

Enhanced delivery of IL-1 receptor antagonist to the central nervous system as a novel anti-transferrin receptor-IL-1RA fusion reverses neuropathic mechanical hypersensitivity

Carl I. Webster^{a,*}, Jon Hatcher^b, Matthew Burrell^a, George Thom^a, Peter Thornton^b, Ian Gurrell^b, Iain Chessell^b

Abstract

Neuropathic pain is a major unmet medical need, with only 30% to 35% of patients responding to the current standard of care. The discovery and development of novel therapeutics to address this unmet need have been hampered by poor target engagement, the selectivity of novel molecules, and limited access to the relevant compartments. Biological therapeutics, either monoclonal antibodies (mAbs) or peptides, offer a solution to the challenge of specificity as the intrinsic selectivity of these kinds of molecules is significantly higher than traditional medicinal chemistry-derived approaches. The interleukin-1 receptor system within the spinal cord has been implicated in the amplification of pain signals, and its central antagonism provides relief of neuropathic pain. Targeting the IL-1 system in the spinal cord with biological drugs, however, raises the even greater challenge of delivery to the central compartment. Targeting the transferrin receptor with monoclonal antibodies has proved successful in traversing the endothelial cell-derived blood-brain barrier and delivering proteins to the central nervous system. In this study, we describe a novel construct exemplifying an engineered solution to overcome these challenges. We have generated a novel anti-transferrin receptor-interleukin-1 receptor antagonist fusion that transports to the central nervous system and delivers efficacy in a model of nerve ligation-induced hypersensitivity. Approaches such as these provide promise for novel and selective analgesics that target the central compartment.

Keywords: Analgesic, Neuropathic pain, Interleukin-1, Blood-brain barrier, Antibody

1. Introduction

Drugs based on biological molecules, such as monoclonal antibodies, peptides, and other proteins have been dramatically successful in the treatment of many diseases. This success is often because of an increased intrinsic selectivity and potency inherent in this class of drugs. Conversely, attrition of novel small molecule entities during the development phase is often because of poor physicochemical properties, selectivity (safety), and pharmacokinetics.^{37,33} These findings are equally applicable to new drugs for the treatment of chronic neuropathic pain, but with one additional challenge: Many novel drug targets for the treatment of this disease are located within the central nervous system (CNS), and thus inside the blood-brain barrier (BBB), an endothelial cell layer which

is notoriously impermeable to biological drugs. Successful intervention in neuropathic pain using biological drugs therefore requires the development of new technologies which are able to transport biological payloads across the BBB.

Neuropathic pain, arising from damage or lesion of the somatosensory nervous system, has a complex pathophysiology and is difficult to treat. The unmet medical need is huge, with responder rates as low as 30% to 35% of patients.⁵ The discovery and development of novel analgesics have been unsuccessful, with no genuinely novel mechanisms launched for many decades. For targets located outside the CNS, biological interventions are showing promise,⁷ but targeting central pathways, which include second-order dorsal horn neurons, central neuroimmune interactions, or indeed manipulation of ascending and descending control, remains challenging. Bringing the selectivity of biological interventions to these targets may bring significant advantages, but the challenge of accessing the central compartment must first be addressed.

Interleukin (IL)-1 is a proinflammatory cytokine involved in both normal homeostasis and in pathological conditions.¹³ There is growing evidence for the role of IL-1-induced signalling in the development, maintenance, and propagation of pain. IL-1 expression is increased in conditions associated with pain and hyperalgesia in the periphery³¹ and the central compartment.^{1,2,11} When applied systemically, IL-1 is a potent hyperalgesic¹⁴ and if administered intrathecally (i.t.), IL-1 drives allodynia and hyperalgesia.³⁰ In the CNS, IL-1 induces COX-2 expression, contributing to central hyperexcitability,³² and increases synaptic activity in superficial spinal cord.¹⁷

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

^a Antibody Discovery and Protein Engineering, MedImmune, Cambridge, United Kingdom, ^b Neuroscience, Innovative Medicines and Early Development, AstraZeneca, Cambridge, United Kingdom

*Corresponding author. Address: Antibody Discovery and Protein Engineering, MedImmune, Milstein Building, Granta Park, Cambridge, CB21 6GH, United Kingdom. Tel.: +44 1223 471471. E-mail address: websterc@medimmune.com (C. I. Webster).

PAIN 158 (2017) 660–668

Copyright © 2016 The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the International Association for the Study of Pain. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

<http://dx.doi.org/10.1097/j.pain.0000000000000810>

We have exploited the naturally occurring proinflammatory cytokine inhibitor, interleukin-1 receptor antagonist (IL-1RA),⁶ coupled to an engineered BBB transport solution to demonstrate analgesia in a mouse model of neuropathic pain. We have found that the neuropathic pain induced in this model is sensitive to central, but not peripheral, antagonism of the IL-1 receptor system. Central penetration IL-1RA was achieved using the anti-mouse transferrin receptor (TfR) antibody 8D3¹⁸ engineered to further enhance central penetration. After subcutaneous (s.c.) administration, we have observed analgesia that is dependent on the dose of the IL-1RA fusion, and the affinity of the anti-TfR antibody for the TfR.

2. Materials and methods

2.1. Antibody and protein manipulation

2.1.1. Cloning, expression, and purification of 8D3 variants

DNA encoding the amino acid sequence of the V_H and V_L of the rat anti-mouse TfR antibody 8D3⁴ was assembled by polymerase extension of overlapping oligonucleotides and cloned into expression vectors containing the appropriate light or heavy chain constant regions.²⁷ Single alanine substitutions were introduced into the V_H and V_L complementarity-determining region 3 (CDR3) of 8D3 by site-directed mutagenesis. 8D3 variants were expressed as chimeric human IgG1 molecules with the S239D/A330L/I332E triple mutation (IgG1 TM).²⁵ Antibodies were expressed in transiently transfected Chinese hamster ovary cells in serum-free media as described previously.¹⁰ Cultures were maintained in a humidified incubator at 37°C, 5% CO₂ for 14 days after which the medium was harvested. Antibodies were purified from cell culture media using protein A affinity chromatography followed by size-exclusion chromatography. The concentration of IgG was determined by A₂₈₀ using an extinction coefficient based on the amino acid sequence of the IgG.²⁶

Plasmids enabling the expression of IL-1RA fused to the C-terminus of the IgG1 TM heavy chain through a (G₄S)₂ flexible linker were assembled by PCR amplification of the IL-1RA gene from complementary DNA obtained from Source Bioscience and subsequent PCR amplification with oligos that overlapped the *IL-1RA* gene and the *IgG1 TM CH3* domain and incorporated the linker. Expression and purification of IgG1 TM-IL-1RA fusions were performed as described above.

2.1.2. Screening of 8D3 variants for reduced binding to mouse transferrin receptor

8D3 variants were screened for reduced affinity to mouse TfR (mTfR) using a bio-layer interferometry assay performed on an Octet RED384 System (ForteBio). Assays were performed in kinetics buffer (PBS containing 1 mg/mL BSA and 0.01% Tween-20). Purified IgGs were immobilised onto antihuman Fc capture biosensors at 20 µg/mL after which association and dissociation of mTfR (Sino Biological) were monitored at concentrations of 0.031 to 2 µM. Data were fitted using a 1:1 binding model to determine kinetic constants. Because of the slow off-rate of the complex between wild-type 8D3 and mTfR, it was not possible to accurately determine kinetic parameters for this interaction using the above assay. In this case, affinity was determined by surface plasmon resonance using a Biacore T100 system. Wild-type 8D3 IgG1 TM was diluted to 20 µg/mL in 10 mM sodium acetate of pH 5.0 and immobilised onto a CM3 Sensor Chip using the Amine Coupling Kit according to the manufacturer's instructions. Binding analysis was performed in HBS-P buffer at 1.6 to 25

nM mTfR with 120 seconds contact time and 600 seconds dissociation time. The surface was regenerated using 10 mM glycine of pH 2.5. A 1:1 binding model was used to fit the data.

2.1.3. In vitro potency testing of anti-transferrin receptor-IL-1RA constructs in cell-based cytokine release assay

The in vitro activity of the antibody-IL-1RA fusions was tested in a cellular cytokine secretion assay. Mouse NIH-3T3 cells were seeded on PDL-coated Greiner tissue culture-treated plates in 50 µL Dulbecco modified Eagle medium containing 10% FCS and 1% pen-strep and incubated for 18 hours at 37°C with 5% CO₂. Anti-TfR-IL-1RA constructs were diluted in Dulbecco modified Eagle medium, applied to cells and incubated further for 30 minutes. NIH-3T3 cells were stimulated with recombinant mouse IL-1β (0.015 ng/mL, R&D Systems). After 18 hours, cell supernatants were collected and levels of secreted IL6 quantified using the MesoScale Discovery (MSD) kit according to the manufacturer's instructions. Levels of secreted IL6 were quantified by reference to standard curves generated using calibrator samples.

2.1.4. Peripheral kinetics and brain exposure

Noncompartmental (NCA) pharmacokinetic (PK) analysis was performed using Phoenix WinNonlin Professional [version 6.3; Pharsight (Certara), Sunnyvale, CA]. Nominal collection times were used for the PK data analyses, with below level of quantification values set to missing for calculation of the concentration means at nominal time points. The below level of quantification values were set as zero at predose, and missing after peak concentrations for the NCA.

The area under the concentration-time curve to the last measurable time point (AUC_{0-t}) was calculated for plasma and brain using the linear trapezoidal method as implemented in WinNonlin Phoenix. In addition, systemic clearance (CL), terminal volume of distribution (V_z), and terminal half-life (t_{1/2}) were determined for plasma. The C_{max} and T_{max} quoted are the observed values based on the mean concentration data at each time point.

All studies to measure antibody exposure in the periphery and brain were performed at Quotient Biosciences (Rushden, United Kingdom). Male C57B/6 mice, age 10 to 12 weeks were intravenously injected with anti-TfR variants or control IgG at 20 mg/kg or molar equivalent. Intravenous doses were administered into a tail vein at a constant dose volume of 10 mL/kg. Antibodies were supplied in D-PBS (Sigma). After dosing, 2 blood plasma samples were collected into individual Li-heparin containers from each of the 6 animals per time point per dose group. The first sample from each animal was collected from the lateral tail vein (ca 200 µL) into a Li-Hep microvette (BD Diagnostic Systems), whereas the second sample (ca 600 µL) was collected by cardiac puncture under isoflurane anaesthesia into a Li-Hep microtainer (BD Diagnostic Systems). After collection, blood samples were allowed to clot for 30 minutes and centrifuged at ×10,000 g for 2 minutes at 4°C and the resultant plasma drawn off. Plasma samples were flash frozen on dry ice for subsequent analysis. After final blood collection, the mice were perfused with D-PBS at a rate of 2 mL/min for 10 minutes until the extremities appeared white. Brains were excised and one hemisphere immediately processed, the other snap frozen in liquid nitrogen. To remove the spinal cord, the spinal column was exposed and severed at both the neck and at the base of the spine. To collect the spinal cord, D-PBS was rapidly forced through the section of spine through a needle and syringe. The excised spinal cord was dried with absorbent paper and samples weighed before homogenisation.

Spinal cord or brain hemisphere was homogenized in 5 volumes of ice-cold PBS containing 1% NP-40 and Complete protease inhibitor cocktail tablets (Roche Diagnostics). Homogenisation was performed in a 10 mL Potter-Elvehjem mortar type glass homogeniser with PTFE pestle, using 2×10 clockwise strokes with 5 seconds rest time. Homogenates were transferred to LoBind tubes (Eppendorf) and rotated at 4°C for 1 hour before centrifuging in a chilled benchtop centrifuge at 13,000 g for 20 minutes. The supernatant was isolated for brain antibody measurement.

2.1.5. Measurement of antibody concentrations in mouse brain, spinal cord, and plasma

Antibody concentrations in mouse plasma, brain, and spinal cord samples were measured through the MSD assay platform. The MSD method uses a plate-based sandwich immunoassay format where antihuman IgG capture antibody binds calibrator or samples, and a specific detection antibody labelled with SULFO-TAG emits light on electrochemical stimulation. Levels of anti-TfR variant and control antibody \pm IL-1RA fusions in plasma, brain, and spinal cord samples were quantified by reference to standard curves generated using calibrator samples with a four-parameter nonlinear regression model.

2.1.6. Partial nerve ligation

All studies were performed using adult female C57Bl/6J mice weighing 18 to 22 g (Charles River, United Kingdom). Female mice were chosen as we have found them to be more suitable for behavioural studies. In addition, they are easier to keep group housed for longer-term studies thus removing the need to isolate them due to fighting. Eight to ten mice were used for each study group. Separate animals were used in each study. Animals were housed in groups of 5 to 6 per cage, in individually ventilated cages with free access to food and water under a 12-hour light/dark cycle (lights on 07:00-19:00). A total of 110 mice were used in these studies. Housing and procedure rooms were maintained at $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a relative humidity of $65\% \pm 10\%$. All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by a local ethics committee. All mice underwent insertion of transponders under anaesthesia (3% isoflurane in oxygen) for identification purposes at least 5 days before the start of each study. Mechanical hyperalgesia was determined using an analgesiometer²⁹ (Ugo Basile, Italy). An increasing force was applied to the dorsal surface of each hind paw in turn until a withdrawal response was observed. The application of force was halted at this point and the weight in grams recorded. Data were expressed as withdrawal threshold in grams for ipsilateral and contralateral paws. After the establishment of baseline readings, mice were divided into 2 groups with approximately equal ipsilateral/contralateral ratios which underwent surgery to partially ligate the sciatic nerve or served as sham-operated controls based on the previously described method of Seltzer et al.³³ Operated mice were anaesthetised with isoflurane. After this, approximately 1 cm of the left sciatic nerve was exposed by blunt dissection through an incision at the level of the mid-thigh. A suture (9/0 Virgin Silk; Ethicon) was then passed through the dorsal third of the nerve and tied tightly. The incision was closed using Vetbond, and the mice were allowed to recover for at least 7 days before commencement of testing. Sham-operated mice underwent the same protocol but after exposure of the nerve, the mice were glued and allowed to recover. Mice were tested for

baseline responses on day 7 and 10 postsurgery. Operated mice showing ipsilateral/contralateral ratios of greater than 80% were classed as nonresponders and were removed from the study. The remaining mice were then randomly allocated into treatment groups of 8 to 10 mice per group with approximately equal ipsilateral/contralateral ratios after which mice were treated with the compound under test. For the IL-1R antagonist Kineret (the commercial form of IL-1RA) study, all mice were anaesthetised using 3% isoflurane in oxygen and were then dosed intrathecally with one of the following treatments: PBS vehicle (5 μL per mouse) or Kineret (10, 13, or 100 μg per mouse). Sham-operated animals received PBS vehicle (5 μL per mouse). To investigate the effects of the transferrin fusions, animals received PBS vehicle (10 mL/kg bodyweight s.c.) or the relevant antibody IL-1RA fusion protein (100 mg/kg s.c.). Sham-operated mice received PBS vehicle (10 mL/kg bodyweight s.c.). Mice were retested for changes in mechanical hyperalgesia 4 hours postdose as described above. Mice were also retested at 1, 2, 3, and 4 days postdose.

2.1.7. Data analysis

Statistical analysis was performed in GraphPad Prism. Only animals which completed the study were included in the analysis. Results were analysed using 2-way analysis of variance. Pairwise comparisons, where appropriate, were made using Tukey test.

3. Results

3.1. Sciatic nerve ligation results in mechanical hyperalgesia that can be reversed by central exposure to IL-1RA

We investigated the partial nerve ligation model of Seltzer et al.³³ for the development of mechanical hyperalgesia. In rats, this procedure results in the development of a neuropathic pain phenotype that can be measured through increased sensitivity to mechanical pressure at the ipsilateral paw. It has been previously shown that mice respond in a similar manner⁸ and it was preferential for these studies to be performed in mice as the antibody 8D3 does not bind to rat TfR. In all studies, C57BL/6 mice subjected to partial ligation of the sciatic nerve exhibited mechanical hyperalgesia that manifested as a significant reduction in the ipsilateral but not contralateral sensitivity to mechanical force on day 7 and 10 postsurgery when compared with sham-operated controls. Having demonstrated a robust reproducible response in mice, we investigated the sensitivity of the model to the administration of drug molecules. When vehicle, PBS, was administered i.t., neither operated mice nor sham-operated mice showed any change in the level of mechanical hyperalgesia from predose levels. This indicated that animal handling and drug administration procedures did not alter response.

To test whether the mechanical hyperalgesia induced by partial ligation of the sciatic nerve was dependant on IL-1R signalling, we investigated the effect of peripheral and i.t. delivery of the IL-1R antagonist Kineret (the commercial form of IL-1RA). The administration of Kineret peripherally at a dose of 100 mg/kg demonstrated no reversal of the mechanical hyperalgesia. However, i.t. delivery of Kineret showed a dose-related reversal that peaked at 2 to 4 hours postinjection and then diminished over time, returning to baseline levels 2 days postdose. A dose of 100 μg of Kineret i.t. returned the ipsilateral/contralateral ratio from around 55 to approximately 80 at 2 hours postdose. This was maintained at 4 hours but had reduced to around 70 at 24 hours. A dose of 30 μg i.t. achieved

a ratio of around 78 at 2 and 4 hours before declining to around 58 at 24 hours. A dose of 10 μg did not result in any significant analgesia at any time point (Fig. 1). This demonstrates that in this model, only central blockade of IL-1R signalling can result in dose-dependent analgesia and that a peripheral dose approximately 80-fold higher than the central dose exhibits no change in the response.

3.2. Identification and characterisation of a blood–brain barrier transmutating anti–transferrin receptor antibody

To investigate whether IL-1RA could be delivered across the BBB in mice, the anti-TfR antibody 8D3 was chosen for its ability to enter the CNS.^{19–21,34,41,42} Despite previous evidence, we were unable to detect significant amounts of 8D3 within the brains of treated animals. It has been reported that the affinity of antitransferrin antibodies for TfR is important for their ability to cross the BBB.³⁹ We therefore generated 8D3 variants with reduced affinity for TfR. Variants with reduced affinity were identified through alanine scanning mutagenesis of either the light or heavy chain CDR3s. Only modest reductions in affinity were achieved by this method, but pairing of the heavy and light chain alanine variants resulted in substantial further reductions in affinity relative to that of the parent antibodies, with K_D values of up to 610 nM being observed. Three mutants which had K_D values of 56, 130, and 610 nM, respectively, were selected for testing for brain exposure and ability to elicit a centrally mediated effect when coupled to IL-1RA. These mutants are referred to herein as 8D3₅₆, 8D3₁₃₀, and 8D3₆₁₀ on the basis of their respective affinities (Table 1).

3.3. Pharmacokinetic properties of anti–transferrin receptor antibodies

3.3.1. Peripheral pharmacokinetics

To confirm that variants of the anti-TfR antibody 8D3 had altered brain exposure dependent on their affinity for TfR, we conducted a series of PK and brain exposure studies in C57BL/6J mice. Studies were performed over a 2-week period with blood and brain homogenate samples taken at regular time points. The sampling procedure in these studies resulted in composite profiles for plasma exposure after a single intravenous dosing at 20 mg/kg, and all parameters were derived using the mean of each data point. A control group received the same dosage of an antibody to an irrelevant antigen of the same isotype as the anti-TfR antibodies (NIP228). Figure 2A shows the mean plasma data \pm SE for the 5 antibodies profiled.

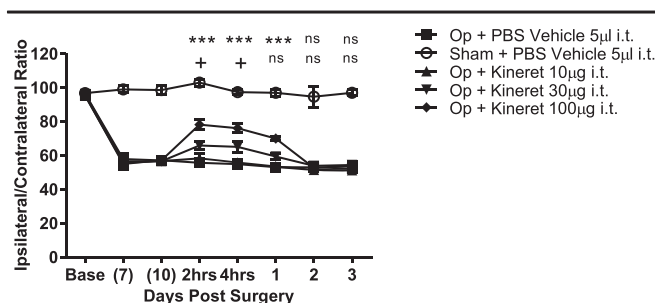


Figure 1. Effect of i.t. administered Kineret on the reversal of partial nerve ligation-induced mechanical hyperalgesia. Data are mean % ipsilateral/contralateral ratio \pm SEM. N = 9 to 10 per group. +P < 0.05 Kineret (30 μg per mouse) vs PBS vehicle control; ***P < 0.001 Kineret (10 μg per mouse) vs PBS vehicle control. i.t., intrathecal; ns, not significant.

Table 1

Kinetic parameters for binding of wild-type 8D3 and variants to mTfR.

	$k_{on}, \text{M}^{-1} \text{s}^{-1}$	k_{off}, s^{-1}	K_D, nM
^a Wild-type 8D3	8.8×10^4	1.1×10^{-4}	1.2
^b 8D3 ₅₆	7.9×10^3	5.3×10^{-4}	56
^b 8D3 ₁₃₀	4.7×10^3	6.3×10^{-4}	130
^b 8D3 ₆₁₀	1.4×10^3	7.9×10^{-4}	610

mTfR, mouse transferrin receptor.

The pharmacokinetic parameters for the lower affinity variants of the anti-TfR antibody 8D3 (8D3₅₆, 8D3₁₃₀, and 8D3₆₁₀) were broadly similar, and within 2-fold of the isotype control antibody (Table 2). Their half-lives ranged from 4.3 to 6.5 days, differences that are driven by differences in $V\beta$ and the rate of CL. However, in comparison, 8D3 had considerably altered systemic pharmacokinetic parameters that were driven by an approximately 4–6-fold higher CL than the lower affinity 8D3 variants or isotype control antibody.

3.3.2. Brain and spinal cord exposure of anti–transferrin receptor antibodies

Measurement of each of the antibodies in homogenate of brain and spinal cord was performed to determine the central exposure. Brain and spinal cord samples were taken at 4, 24, 48, 96, 168, and 336 hours after intravenous administration and processed to homogenate for analysis through the MSD assay (Fig. 2B, C). For all 8D3 anti-TfR antibodies, T_{max} occurred at 24 hours after administration (Table 3). The isotype control antibody reached T_{max} much later at 4 days after administration. Brain C_{max} and $AUC_{(0-t)}$ values observed for the anti-TfR antibodies indicated that there was a relationship between affinity and brain exposure similar to data that were previously reported by Yu et al.³⁹ and Couch et al.⁹ Lower affinity variants of 8D3 (8D3₅₆, 8D3₁₃₀, and 8D3₆₁₀) demonstrated significantly more brain and spinal cord exposure than 8D3 (Fig. 2B, C and Table 3). Pharmacokinetic parameter analysis indicated that 8D3 possessed the poorest brain exposure, as described by the mean C_{max} . This parameter was 18.7 times less than the best brain exposure of lower affinity variant—8D3₁₃₀. In addition, $AUC_{(0-t)}$ for 8D3 was 44 times less than that achieved for 8D3₁₃₀ (Table 3). Notably, 8D3 could only be detected in brain and spinal cord samples for the first 96 hours of the study (Fig. 2B, C). Assay readings for later time points were below the lower limit of quantification for the assay. All PK parameters were derived using mean data (Table 3).

3.4. Pharmacokinetics for anti–transferrin receptor hlgG1-IL-1RA fusion proteins

3.4.1. Peripheral pharmacokinetics

Peripheral PK analysis was performed on the antibody IL-1RA fusions over a 2-week period. The IL-1RA antibody fusions demonstrated significantly reduced plasma exposure, with a 1.8 and 2.7 times higher CL than the lower affinity anti-TfR antibodies (8D3₅₆, 8D3₁₃₀, and 8D3₆₁₀) (Fig. 3A and Table 4). There was also a 3.6 to 11.4 fold reduction in plasma exposure, measured by $AUC_{(0-t)}$, for all IL-1RA antibody fusions tested, when compared with anti-TfR antibodies without IL-1RA (Tables 2 and 4). This suggests that IL-1RA also played a significant role

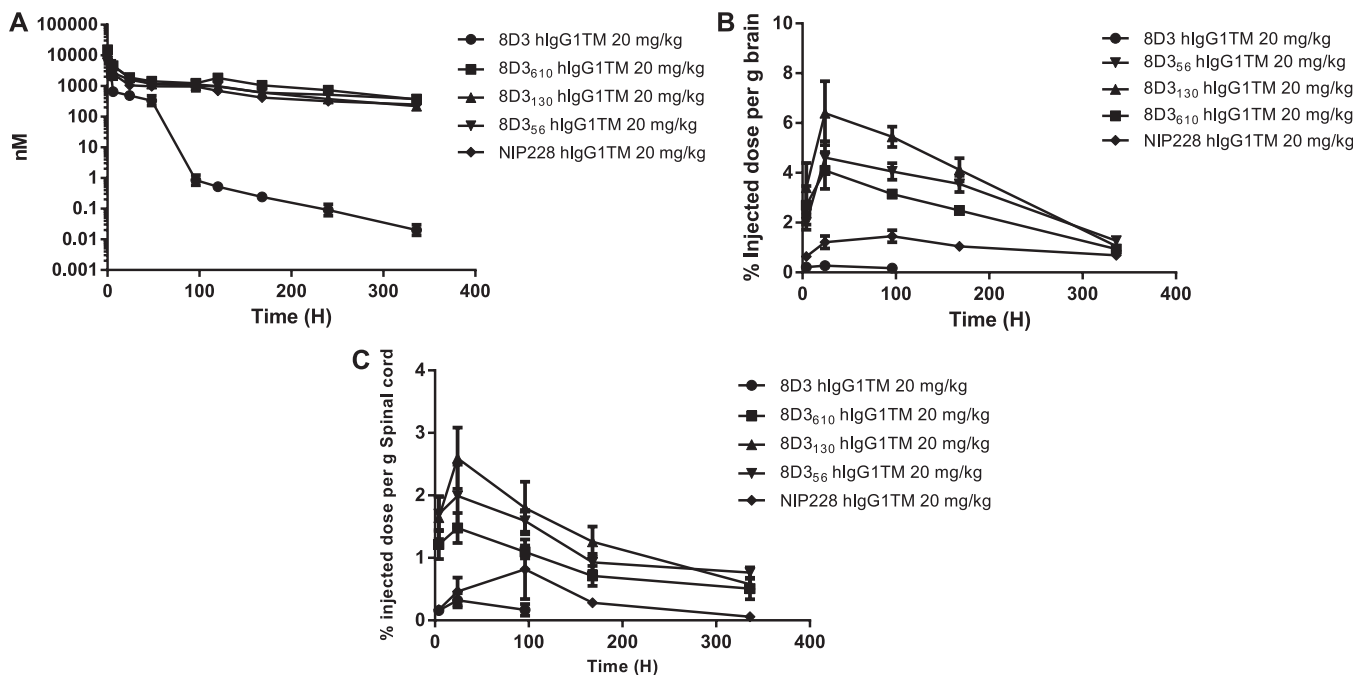


Figure 2. Plasma and brain exposure of anti-transferrin variants in a mouse PK assay. (A) Plasma PK of anti-TfR variants and isotype control (NIP228) over a 2-week period. N = 6 per group (B) Brain exposure as a measure of % injected dose per gram of brain. (C) Spinal cord exposure as a measure of % injected dose per gram of spinal cord. PK, pharmacokinetic; TfR, transferrin receptor.

in plasma CL for these proteins. C_{max} for all fusion proteins occurred at the first measured time point, 10 minutes after administration.

3.4.2. Brain and spinal cord exposure

Brain and spinal cord exposure was measured for the antibodies fused to IL-1RA. T_{max} for each of the antibody-IL-1RA fusions tested was at the first measured time point (4 hours), which is somewhat earlier than that observed for the antibodies alone at 24 hours (Fig. 3B, C and Table 5). 8D3 was only detectable in the brain and spinal cord for the first 24 hours of the study, after which it was below the lower limit of quantification for the assay (Fig. 3B, C). Brain exposure as measured by $AUC_{(0-t)}$ was between 22.6- and 69-fold higher for the lower affinity 8D3 variants compared with 8D3 (Table 5).

The lower central exposure of 8D3-IL-1RA was likely due to faster peripheral CL, but this fusion protein also had 12- to 25-fold lower brain:plasma $AUC_{(0-t)}$ ratio than the lower affinity 8D3 variant antibody IL-1RA fusions (Table 5).

Table 2
Plasma PK parameters of anti-TfR variants and isotype control in hlgG1TM format.

Variant	C_{max} , $\mu\text{g/mL}$	$AUC_{(0-t)}$, $\text{d}\cdot\mu\text{g/mL}$	CL, mL/h/kg	Vss, mL/kg	Vz, mL/kg	Terminal half-life, h
8D3	313	11,606	1.7	20	110	44
8D3 ₆₁₀	2301	64,968	0.3	45	53	141
8D3 ₁₃₀	1966	48,310	0.4	47	56	103
8D3 ₅₆	838	45,129	0.3	73	78	156
NIP228	1239	35,022	0.5	82	92	134

AUC_{0-t} , area under the curve to last measurable time point; CL, clearance; C_{max} , maximum observed concentration; PK, pharmacokinetic; TfR, transferrin receptor; Vss, steady state volume of distribution; Vz, terminal phase volume of distribution.

3.5. In vitro activity of antibody-IL-1RA fusions

To test whether the fusion of IL-1RA to the antibodies resulted in any change in its ability to inhibit the action of IL-1, we measured potency of these fusion proteins in an IL-1 β -driven in vitro-cell-based assay. Pretreatment of NIH-3T3 cells for 30 minutes with IL-1RA-anti-TfR fusions potently inhibited IL-1 β -induced IL6 secretion (Fig. 4). 8D3-IL-1RA displayed a 3-fold increase in potency when compared with IL-1RA alone (Fig. 4). 8D3₆₁₀-IL-1RA, 8D3₁₃₀-IL-1RA, 8D3₅₆-IL-1RA, and the isotype control NIP228-IL-1RA displayed an ~10-fold reduction in potency compared with IL-1RA alone (Fig. 4). NIP228 isotype control with no IL-1RA fusion protein had no effect on IL-1 β activity.

3.6. Reversal of mechanical hyperalgesia by blood-brain barrier transmutating anti-TfR-IL-1RA fusions

We next tested whether fusions of IL-1RA to the anti-TfR antibodies were able to reverse the mechanical hyperalgesia. Antibodies were administered s.c. at doses of 100 mg/kg, and

Table 3
Brain PK parameters for anti-TfR variants and isotype control in hlgG1TM format.

Variant	T_{max} , h	C_{max} , ng/mg	$AUC_{(0-t)}$, $\text{h}\cdot\mu\text{g/g tissue}$	Brain:plasma ratio (AUC ratios)
8D3	24	1.2	95	0.0008
8D3 ₆₁₀	24	11.4	2827	0.044
8D3 ₁₃₀	24	22.4	4182	0.087
8D3 ₅₆	24	14.4	3250	0.072
NIP228	96	6.3	1582	0.045

AUC_{0-t} , area under the curve to last measurable time point; PK, pharmacokinetic; C_{max} , maximum observed concentration; T_{max} , time at which maximum concentration is observed; TfR, transferrin receptor.

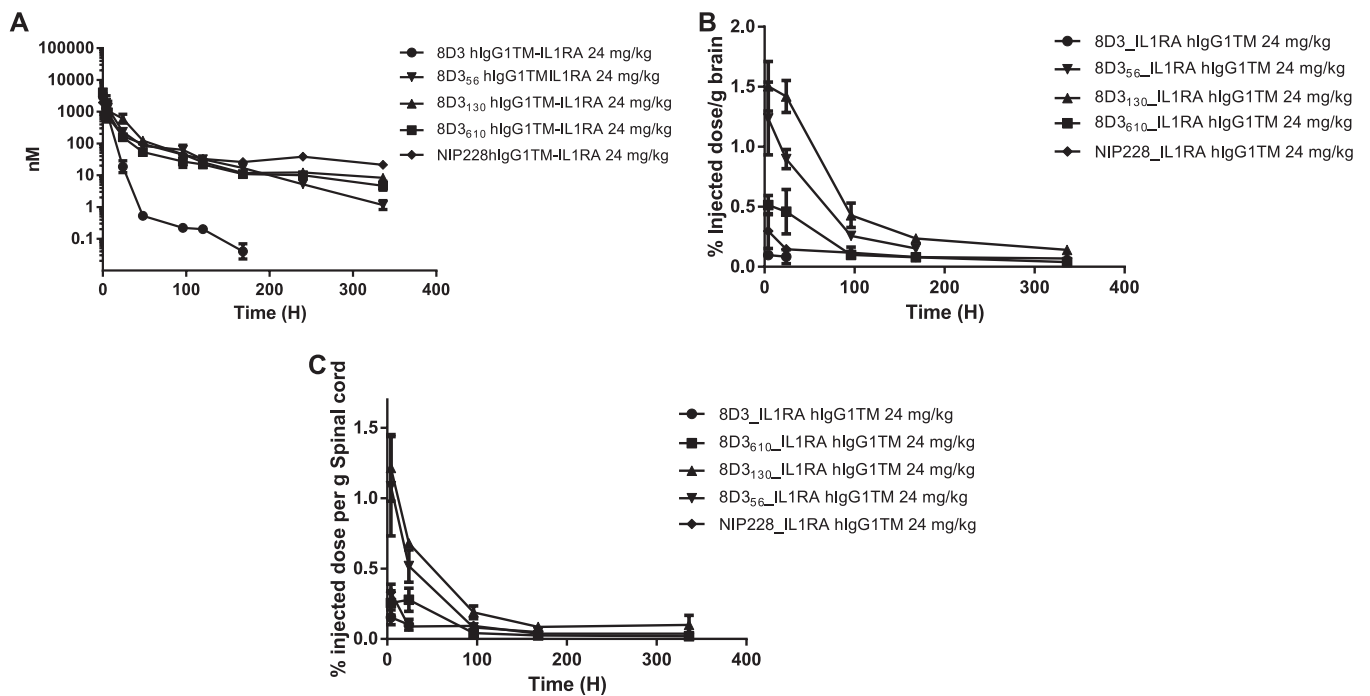


Figure 3. Plasma, brain, and spinal cord exposure of anti-transferrin variant-IL-1RA fusion in mouse PK assay. (A) Plasma PK of anti-TfR-IL-1RA variants and isotype control (NIP228) over a 2 week period. N = 6 per group (B) Brain exposure as a measure of % injected dose per gram of brain. (C) Spinal cord exposure as a measure of % injected dose per gram of spinal cord. PK, pharmacokinetic; TfR, transferrin receptor.

the mice monitored for 7 days postdose. Treatment with vehicle, PBS, or NIP228-IL-1RA had no effect on mechanical hypersensitivity in mice indicating that NIP228-IL-1RA was not able to access IL-1R in the central compartment. Administration of the 8D3-IL-1RA fusion also showed no reversal of the mechanical hyperalgesia throughout the study. However, reversal of the mechanical hyperalgesia was seen with the reduced affinity variants of 8D3 fused to IL-1RA. 8D3₆₁₀-IL-1RA showed a significant reversal at all time points from 4 hours to 7 days postdose. The response was constant through the first 4 days and only started to decline on day 7. 8D3₁₃₀-IL-1RA produced a significant reversal from 4 hours to 4 days postdose but no significance was seen at 7 days postdose. The magnitude of response was greater than that seen for 8D3₆₁₀-IL-1RA during the first 4 days but the loss of effect at day 7 resulted in a greater response from 8D3₆₁₀-IL-1RA at this time point. (Fig. 5).

To investigate the relationship between dose and the reversal of mechanical hyperalgesia, we administered 8D3₁₃₀-IL-1RA at

doses of 20, 50, and 100 mg/kg. The different dose levels showed differences in both the magnitude and duration of the response. The 20 mg/kg dose showed significant reversal of mechanical hyperalgesia for only 24 hours postdose, the 50 mg/kg for up to 4 days postdose, and the 100 mg/kg for up to 7 days postdose (Fig. 6).

4. Discussion

The expression of IL-1β by glia in the spinal cord and its direct action on spinal neurons potentiates nociceptive signalling through IL-1-receptor-dependent signalling and *N*-methyl-*D*-aspartate receptor phosphorylation. Furthermore, central administration of IL-1RA reduces inflammatory hyperalgesia by blocking this central mechanism of nociceptive processing.⁴⁰ In rodent models of neuropathic pain including chronic constriction injury and spared nerve injury, central IL-1β levels correlate with nociceptive responses¹² and blockade of IL-1 signalling with

Table 4
Plasma PK parameters for anti-TfR variants and isotype control hlgG1-IL-1RA fusion proteins.

Variant	C _{max} , μg/mL	AUC _(0-t) , h·μg/mL	CL, mL/h/kg	V _{ss} , mL/kg	V _z , mL/kg	Terminal half-life, h
8D3-IL-1RA	531	3212	7.5	31	526	49
8D3 ₆₁₀ -IL-1RA	736	5700	4.1	141	522	87
8D3 ₁₃₀ -IL-1RA	811	8371	2.8	139	629	158
8D3 ₅₆ -IL-1RA	515	6778	3.5	98	218	43
NIP228-IL-1RA	351	5204	3.9	593	1003	180

AUC_(0-t), area under the curve to last measurable time point; CL, clearance; C_{max}, maximum observed concentration; PK, pharmacokinetic; TfR, transferrin receptor; V_{ss}, steady state volume of distribution; V_z, terminal phase volume of distribution.

Table 5
Brain PK parameters for anti-TfR variants and isotype control hlgG1-IL-1RA fusion proteins.

Variant	T _{max} , h	C _{max} , ng/mg	AUC _(0-t) , h·μg/g tissue	Brain:plasma ratio (AUC ratios)
8D3-IL-1RA	4.0	0.4	9.0	0.003
8D3 ₆₁₀ -IL-1RA	4.0	2.3	204	0.036
8D3 ₁₃₀ -IL-1RA	4.0	5.2	626	0.075
8D3 ₅₆ -IL-1RA	4.0	5.7	364	0.054
NIP228-IL-1RA	4.0	1.7	163	0.031

AUC_(0-t), area under the curve to last measurable time point; PK, pharmacokinetic; C_{max}, maximum observed concentration; T_{max}, time at which maximum concentration is observed; TfR, transferrin receptor.

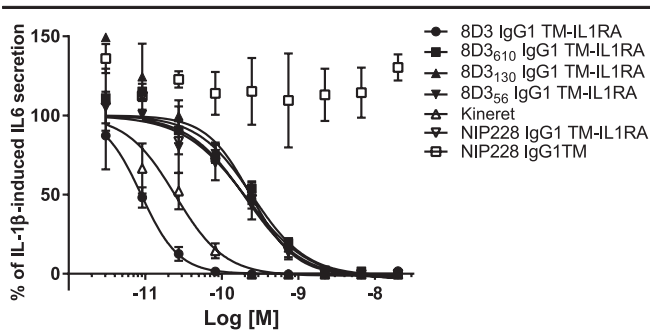


Figure 4. Effect of IL-1RA-anti-TfR fusions on IL-1 β -induced IL6 secretion from NIH-3T3 cells in culture. Data are expressed as % IL-1 β -induced IL6 mean \pm SEM. Cells were pretreated with IL-1RA-anti-TfR fusions for 30 minutes before IL-1 β stimulation. TfR, transferrin receptor.

IL-1RA provides analgesia in part due to a reduction in ectopic neuronal discharge.¹⁶

Our results have confirmed that the symptoms of neuropathic pain can be relieved through central, but not peripheral, blockade of IL-1R signalling. Central IL-1R blockade was achieved by direct i.t. IL-1RA delivery or through peripheral delivery of an IL-1RA-anti-TfR fusion protein. However, such responses do not necessarily indicate the site of action as behind the BBB in the brain or spinal cord. Centrally delivered antibodies and other vectors may access the dorsal root ganglion (DRG) through the cerebrospinal fluid, which is known to reach the DRG.^{28,36} Furthermore, some of the changes that may contribute to neuropathic pain occur in the DRG including an increase in the expression of the IL-1 β and IL-1R.^{22,35} We cannot rule out the possibility that peripherally administered IL-1RA-anti-TfR antibodies penetrate the DRG secondary to crossing the BBB into the spinal cord. However, our data indicate that CNS delivery of the IL-1R1 antagonist is a fundamental requirement in driving efficacy in this neuropathic pain model.

Antibodies have become an important class of drug molecules over recent years, demonstrating strong clinical efficacy in a variety of indications.³ However, there are few antibodies entering clinical development with a centrally driven mechanism of action. Development of effective antibody-based treatments for CNS disorders will require the codevelopment of technologies that enhance their BBB penetration. In vitro models of the BBB have proven useful for the measurement of small molecule transport and can help drive in vivo exposure prediction.³⁸ However, such in vitro studies are far less common for antibodies

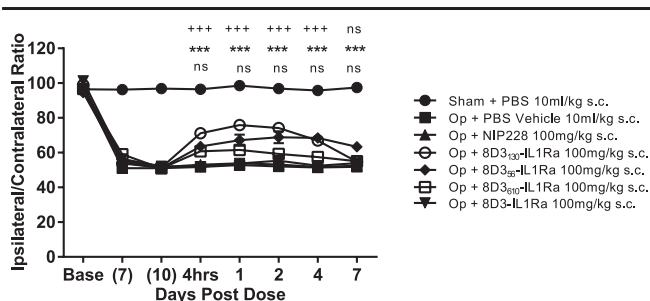


Figure 5. Effect of anti-transferrin-IL-1RA fusions on the reversal of partial nerve ligation induced mechanical hyperalgesia. N = 8 to 9 per group. ** P < 0.01; *** P < 0.001 Op + NIP228 vs Op + 8D3₆₁₀-IL-1RA; +++ P < 0.001 Op + NIP228 vs Op + 8D3₁₃₀-IL-1RA; ns, not significant (P > 0.5). s.c, subcutaneous.

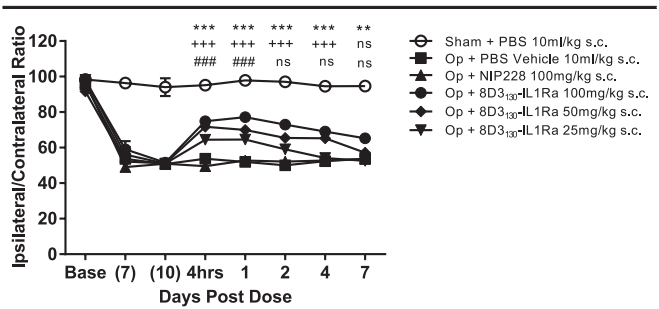


Figure 6. Dose response of 8D3₁₃₀-IL-1RA fusions on the reversal of partial nerve ligation induced mechanical hyperalgesia. N = 8 to 9 per group. ** P < 0.01; *** P < 0.001 Op + NIP228 vs Op + 8D3₁₃₀-IL-1RA (100 mg/kg); +++ P < 0.001 Op + NIP228 vs Op + 8D3₁₃₀-IL-1RA (50 mg/kg); ### P < 0.001 Op + NIP228 vs Op + 8D3₁₃₀-IL-1RA (20 mg/kg); ns not significant (P > 0.5). s.c, subcutaneous.

with the majority of evidence supporting antibody-BBB delivery coming from in vivo studies.

IL-1RA is amenable to genetic manipulation and we have found it can be successfully fused at the C-terminus of the Fc domain of an IgG and maintaining sufficient activity for further study (Fig. 4). Such fusions do not influence the activity of the Fab domain of the antibody allowing molecules with differing antigen specificities or affinities to be generated all having near identical specific activity at IL-1R, with the exception of the fusion to 8D3. The high affinity antibody 8D3 when fused to IL-1RA had a higher in vitro IL-1 neutralising activity. We attribute this to be due to the high affinity anti-TfR antibody creating a higher local concentration of the fusion protein at the cell surface, thereby generating an apparently higher neutralising capability. As this fusion protein had undetectable activity in vivo, in contrast to its in vitro behaviour, we do not believe this anomalous behaviour had any bearing on the conclusion that a lower affinity for TfR enhances brain penetration. Thus the magnitude of any pharmacological response resulting from IL-1R blockade in vivo is dependent only on changes resulting from alteration in the antibody affinity for TfR (and not on any modulation of the IL-1RA).

Blood-brain barrier transport of antibodies through the TfR has been previously described.^{19,23,24,39} Transport of anti-TfR antibodies into the CNS is dependent on their affinity for TfR, with lower affinity antibodies demonstrating greater BBB transport.³⁹ The variants of the antimouse transferrin receptor antibody 8D3 described in this article also have lowered affinities for TfR and demonstrate markedly differing abilities to cross the BBB (Fig. 2). The magnitude of brain exposure is similar to the findings of Yu et al.³⁹ However, the temporal pattern is markedly different. Yu et al observed peak brain exposure at 4 to 5 hours after administration, whereas we see peak exposure 24 hours after administration. There are a number of possible reasons for these differences: Yu et al do not report the absolute affinities of their anti-TfR antibodies but only the differences relative to each other. We are not therefore able to say whether or not we are working in the same affinity range. The anti-TfR described by Yu et al³⁹ may also have a distinct epitope to the 8D3 antibody we describe which could drive differing transport properties.

On fusion of IL-1RA to the antibodies, a slightly reduced peripheral half-life was noted, whereas the CNS exposure profile was markedly different (Fig. 3B, C). Peak brain exposure was observed at the first sample postinjection and decayed rapidly before plateauing at around 4 days. This suggests that IL-1RA affects the distribution of these antibodies and this effect is mostly likely due to binding of the IL-1RA component of the fusion to

IL-1R in the peripheral and central compartments. The more marked change in the exposure profile in the brain may be due to the presence of IL-1R on endothelial cells of the BBB. The presence of both IL-1R and TfR on these cells may result in an avid interaction of the IL-1RA-anti-TfR fusions with the receptors on these cells and alter their ability to cross the BBB. An increase in avidity would increase the apparent affinity of these fusions for the endothelial cells and, as it has been demonstrated that affinity plays an important role in BBB transport, the result would likely be reduced transport across these cells. The more rapid CL from the blood would also reduce the further uptake of antibody across the BBB.

In agreement with previous studies,^{15,40} our data suggest a critical role for central IL-1 β in nociceptive signalling during chronic pain states. Greater IL-1RA penetration of the CNS results in greater analgesia and prolonged exposure in the central compartment results in a longer duration of the analgesia induced by the IL-1RA fusion. Using a disease-relevant model of chronic pain, we have successfully shown for the first time that a biological peptide antagonist can be re-engineered, facilitating its CNS delivery and enhancing its analgesic properties.

Conflict of interest statement

All authors were employees of MedImmune at the time of the work and therefore have a theoretical conflict of interest through being employed by the organisation that both funded the work and has a potential commercial interest in the findings.

Acknowledgements

The authors thank all the staff of MedImmune's Biological Services unit for their invaluable help and technical assistance in performing the mechanical hyperalgesia studies. All work described in this manuscript was funded by MedImmune.

Article history:

Received 12 September 2016

Received in revised form 21 November 2016

Accepted 12 December 2016

Available online 20 December 2016

References

- Alexander GM, van Rijn MA, van Hilten JJ, Perreault MJ, Schwartzman RJ. Changes in cerebrospinal fluid levels of pro-inflammatory cytokines in CRPS. *PAIN* 2005;116:213–19.
- Apkarian AV, Lavarello S, Randolph A, Berra HH, Chialvo DR, Besedovsky HO, del Rey A. Expression of IL-1beta in supraspinal brain regions in rats with neuropathic pain. *Neurosci Lett* 2006;407:176–81.
- Beck A, Wurch T, Bailly C, Corvaia N. Strategies and challenges for the next generation of therapeutic antibodies. *Nat Rev Immunol* 2010;10:345–52.
- Boado RJ, Zhang Y, Wang Y, Pardridge WM. Engineering and expression of a chimeric transferrin receptor monoclonal antibody for blood-brain barrier delivery in the mouse. *Biotech Bioeng* 2009;102:1251–8.
- Burgess G, Williams D. The discovery and development of analgesics: new mechanisms, new modalities. *J Clin Invest* 2010;120:3753–9.
- Carter DB, Deibel MRJ, Dunn CJ, Tomich CS, Laborde AL, Slightom JL, Berger AE, Bienkowski MJ, Sun FF, McEwan RN. Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. *Nature* 1990;344:633–8.
- Chessell IP, Dudley A, Billinton A. Biologics: the next generation of analgesic drugs? *Drug Discov Today* 2012;17:875–9.
- Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CBA, Casula MA, Yangou Y, Birch R, Anand P, Buell GN. Disruption of the P2X7 purinoreceptor gene abolishes chronic inflammatory and neuropathic pain. *PAIN* 2005;114:386–96.
- Couch JA, Yu YJ, Zhang Y, Tarrant JM, Fuji RN, Meilandt WJ, Solanoy H, Tong RK, Hoyte K, Luk W, Lu Y, Gadkar K, Prabhu S, Ordonia BA, Nguyen Q, Lin Y, Lin Z, Balazs M, Scearce-Levie K, Ernst JA, Dennis MS, Watts RJ. Addressing safety liabilities of TfR bispecific antibodies that cross the blood-brain barrier. *Sci Transl Med* 2013;5:183ra57.
- Daramola O, Stevenson J, Dean G, Hatton D, Pettman G, Holmes W, Field R. A high-yielding CHO transient system: coexpression of genes encoding EBNA-1 and GS enhances transient protein expression. *Biotechnol Prog* 2014;30:132–41.
- del Rey A, Apkarian AV, Martina M, Besedovsky HO. Chronic neuropathic pain-like behavior and brain-borne IL-1beta. *Ann N Y Acad Sci* 2012;1262:101–7.
- del Rey A, Yau H, Randolph A, Centeno MV, Wildmann J, Martina M, Besedovsky HO, Apkarian AV. Chronic neuropathic pain-like behavior correlates with IL-1beta expression and disrupts cytokine interactions in the hippocampus. *PAIN* 2011;152:2827–35.
- Dinarello CA. Biologic basis for interleukin-1 in disease. *Blood* 1996;87:2095–147.
- Ferreira SH, Lorenzetti BB, Bristow AF, Poole S. Interleukin-1 beta as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature* 1988;334:698–700.
- Florentino PM, Tallents RH, Miller JH, Brouxhon SM, O'Banion MK, Puzas JE, Kyrkanides S. Spinal interleukin-1beta in a mouse model of arthritis and joint pain. *Arthritis Rheum* 2008;58:3100–9.
- Gabay E, Wolf G, Shavit Y, Yirmiya R, Tal M. Chronic blockade of interleukin-1 (IL-1) prevents and attenuates neuropathic pain behavior and spontaneous ectopic neuronal activity following nerve injury. *Eur J Pain* 2011;15:242–8.
- Kawasaki Y, Zhang L, Cheng J, Ji R. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci* 2008;28:5189–94.
- Kissel K, Hamm S, Schulz M, Vecchi A, Garlanda C, Engelhardt B. Immunohistochemical localization of the murine transferrin receptor (TfR) on blood-tissue barriers using a novel anti-TfR monoclonal antibody. *Histochem Cell Biol* 1998;110:63–72.
- Lee HJ, Engelhardt B, Lesley J, Bickel U, Pardridge WM. Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-brain barrier in mouse. *J Pharmacol Exp Ther* 2000;292:1048–52.
- Lee HJ, Boado RJ, Braasch DA, Corey DR, Pardridge WM. Imaging gene expression in the brain in vivo in a transgenic mouse model of Huntington's disease with an antisense radiopharmaceutical and drug-targeting technology. *J Nucl Med* 2002;43:948–56.
- Lee HJ, Zhang Y, Zhu C, Duff K, Pardridge WM. Imaging brain amyloid of Alzheimer disease in vivo in transgenic mice with an abeta peptide radiopharmaceutical. *J Cereb Blood Flow Metab* 2002;22:223–31.
- Lee H, Lee K, Son S, Hwang S, Cho H. Temporal expression of cytokines and their receptors mRNAs in a neuropathic pain model. *Neuroreport* 2004;15:2807–11.
- Moos T, Morgan EH. Evidence for low molecular weight, non-transferrin-bound iron in rat brain and cerebrospinal fluid. *J Neurosci Res* 1998;54:486–94.
- Niewoehner J, Bohrmann B, Collin L, Urich E, Sade H, Maier P, Rueger P, Stracke J, Lau W, Tissot A, Loetscher H, Ghosh A, Freskgard PO. Increased brain penetration and potency of a therapeutic antibody using a monovalent molecular shuttle. *Neuron* 2014;81:49–60.
- Oganesyan V, Gao C, Shirinian L, Wu H, Dall'Acqua WF. Structural characterization of a human Fc fragment engineered for lack of effector functions. *Acta Crystallogr D Biol Crystallogr* 2008;64:700–4.
- Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 1995;4:2411–23.
- Persic L, Roberts A, Wilton J, Cattaneo A, Bradbury A, Hoogenboom HR. An integrated vector system for the eukaryotic expression of antibodies or their fragments after selection from phage display libraries. *Gene* 1997;187:9–18.
- Petersson LME, Geremia NM, Ying Z, Verge VMK. Injury-associated PACAP expression in rat sensory and motor neurons is induced by endogenous BDNF. *PLoS One* 2014;9:e100730.
- Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther* 1957;111:409–19.
- Reeve AJ, Patel S, Fox A, Walker K, Urban L. Intrathecally administered endotoxin or cytokines produce allodynia, hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli in the rat. *Eur J Pain* 2000;4:247–57.

- [31] Ren K, Torres R. Role of interleukin-1beta during pain and inflammation. *Brain Res Rev* 2009;60:57–64.
- [32] Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ. Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 2001;410:471–5.
- [33] Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *PAIN* 1990;43:205–18.
- [34] Shi N, Zhang Y, Zhu C, Boado RJ, Pardridge WM. Brain-specific expression of an exogenous gene after i.v. administration. *Proc Natl Acad Sci U S A* 2001;98:12754–9.
- [35] Uceyler N, Tschärke A, Sommer C. Early cytokine expression in mouse sciatic nerve after chronic constriction nerve injury depends on calcitonin. *Brain Behav Immun* 2007;21:553–60.
- [36] Wang X, Wang C, Zeng J, Xu X, Hwang PYK, Yee W, Ng Y, Wang S. Gene transfer to dorsal root ganglia by intrathecal injection: effects on regeneration of peripheral nerves. *Mol Ther* 2005;12:314–20.
- [37] Waring MJ, Arrowsmith J, Leach AR, Leeson PD, Mandrell S, Owen RM, Pairaudeau G, Pennie WD, Pickett SD, Wang J, Wallace O, Weir A. An analysis of the attrition of drug candidates from four major pharmaceutical companies. *Nat Rev Drug Discov* 2015;14:475–86.
- [38] Watson PM, Paterson JC, Thom G, Ginman U, Lundquist S, Webster CI. Modelling the endothelial blood-CNS barriers: a method for the production of robust in vitro models of the rat blood-brain barrier and blood-spinal cord barrier. *BMC Neurosci* 2013;14:59.
- [39] Yu YJ, Zhang Y, Kenrick M, Hoyte K, Luk W, Lu Y, Atwal J, Elliott JM, Prabhu S, Watts RJ, Dennis MS. Boosting brain uptake of a therapeutic antibody by reducing its affinity for a transcytosis target. *Sci Trans Med* 2011;3:84ra44.
- [40] Zhang R, Li A, Liu B, Wang L, Ren K, Zhang H, Berman BM, Lao L. IL-1ra alleviates inflammatory hyperalgesia through preventing phosphorylation of NMDA receptor NR-1 subunit in rats. *PAIN* 2008;135:232–9.
- [41] Zhang Y, Pardridge WM. Delivery of beta-galactosidase to mouse brain via the blood-brain barrier transferrin receptor. *J Pharmacol Exp Ther* 2005;313:1075–81.
- [42] Zhu C, Zhang Y, Pardridge WM. Widespread expression of an exogenous gene in the eye after intravenous administration. *Invest Ophthalmol Vis Sci* 2002;43:3075–80.