Antinociceptive potentiation and attenuation of tolerance by intrathecal β-arrestin 2 small interfering RNA in rats†

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Editor’s key points
- In chronic pain, analgesic tolerance to opioids and physical dependence are significant clinical problems.
- Opioid and glutaminergic receptors are involved in the neural mechanisms of tolerance and dependence.
- The role of β-arrestin 2 was examined in a rodent model of opioid tolerance.
- Intrathecal β-arrestin 2 siRNA significantly reduced both opioid tolerance and physical dependence.

Background. Tolerance to the analgesic effect of opioids complicates the management of persistent pain states. We tested whether the intrathecal infusion of small interfering RNA (siRNA) against β-arrestin 2 would reduce tolerance to chronic morphine use and the severity of precipitated morphine withdrawal.

Methods. Intrathecal β-arrestin 2 (2 μg siRNA per 10 μl per rat) was injected once daily for 3 days. Rats then received a continuous intrathecal infusion of morphine (2 nmol h⁻¹) or saline for 7 days. Daily tail-flick (TF) and intrathecal morphine challenge tests were performed to assess the effect of intrathecal β-arrestin 2 siRNA on antinociception and tolerance to morphine. Naloxone withdrawal (2 mg kg⁻¹) was performed to assess morphine dependence.

Results. In the daily TF test, the antinociception of intrathecal morphine was increased and maintained in rats receiving β-arrestin 2 siRNA compared with the control group (morphine alone). In the probe response test, rats receiving morphine infusion with β-arrestin 2 siRNA treatment showed a significant left shift in their dose–response curve, as measured by percent maximal possible effect (MPE), such that the AD₅₀ was significantly decreased by a factor of 5.6 when compared with that of morphine-infused rats. In the naloxone-induced withdrawal tests, rats receiving β-arrestin 2 siRNA injection with morphine infusion showed a significant reduction in four of the six signs of withdrawal.

Conclusions. We show here that intrathecal β-arrestin 2 siRNA in rats enhances analgesia and attenuates naloxone-induced withdrawal symptoms. This may warrant further investigation in the context of long-term use of intrathecal opioids for controlling chronic pain.

Keywords: analgesics opioid, morphine; β-arrestin 2; injections, spinal; substance withdrawal syndrome

Accepted for publication: 15 July 2011

Opioids such as morphine are widely used in the clinical management of acute or chronic pain. However, i.v. or oral morphine is only partially effective and is accompanied by side-effects such as constipation, sedation, and respiratory depression. Furthermore, the clinical usefulness of such opioids is often limited by tolerance and dependence. Catheters can be implanted into the subarachnoid space¹ and connected to infusion pumps to localize delivery of opioids and reduce the side-effects, but the problems of tolerance and withdrawal remain unsolved.

Tolerance and dependence are thought to result from neuronal adaptations produced by repeated drug exposure. Considerable progress has been made concerning mechanisms underlying opioid tolerance. Multiple factors are known to be involved in opioid tolerance, including desensitization of opioid receptors,² functional changes in glutamate receptors³ ⁴ and transporters,⁵ and uncoupling of G-protein from opioid receptors.⁶ In addition, β-arrestin has been demonstrated as playing an important role in regulating opioid receptors.⁷ ⁸

β-arrestins, including β-arrestin 1 and β-arrestin 2, are predominantly expressed in neuronal tissues (low expression can be detected in most tissues)⁹ and regulate G-protein-coupled receptor coupling and signalling.¹⁰ In β-arrestin 2 knockout mice, the tolerance to the antinociceptive effects was significantly attenuated in the tail-flick (TF)
test. Intrathecal pretreatment with β-arrestin 2 antibody potentiated the antinociception induced by administered μ-opioid receptor agonists. Furthermore, intrathecal administration of β-arrestin 2 antisenses delays the development of tolerance to morphine; thus, regulating the expression of β-arrestin 2 in the spinal cord may be one approach to improving the antinociceptive effect of intrathecal morphine and delaying tolerance. In the present study, we investigated the effects of β-arrestin 2 on morphine-induced analgesia and tolerance in rats by intrathecally delivering small interfering RNA (siRNA) against β-arrestin 2.

**Methods**

**Animals**

The study was approved by the local ethics committee and the guidelines of the International Association for the Study of Pain were obeyed. Male Sprague–Dawley rats (NSC, Taiwan), weighing 250–300 g, were used in our study. They were housed in standard cages in climate- and light-controlled rooms (12 h light/dark cycles) at a temperature of 22 °C with free access to food and water. To reduce the influence from handling on nociceptive responses, all animals were handled and trained for at least 4–6 days before intrathecal catheterization and testing. With rats under 2.5% isoflurane anaesthesia, the intrathecal catheter was implanted into the rats’ intrathecal lumbar (L3–4) space through a cisternal incision as previously described. Buprenorphine 0.02 mg kg⁻¹ was given subcutaneously for postoperative analgesia. Each catheter was connected to a T-connector (PE60), one end for daily siRNA injection and the other end for osmotic pumps (Model 2001 delivering 1 μl h⁻¹; Alza, Palo Alto, CA, USA).

**Experimental design**

The experimental design and silencing strategy are shown in Figure 1. Rats were randomly assigned to eight groups as follows:

- **Group 1 (M0+NS):** 10 μl normal saline injection and infusion with vehicle.
- **Group 2 (M0+TR):** 10 μl transfection reagent (i-Fect, Neuromics Antibodies, Northfield, MN, USA) injection and infusion with vehicle.
- **Group 3 (M0+ mismatch):** 2 μg 10 μl⁻¹ mismatch siRNA + i-Fect injection and infusion with vehicle.
- **Group 4 (M0+β-arrestin 2):** 2 μg 10 μl⁻¹ β-arrestin 2 siRNA + i-Fect injection and infusion with vehicle.
- **Group 5 (M2+NS):** 10 μl normal saline injection and infusion with 2 nmol h⁻¹ of morphine.
- **Group 6 (M2+TR):** 10 μl transfection reagent (i-Fect) injection and infusion with 2 nmol h⁻¹ of morphine.
- **Group 7 (M2+ mismatch):** 2 μg 10 μl⁻¹ mismatch siRNA + transfection reagent injection and infusion with 2 nmol h⁻¹ of morphine.
- **Group 8 (M2+β-arrestin 2):** 2 μg 10 μl⁻¹ β-arrestin 2 siRNA + transfection reagent injection and infusion with 2 nmol h⁻¹ of morphine.

![Fig 1](https://academic.oup.com/bja/article-abstract/107/5/774/301920)
Behavioural assessment
The TF test was used to measure responses to noxious somatic stimuli by monitoring the rat's latency to withdrawal from a heat source (54 °C) which was focused on the dorsal surface of the tail ∼5 cm from the tip. If there was no TF response within 20 s, the stimulus was terminated. The 20 s interval was set as the cutoff time to avoid damage to the tail. Measurements were performed daily to assess each animal's response to noxious somatic stimuli for 7 days during infusion. The investigators were prevented from knowledge of the treatment results in accordance with a blinded experiment.

On day 7, a challenge test with morphine was performed to assess the development of tolerance to morphine. In this test, morphine (1, 10, or 100 nmol 10 μl−1) was intrathecally administered after the determination of the baseline values, and TF tests were given 30 min after injection.

Naloxone challenge test
To assess dependency in these animals, 36 rats entered into the withdrawal study. On day 7 at noon, rats from each group were given 2 mg kg−1 of intraperitoneal naloxone and observed for 1 h in a circular transparent observation chamber for the presence or absence of signs of withdrawal: vocalization in response to light touching with a piece of polyethylene tubing, spontaneous vocalization, abnormal posture, ejaculation, ‘wet dog’ head shakes, or attempts to escape. Each group contained five rats. The observer was blinded to group identity.

Real-time reverse transcription–polymerase chain reaction
For real-time reverse transcription–polymerase chain reaction (RT–PCR), researchers pooled spinal cords and dorsal root ganglia (DRGs) from experimental rats in order to minimize inter-animal variability and to prevent significant loss of RNA during the extraction procedure. Total RNA was extracted from spinal cords and DRGs by using Trizol (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized by using 2 μg of total RNA in the presence of Ready-To-Go You Prime First-Strand Beads (Amersham Pharmacia, Piscataway, NJ, USA) and random primers (Invitrogen). Measurements of mRNA levels were performed by real-time RT–PCR by using a DNA Engine Opticon System (M.J. Research, Waltham, MA, USA), and the relative amount of the gene of interest (β-arrestin 1 or β-arrestin 2) was normalized to the mRNA amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). To avoid the possibility of amplifying or contaminating DNA, all of the primers for real-time RT–PCR were designed with an intron sequence inside the cDNA to be amplified; reactions were performed with appropriate negative control samples (template-free control samples); a uniform amplification of the products was rechecked by analysing the melting curves of the amplified products (dissociation graphs); the melting temperature (Tm) was 57–60 °C; the probe Tm was at least 10 °C higher than the primer Tm, and gel electrophoresis was performed to confirm the correct size of the amplification and the absence of non-specific bands. The primer sequences were: β-arrestin 1, AAGGGACAC-GAGTTTCAAG (forward), GACTCCGTTTCTTGGATAC (reverse); β-arrestin 2, AGCACCGCAGTACAAGT (forward), CAGCCTTCTCTGTTGTA (reverse).

Western blots
Total protein was prepared by the addition of 500 μl of ice-cold solubilization buffer (150 mM NaCl, 50 mM Tris–HCl, pH=8.0, 5 mM EDTA, and 1% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride and 5 μg ml−1 each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin). The tissue was homogenized with a homogenizer, and subsequent to being placed on ice for 30 min, the homogenate was centrifuged at 10 000g for 5 min at 4 °C. The supernatant was then collected and assayed for the protein content using the bicinchoinic acid assay method (Pierce, Rockford, IL, USA), and stored at −70 °C for further use. A total of 15 μg of total protein were electrophoresed on an 8% sodium dodecylsulphate–polyacrylamide gel using a sample buffer, running buffer, and molecular-weight standards, as suggested by the manufacturer. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane and blocked with 5% non-fat dry milk. The primary antibody for anti-β-arrestin 1 (a 1:1000 dilution of goat polyclonal antibody; Abcam, USA) and β-arrestin 2 (a 1:600 dilution of goat polyclonal antibody; Abcam) was added for a period of 2 h at room temperature in fresh blocking buffer. The membranes were then washed for 30 min in washing buffer at room temperature, before the secondary antibody (a 1:6000 dilution of horseradish peroxidase-coupled rabbit anti-goat immunoglobulin G; Chemicon, Temecula, CA, USA) was added for a 1 h period at room temperature in the blocking buffer. The membranes were washed in the washing buffer for another 30 min, and the antibodies were then revealed using Western Blot Chemiluminescence Reagent Plus (NEN, Boston, MA, USA). For densitometric analyses, the blots were scanned and quantified using Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Statistical analysis
All data were analysed by using commercially available computer software (SPSS Version 12.0; SPSS Inc., Chicago, IL, USA). Behavioural and biochemical results were analysed by using a non-parametric analysis of variance (ANOVA) (Kruskal–Wallis test followed by the Mann–Whitney U-test). Comparisons between individual treatment groups and time points were performed with the Wilcoxon signed-rank test. To compare groups, a post hoc Fisher's least significant difference test was used. A P-value of <0.05 was considered significant. Spinal neurochemical data were expressed as a percentage change from the baseline (SD). Where applicable,
data from antinociceptive testing, that is, absolute latencies or calculated maximal possible effects [%MPE=(post-drug value–baseline value)/(cutoff value–baseline value)×100], were analysed with one- or two-way ANOVA to detect differences between the groups. When differences were found, these findings were subjected to the Scheffe’ F-test (significant at 95%). Unless stated otherwise, single points of comparison were made by using a standard paired or unpaired Student’s t-test. By using linear regression, calculation of the AD50 (95% confidence intervals (CIs)) test for relative potency was done. The tolerance ratio (the ratio of AD50 in treated animals to AD50 of saline-infused animals) and 95% CI were calculated. Differences yielding critical values corresponding to $P<0.05$ were considered statistically significant.

Results

siRNA duplexes (Sigma) with sequences specifically targeting β-arrestin 2 RNA was 5’AAGGACCGAAAGUGUUGUG-3’, as reported previously.15 16 These sequences have been extensively validated with regard to specificity for β-arrestin 2 knockdown, effects on signalling and extracellular signal-regulated kinase (ERK) phosphorylation, and by similarity of results with siRNA to those obtained in mouse embryo fibroblasts from β-arrestin 1- and 2-knockout mice.16 A scrambled RNA duplex (sc-37007; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control. The efficiency of the inhibition of β-arrestin 1 and 2 expression in injected rats was investigated by quantitative RT–PCR (qRT–PCR) and western blot over 9 days. For each time point, qRT–PCR and western blots were performed on triplicates (three pools of 7–10 injected rats). As a negative control, rats were injected with a scrambled RNA duplex. qRT–PCR or western blot data were expressed relatively to the GAPDH internal control gene. Results of the β-arrestin 1 and 2 expression in β-arrestin 2 siRNA and control-injected rats are presented in Figure 2. Despite a moderate variability between the replicates, an unambiguous decrease in β-arrestin 2 transcripts was observed in rats injected with β-arrestin 2 siRNA. β-Arrestin 2 expression was reduced by 57% and 29% of transcript and protein level 1 day after injection (Fig. 2B and D). The maximum reduction of 82% of the transcript level occurred at 5 days after injection. The maximum reduction of 71% of the protein level occurred at 3 days after injection. The dsRNAi had less effect (35% and 39% reduction of transcript and protein, respectively) at 9 days after injection. No significant change of β-arrestin 1 expression was observed. The β-arrestin 2 expression in β-arrestin 2 siRNA-injected rats was expressed as the proportion of the expression recorded in the control. Average percentages of mRNA depletion are indicated. Data are presented as mean (SD), $*P<0.05$. 

Fig 2 Real-time RT–PCR and western blot analysis showing a decrease in β-arrestin 2 but not β-arrestin 1 mRNA and protein level in the spinal cord after intrathecal injection with β-arrestin 2 siRNA. β-arrestin 1 and 2 mRNA and protein level in control and β-arrestin 2 siRNA-injected rats were monitored by qRT–PCR over 9 days after injection. Each kinetic point was performed in triplicate on three to seven pooled rats. For each sample, β-arrestin 1 and 2 transcripts or protein level was normalized against GAPDH. Normalized β-arrestin 1 and 2 expression in β-arrestin 2 siRNA-injected rats was expressed as the proportion of the expression recorded in the control. Average percentages of mRNA depletion are indicated. Data are presented as mean (SD), $*P<0.05$. 

Intrathecal β-arrestin 2 siRNA and morphine tolerance
expression was observed in β-arrestin 2 siRNA-injected rats (Fig. 2a and c).

Baseline latencies in the TF were not statistically different among the groups. None of scrambled siRNA with/without transfection reagent had any effect on the baseline TF threshold when compared with that of the vehicle control group. Neither vehicle nor siRNA treatment precipitated overt signs of behavioural toxicity or motor impairment in the animals. Rats receiving β-arrestin 2 siRNA injection for 3 days experienced a significantly greater delay in tail withdrawal than the control group (Fig. 3a), suggesting the importance of the concentration of β-arrestin 2 in regulating μ-opioid receptor responsiveness. Increased spinal μ-opioid receptor–G protein coupling has been shown in β-arrestin 2 −/− mice. The extended response latencies in a TF test may be caused by increased activity at spinal μ-opioid receptor.

After osmotic minipump implantation, rats receiving normal saline infusion showed no significant difference between %MPE on days 1 and 7 in rats that were tested daily (4.7 vs 2.1 s, n=5, P>0.1). Nor was there a significant difference among these four saline-infused groups (ANOVA, P>0.1), demonstrating no significant effect after day 1 from implantation among normal saline, transfection reagent, control siRNA, or β-arrestin 2 siRNA (Fig. 2).

In morphine-infused groups, the response to morphine at 2 nmol h−1 of rats receiving normal saline injection for 3 days (M2+NS assessed by %MPE) reflected 66% antinociception on day 1, but by days 3–7, antinociception was significantly reduced compared with day 1 (Fig. 3). Relative to day 1, the combination of β-arrestin 2 siRNA injection and morphine infusion (M2+β-arrestin 2) maintained antinociception through day 7, whereas the combination of transfection reagent or scrambled RNA with morphine infusion did not maintain antinociception. Compared with M2+NS, the combination of β-arrestin 2 siRNA and morphine infusion (M2+β-arrestin 2) resulted in a significant increase in %MPE on days 1–7, whereas the combination of transfection reagent, scrambled RNA, or saline with morphine infusion did not show a significant difference compared with the morphine infusion with a saline injection (Fig. 3a).

In the naloxone-induced withdrawal tests, none of the rats infused with saline, control siRNA, or transfection reagent (n=5 per group) showed any of the six withdrawal signs examined. In contrast, morphine-infused rats (n=5), all exhibited three or more of the six signs of withdrawal assessed. Rats receiving β-arrestin 2 siRNA injection with morphine infusion (n=5) showed a significant reduction in four of the six signs of withdrawal: vocalization to light touch, spontaneous vocalization, ejaculation, and head shaking, when compared with the saline control with morphine-infused rats (P<0.05) (Fig. 4).

In the probe response test, the curve in rats treated previously with 2 nmol h−1 intrathecal morphine infusions displayed a significant right shift, and this was evident because the AD50 was increased by a factor of 25.3 when compared with saline-infused rats. This suggests that in morphine-tolerant animals, the potency of the probe dose of spinal morphine is considerably reduced (Fig. 5).

Rats receiving morphine infusion with β-arrestin 2 siRNA treatment showed a significant left shift in their dose–response curve, as measured by %MPE, and this was evident because the AD50 was significantly decreased by a factor of 5.6 when compared with that of morphine-infused rats.

Light microscopy analysis showed no evidence of pathological changes. All neuronal pools including large A-motor neurones and small- and medium-sized interneurones showed fully preserved nuclei and nucleoli, with no detectable changes in neuropils (data not shown).

**Discussion**

The main objective of this study was to determine if delivery of encapsulated siRNA using intrathecal injection would
result in tolerance attenuation in rats receiving chronic spinal morphine infusion. We chose to target β-arrestin 2, a well-characterized gene that is critically important in opioid tolerance, for which a genetic knockout exists. We obtained several lines of evidence indicating that delivery of β-arrestin 2 siRNA induced target-specific inhibition. First, quantification of β-arrestin 2 mRNA levels by RT–qPCR resulted in a significant reduction in rats receiving β-arrestin 2 siRNA compared with those receiving a control. Secondly, the transcriptional changes were maintained for at least 7 days and were previously validated. Thirdly, β-arrestin 1 mRNA expression was not regulated in β-arrestin 2 siRNA-treated rats compared with the control group. Finally, rats treated with β-arrestin 2 siRNA displayed phenotypes similar to those observed in β-arrestin 2–/– mice, namely the attenuation of morphine tolerance. Thus, both molecular and phenotypic data indicate that functional delivery of β-arrestin 2 siRNA to the spinal cord was achieved.

Although the behavioural alterations in rats treated with β-arrestin 2 siRNA were similar to those reported for β-arrestin 2–/– mice, important differences were also noted. The most significant difference was the attenuation of withdrawal symptoms in β-arrestin 2 siRNA-treated rats in the naloxone challenge tests. This was an unexpected finding, given the lack of attenuation of withdrawal symptoms phenotypes in β-arrestin 2–/– mice. This is possible that compensatory mechanisms are induced in β-arrestin 2–/– mice, which were devoid of β-arrestin 2 function throughout development. In the absence of β-arrestin 2, β-arrestin 1 may compensate inducing receptor desensitization and antinociceptive tolerance. In rats treated with β-arrestin 2 siRNA, it is possible that β-arrestin 2 expressions are inhibited before the putative compensatory mechanisms can be fully induced. Alternatively, it is possible that the lack of complete knockdown or a different β-arrestin 1/β-arrestin 2 ratio impacted the phenotypes that were observed in the knockdown rats vs those of the β-arrestin 2–/– mice. These possibilities cannot be differentiated based on the data presented in this report.

Morphine tolerance and dependence is a serious problem that is often refractory to the best available conventional therapies. Advances over the past decades have provided substantial insight into the anatomy, physiology, and molecular biology alterations responsible for the state of tolerance/dependence. As there is a limited repertoire of ion channels, neurotransmitters, and neurotransmitter receptors used in nervous system pathways, it has been difficult to identify small molecules that selectively target tolerance or dependence pathways. The neuroanatomic sites involved in tolerance/dependence include nuclei in the central nervous system such as the locus coeruleus, periaqueductal gray, and the ventral tegmental area. In addition to central nuclei, neuroadaptation in the spinal cord also appears to
play a significant role in development of tolerance and dependence.\textsuperscript{30} \textsuperscript{31} Pharmacological\textsuperscript{31} and electrophysiological studies\textsuperscript{32} suggest that the opioid-dependent spinal cord is capable of generating withdrawal symptoms in the absence of participation of supraspinal centres. The AD\textsubscript{50} values of naloxone in precipitating withdrawal signs for intrathecal injection are 25 times lower than for intraventricular injection, suggesting that spinal sites appear to be more important than the supraspinal sites.\textsuperscript{30} Intrathecal delivery of an N-methyl-D-aspartate antagonist (e.g. ketamine) has been used for sometime to decrease the tolerance/dependence of morphine by physically directing the drug in highest concentration to the spinal cord. Hence, the first synapse in the pain pathway between nociceptive neurons terminals of the DRG and second-order neurones in the spinal cord provide an attractive target for modulating neurotransmission involving morphine tolerance/dependence.

The use of gene transfer in place of drug delivery to achieve the continuous release of short-lived bioactive peptides in or near the spinal dorsal horn exemplifies the most common experimental strategy for gene therapy of tolerance/dependence. There are two principal models. In the first approach, neurones of the DRG or spinal cord are transduced by expression of anti-tolerance/dependence genes. Hao and colleagues\textsuperscript{13} reported herpes simplex virus vectormediated overexpression of proenkephalin in lumbar DRGs in rats with neuropathic pain treated with morphine. Rats with neuropathic pain inoculated subcutaneously with the vector-mediated overexpression of proenkephalin showed a significant reduction in withdrawal signs precipitated by naloxone after 2 weeks of morphine treatment. In the second approach, neurones of the DRG or spinal cord were transduced by knockdown of pro-tolerance/dependence genes.\textsuperscript{13} \textsuperscript{34} In the current report, it was demonstrated that intrathecal injection of an i-Fect encapsulated siRNA in knocking down the expression of β-arrestin 2 siRNA enhances analgesia and attenuates naloxone-induced withdrawal symptoms in rats receiving chronic intrathecal morphine infusion. There are some important issues that need to be explored further: (i) what is the rostro-caudal extent of encapsulated siRNA uptake after intrathecal injection with variable doses? (ii) What is the effect if the siRNA is injected at thoracic or higher levels of the spinal cord? (iii) Which regions of the brain (higher dose or thoracic injection) are affected? (iv) Do multiple injections of siRNA intrathecally cause any toxicity to the central nervous system?

**Conclusion**

We have shown that intrathecal injections of siRNA lead to a decrease in β-arrestin 2 expression in the spinal cord and attenuate morphine tolerance and dependence in rats. Since patients suffering from moderate-to-severe pain could be treated by infusing morphine into the spinal fluid, a continuous intrathecal infusion paradigm similar to that used in the study may closely mimic the exposure to opioid drugs in the clinical setting.

**Conflict of interest**

None declared.

**Funding**

This work was supported in part by grant nos 870641, 860231, and 860232 from Chang Gung Memorial Hospital Research, Kaohsiung, Taiwan, and by grant nos 95-2745-B-182A-004, 96-2628-B-182A-005-MY3, and 98-2314-B-182A-035-MY2 from the Taiwan National Science Council Research, Taipei, Taiwan.

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