., Slavc I., *Pediatr. Neurol.*, **67**, 23–35 (2017).
101) Chamberlain M., Soffietti R., Raizer J., Ruda R., Brandsma D., Boogerd W., Taillibert S., Groves M. D., Le Rhun E., Junck L., van den Bent M., Wen P. Y., Jaeckle K. A., *Neuro-oncol.*, **16**, 1176–1185 (2014).
102) Doetsch F., Caille I., Lim D. A., Garcia-Verdugo J. M., Alvarez-Buylla A., *Cell*, **97**, 703–716 (1999).
103) Ochi T., Nakatomi H., Ito A., Imai H., Okabe S., Saito N., *Brain Res.*, **1636**, 118–129 (2016).
104) Lemkine G. F., Mantero S., Migne C., Raji A., Goula D., Normandie P., Levi G., Demeneix B. A., *Mol. Cell. Neurosci.*, **19**, 165–174 (2002).
105) Newland B., Moloney T. C., Fontana G., Browne S., Abu-Rub M. T., Dowd E., Pandit A. S., *Biomaterials*, **34**, 2130–2141 (2013).
106) Bugeon S., de Chevigny A., Boutin C., Core N., Wild S., Bosio A., Cremer H., Beclin C., *Development*, **144**, 3968–3977 (2017).
107) Tan J. K., Pham B., Zong Y., Perez C., Maris D. O., Hemphill A., Miao C. H., Matula T. J., Mourad P. D., Wei H., Sellers D. L., Horner P. J., Pun S. H., *J. Control. Release*, **231**, 86–93 (2016).
108) Ogawa K., Fuchigami Y., Hagimori M., Fumoto S., Maruyama K., Kawakami S., *Eur. J. Pharm. Biopharm.*, **137**, 1–8 (2019).
109) Fazil M., Md S., Haque S., Kumar M., Baboota S., Sahni J. K., Ali J., *Eur. J. Pharm. Sci.*, **47**, 6–15 (2012).
110) Musumeci T., Serapide M. F., Pellitteri R., Dalpiaz A., Ferraro L., Dal Magro R., Bonaccorso A., Carbone C., Veiga F., Sancini G., Puglisi G., *Eur. J. Pharm. Biopharm.*, **133**, 309–320 (2018).
111) Kumar N. N., Lochhead J. J., Pizzo M. E., Nehra G., Boroumand S., Greene G., Thorne R. G., *J. Control. Release*, **286**, 467–484 (2018).
112) Lochhead J. J., Kellohen K. L., Ronaldson P. T., Davis T. P., *Sci. Rep.*, **9**, 2621 (2019).
113) Van Woensel M., Wauthoz N., Rosiere R., Mathieu V., Kiss R., Lefranc F., Steelant B., Dilissen E., Van Gool S. W., Mathivet T., Gerhardt H., Amighi K., De Vleeschouwer S., *J. Control. Release*, **227**, 71–81 (2016).
114) Rodriguez M., Lapierre J., Ojha C. R., Kaushik A., Batrakova E., Kashanchi F., Dever S. M., Nair M., El-Hage N., *Sci. Rep.*, **7**, 1862 (2017).
115) Kanazawa T., Morisaki K., Suzuki S., Takashima Y., *Mol. Pharm.*, **11**, 1471–1478 (2014).
116) Belur L. R., Temme A., Podetz-Pedersen K. M., Riedl M., Vulchanova L., Robinson N., Hanson L. R., Kozarsky K. F., Orchard P. J., Frey W. H. 2nd, Low W. C., McIvor R. S., *Hum. Gene Ther.*, **28**, 576–587 (2017).
117) Aly A. E., Harmon B., Padegimas L., Sesenoglu-Laird O., Cooper M. J., Yurek D. M., Waszczak B. L., *Nanomedicine*, **16**, 20–33 (2019).
118) Reger M. A., Watson G. S., Green P. S., Baker L. D., Cholerton B.,

Fishel M. A., Plymate S. R., Cherrier M. M., Schellenberg G. D., Frey I. W. H. 2nd, Craft S., *J. Alzheimers Dis.*, **13**, 323–331 (2008).
119) Kamei N., Okada N., Ikeda T., Choi H., Fujiwara Y., Okumura H., Takeda-Morishita M., *Sci. Rep.*, **8**, 17641 (2018).
120) Kanazawa T., Akiyama F., Kakizaki S., Takashima Y., Seta Y., *Biomaterials*, **34**, 9220–9226 (2013).
121) Mukai H., Ozaki D., Cui Y., Kuboyama T., Yamato-Nagata H., Onoe K., Takahashi M., Wada Y., Imanishi T., Kodama T., Obika S., Suzuki M., Doi H., Watanabe Y., *J. Control. Release*, **180**, 92–99 (2014).
122) Oku N., Yamashita M., Katayama Y., Urakami T., Hatanaka K., Shimizu K., Asai T., Tsukada H., Akai S., Kanazawa H., *Int. J. Pharm.*, **403**, 170–177 (2011).
123) Man F., Gawne P. J., de Rosales R. T. M., *Adv. Drug Deliv. Rev.*, **143**, 134–160 (2019).
124) Seo J. W., Ang J., Mahakian L. M., Tam S., Fite B., Ingham E. S., Beyer J., Forsayeth J., Bankiewicz K. S., Xu T., Ferrara K. W., *J. Control. Release*, **220** (Pt A), 51–60 (2015).
125) Miura Y., Fuchigami Y., Hagimori M., Sato H., Ogawa K., Munakata C., Wada M., Maruyama K., Kawakami S., *J. Drug Target.*, **26**, 684–691 (2018).
126) Lin Y. L., Wu M. T., Yang F. Y., *J. Pharm. Biomed. Anal.*, **149**, 482–487 (2018).
127) Jadhav S. B., Khaowroongrueng V., Derendorf H., *J. Pharm. Sci.*, **105**, 3233–3242 (2016).
128) Susaki E. A., Tainaka K., Perrin D., Kishino F., Tawara T., Watanabe T. M., Yokoyama C., Onoe H., Eguchi M., Yamaguchi S., Abe T., Kiyonari H., Shimizu Y., Miyawaki A., Yokota H., Ueda H. R., *Cell*, **157**, 726–739 (2014).
129) Hama H., Hioki H., Namiki K., Hoshida T., Kurokawa H., Ishidate F., Kaneko T., Akagi T., Saito T., Saido T., Miyawaki A., *Nat. Neurosci.*, **18**, 1518–1529 (2015).
130) Kuwajima T., Sitko A. A., Bhansali P., Jurgens C., Guido W., Mason C., *Development*, **140**, 1364–1368 (2013).
131) Chung K., Wallace J., Kim S. Y., Kalyanasundaram S., Andalman A. S., Davidson T. J., Mirzabekov J. J., Zalocusky K. A., Mattis J., Denisin A. K., Pak S., Bernstein H., Ramakrishnan C., Grosenick L., Gradinaru V., Deisseroth K., *Nature* (London), **497**, 332–337 (2013).
132) Hama H., Kurokawa H., Kawano H., Ando R., Shimogori T., Noda H., Fukami K., Sakaue-Sawano A., Miyawaki A., *Nat. Neurosci.*, **14**, 1481–1488 (2011).
133) Nishimura K., Yonezawa K., Fumoto S., Miura Y., Hagimori M., Nishida K., Kawakami S., *Pharmaceutics*, **11**, 244 (2019).
134) Oyama N., Fuchigami Y., Fumoto S., Sato M., Hagimori M., Shimizu K., Kawakami S., *Drug Deliv.*, **24**, 906–917 (2017).
135) Haraguchi A., Fuchigami Y., Kawaguchi M., Fumoto S., Ohyama K., Shimizu K., Hagimori M., Kawakami S., *Biol. Pharm. Bull.*, **41**, 944–950 (2018).
136) Suga T., Kato N., Hagimori M., Fuchigami Y., Kuroda N., Kodama Y., Sasaki H., Kawakami S., *Mol. Pharm.*, **15**, 4481–4490 (2018).
137) Fumoto S., Nishimura K., Nishida K., Kawakami S., *PLOS ONE*, **11**, e0148233 (2016).
138) Arvanitis C. D., Ferraro G. B., Jain R. K., *Nat. Rev. Cancer*, **20**, 26–41 (2020).
139) Wilhelm S., Tavares A. J., Dai Q., Ohta S., Audet J., Dvorak H. F., Chan W. C. W., *Nat. Rev. Mater.*, **1**, 16014 (2016).
140) Zong T., Mei L., Gao H., Cai W., Zhu P., Shi K., Chen J., Wang Y., Gao F., He Q., *Mol. Pharm.*, **11**, 2346–2357 (2014).
141) Liu H. L., Huang C. Y., Chen J. Y., Wang H. Y., Chen P. Y., Wei K. C., *PLOS ONE*, **9**, e114311 (2014).
142) Aryal M., Vykhodtseva N., Zhang Y. Z., Park J., McDannold N., *J. Control. Release*, **169**, 103–111 (2013).
143) Suzuki R., Oda Y., Omata D., Nishiie N., Koshima R., Shiono Y., Sawaguchi Y., Unga J., Naoi T., Negishi Y., Kawakami S., Hashida M., Maruyama K., *Cancer Sci.*, **107**, 217–223 (2016).
144) Alkins R. D., Mainprize T. G., *Prog. Neurol. Surg.*, **32**, 39–47 (2018).
145) Liu S., Guo Y., Huang R., Li J., Huang S., Kuang Y., Han L., Jiang C., *Biomaterials*, **33**, 4907–4916 (2012).
146) Wang J., Lei Y., Xie C., Lu W., Wagner E., Xie Z., Gao J., Zhang X., Yan Z., Liu M., *Bioconjug. Chem.*, **25**, 414–423 (2014).
147) Tominaga N., Kosaka N., Ono M., Katsuda T., Yoshioka Y., Tamura K., Lotvall J., Nakagama H., Ochiya T., *Nat. Commun.*, **6**, 6716 (2015).
148) Tagalakis A. D., Lee D. H., Bienemann A. S., Zhou H., Munye M. M., Saraiva L., McCarthy D., Du Z., Vink C. A., Maeshima R., White E. A., Gustafsson K., Hart S. L., *Biomaterials*, **35**, 8406–8415 (2014).
149) Shyam R., Ren Y., Lee J., Braunstein K. E., Mao H. Q., Wong P. C., *Mol. Ther. Nucleic Acids*, **4**, e242 (2015).
150) Liu Y., An S., Li J., Kuang Y., He X., Guo Y., Ma H., Zhang Y., Ji B., Jiang C., *Biomaterials*, **80**, 33–45 (2016).
151) Wang P., Zheng X., Guo Q., Yang P., Pang X., Qian K., Lu W., Zhang Q., Jiang X., *J. Control. Release*, **279**, 220–233 (2018).
152) Barua N. U., Miners J. S., Bienemann A. S., Wyatt M. J., Welser K., Tabor A. B., Hailes H. C., Love S., Gill S. S., *J. Alzheimers Dis.*, **32**, 43–56 (2012).
153) Iwata N., Sekiguchi M., Hattori Y., Takahashi A., Asai M., Ji B., Higuchi M., Staufenbiel M., Muramatsu S., Saido T. C., *Sci. Rep.*, **3**, 1472 (2013).
154) Lin C. Y., Perche F., Ikegami M., Uchida S., Kataoka K., Itaka K., *J. Control. Release*, **235**, 268–275 (2016).
155) Pahuja R., Seth K., Shukla A., Shukla R. K., Bhatnagar P., Chauhan L. K., Saxena P. N., Arun J., Chaudhari B. P., Patel D. K., Singh S. P., Shukla R., Khanna V. K., Kumar P., Chaturvedi R. K., Gupta K. C., *ACS Nano*, **9**, 4850–4871 (2015).
156) Helmschrodt C., Hobel S., Schoniger S., Bauer A., Bonicelli J., Gringmuth M., Fietz S. A., Aigner A., Richter A., Richter F., *Mol. Ther. Nucleic Acids*, **9**, 57–68 (2017).
157) Alarcon-Aris D., Recasens A., Galofre M., Carballo-Carbajal I., Zacchi N., Ruiz-Bronchal E., Pavia-Collado R., Chica R., Ferres-Coy A., Santos M., Revilla R., Montefeltro A., Farinas I., Artigas F., Vila M., Bortolozzi A., *Mol. Ther.*, **26**, 550–567 (2018).
158) Chen Z.-Y., He C.-Y., Ehrhardt A., Kay M. A., *Mol. Ther.*, **8**, 495–500 (2003).
159) Izco M., Blesa J., Schleef M., Schmeer M., Porcari R., Al-Shawi R., Ellmerich S., de Toro M., Gardiner C., Seow Y., Reinares-Sebastian A., Forcen R., Simons J. P., Bellotti V., Cooper J. M., Alvarez-Erviti L., *Mol. Ther.*, **27**, 2111–2122 (2019).
160) Garbayo E., Montero-Menei C. N., Ansorena E., Lanciego J. L., Aymerich M. S., Blanco-Prieto M. J., *J. Control. Release*, **135**, 119–126 (2009).
161) Lin C. Y., Hsieh H. Y., Chen C. M., Wu S. R., Tsai C. H., Huang C. Y., Hua M. Y., Wei K. C., Yeh C. K., Liu H. L., *J. Control. Release*, **235**, 72–81 (2016).
162) Aly A. E., Harmon B. T., Padegimas L., Sesenoglu-Laird O., Cooper M. J., Waszczak B. L., *Mol. Neurobiol.*, **56**, 688–701 (2019).
163) Ishii T., Asai T., Oyama D., Fukuta T., Yasuda N., Shimizu K., Minamino T., Oku N., *J. Control. Release*, **160**, 81–87 (2012).
164) Fukuta T., Asai T., Yanagida Y., Namba M., Koide H., Shimizu K., Oku N., *FASEB J.*, **31**, 1879–1890 (2017).
165) Xu J., Wang X., Yin H., Cao X., Hu Q., Lv W., Xu Q., Gu Z., Xin H., *ACS Nano*, **13**, 8577–8588 (2019).
166) Fernandez-Susavila H., Bugallo-Casal A., Castillo J., Campos F., *Front. Neurol.*, **10**, 908 (2019).
167) Borlongan C. V., *Stem Cells Transl. Med.*, **8**, 983–988 (2019).
168) Nakaguchi K., Jinnou H., Kaneko N., Sawada M., Hikita T., Saitoh S., Tabata Y., Sawamoto K., *Stem Cells Int.*, **2012**, 915160 (2012).
169) Rodriguez-Frutos B., Otero-Ortega L., Ramos-Cejudo J., Martinez-Sanchez P., Barahona-Sanz I., Navarro-Hernanz T., Gomez-de Frutos Mdel C., Diez-Tejedor E., Gutierrez-Fernandez M.,

Biomaterials, **100**, 41–52 (2016).
170) Yang J., Zhang X., Chen X., Wang L., Yang G., *Mol. Ther. Nucleic Acids*, **7**, 278–287 (2017).
171) Lee H. J., Engelhardt B., Lesley J., Bickel U., Pardridge W. M., *J. Pharmacol. Exp. Ther.*, **292**, 1048–1052 (2000).
172) Negishi Y., Yamane M., Kurihara N., Endo-Takahashi Y., Sashida S., Takagi N., Suzuki R., Maruyama K., *Pharmaceutics*, **7**, 344–362 (2015).
173) Burgess A., Huang Y., Querbes W., Sah D. W., Hynynen K., *J. Control. Release*, **163**, 125–129 (2012).
174) Liu H. L., Hsu P. H., Lin C. Y., Huang C. W., Chai W. Y., Chu P. C., Huang C. Y., Chen P. Y., Yang L. Y., Kuo J. S., Wei K. C., *Radiology*, **281**, 99–108 (2016).
175) Park E. J., Zhang Y. Z., Vykhodtseva N., McDannold N., *J. Control. Release*, **163**, 277–284 (2012).
176) Janowicz P. W., Leinenga G., Gotz J., Nisbet R. M., *Sci. Rep.*, **9**, 9255 (2019).
177) Chen P. Y., Hsieh H. Y., Huang C. Y., Lin C. Y., Wei K. C., Liu H. L., *J. Transl. Med.*, **13**, 93 (2015).
178) Zhao G., Huang Q., Wang F., Zhang X., Hu J., Tan Y., Huang N., Wang Z., Wang Z., Cheng Y., *Cancer Lett.*, **418**, 147–158 (2018).
179) Shen Y., Guo J., Chen G., Chin C. T., Chen X., Chen J., Wang F., Chen S., Dan G., *Ultrasound Med. Biol.*, **42**, 1499–1511 (2016).
180) Chan T. G., Morse S. V., Copping M. J., Choi J. J., Vilar R., *ChemMedChem*, **13**, 1311–1314 (2018).
181) Li Y., Zheng X., Gong M., Zhang J., *Oncotarget*, **7**, 79401–79407 (2016).
182) Zhang C. X., Zhao W. Y., Liu L., Ju R. J., Mu L. M., Zhao Y., Zeng F., Xie H. J., Yan Y., Lu W. L., *Oncotarget*, **6**, 32681–32700 (2015).
183) Wei X., Gao J., Zhan C., Xie C., Chai Z., Ran D., Ying M., Zheng P., Lu W., *J. Control. Release*, **218**, 13–21 (2015).
184) Liu Y., Ran R., Chen J., Kuang Q., Tang J., Mei L., Zhang Q., Gao H., Zhang Z., He Q., *Biomaterials*, **35**, 4835–4847 (2014).
185) Gao H., Xiong Y., Zhang S., Yang Z., Cao S., Jiang X., *Mol. Pharm.*, **11**, 1042–1052 (2014).
186) Suga T., Watanabe M., Sugimoto Y., Masuda T., Kuroda N., Hagimori M., Kawakami S., *J. Drug Deliv. Sci. Technol.*, **49**, 668–673 (2019).
187) Petri B., Bootz A., Khalansky A., Hekmatara T., Müller R., Uhl R., Kreuter J., Gelperina S., *J. Control. Release*, **117**, 51–58 (2007).