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Single intrathecal administration of the transcription factor decoy AYX1 prevents acute and chronic pain after incisional, inflammatory, or neuropathic injury

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**Article info**

**Abstract**

The persistence of pain after surgery increases the recovery interval from surgery to a normal quality of life. AYX1 is a DNA-decoy drug candidate designed to prevent post-surgical pain following a single intrathecal injection. Tissue injury causes a transient activation of the transcription factor EGR1 in the dorsal root ganglia–dorsal horn network, which then triggers changes in gene expression that induce neuronal hypersensitivity. AYX1 is a potent, specific inhibitor of EGR1 activity that mimics the genomic EGR1-binding sequence. Administered in the peri-operative period, AYX1 dose dependently prevents mechanical hypersensitivity in models of acute incisional (plantar), inflammatory (CFA), and chronic neuropathic pain (SNI) in rats. Furthermore, in a knee surgery model evaluating functional measures of postoperative pain, AYX1 improved weight-bearing incapacitance and spontaneous rearing compared to control. These data illustrate the potential clinical therapeutic benefits of AYX1 for preventing the transition of acute to chronic post-surgical pain.

**Keywords:**
AYX1
Oligonucleotide
Post-surgical pain
Prevention
Acute
Chronic

**1. Introduction**

With 30 million surgical procedures performed annually in the United States, post-surgical pain constitutes a public health issue \cite{11}. Its most debilitating component is the mechanical hypersensitivity associated with movement-evoked pain, which is poorly treated by current therapeutics \cite{34,35}. Consequently, ~80% of patients suffer moderate to severe acute perioperative pain for weeks after hospital discharge, and approximately 10% to 50% develop chronic pain \cite{2,17,19,23}. Mechanical hypersensitivity after surgery results from a pathophysiological gain in excitability of the dorsal root ganglia (DRG) and dorsal horn neuronal network. It is a dynamic phenomenon initiated within minutes after injury, with the launch of multi-phasic, genome-wide waves of gene regulation driving long-term neuronal hypersensitivity through a variety of cellular mechanisms. These gene-regulatory events follow a temporal hierarchy: an immediate–early transcriptional phase is first induced after injury, and triggers delayed transcription waves responsible for sustained neuronal sensitization \cite{9,15,22,30,36}.

Early growth response 1 (EGR1) is a transcription factor essential for the early transcriptional response to a noxious injury: its immediate activation after injury triggers delayed, long-term transcriptional and neuronal excitability changes necessary to the development of pain and mechanical hypersensitivity. EGR1 is transiently induced in DRG and dorsal horn neurons in response to surgical incision and a wide range of other painful injuries, including thermal, biochemical/chemical, ischemic, inflammatory, and neuropathic \cite{3,13,14,21,30}. Microarray studies established that EGR1 target genes encompass most of the molecular players involved in pain control, including immune response, translation, apoptosis,
transporters, cell cycle, metabolism, ion channels/receptors, signaling enzymes, structural, transcription, cell–cell communication, and trafficking encoding genes [16]. At a physiological level, EGR1 is required for the maintenance of long-term potentiation [18]. These findings suggest a deep and broad role for EGR1 in sensitization of DRG and dorsal horn neurons: a molecular switch converting transient forms of neuronal sensitization and nociception into a sustained, chronic pain state. EGR1 knockout and antisense studies in which EGR1 expression is impaired have partially confirmed this function by demonstrating an absence of pain maintenance in short-term, acute models of inflammatory pain [21,30]. We hypothesized that the local interruption of EGR1 activity at the DRG–dorsal horn synapse around the time of an injury would not only prevent acute inflammatory pain responses but also attenuate other peri-injury and chronic forms of post-surgical pain.

To this end, we developed AYX1, a 23-bp DNA decoy with high affinity and specificity for EGR1. Decoys are small, double-stranded oligonucleotides inhibiting the activity of transcription factors by mimicking their endogenous DNA-binding sequence. To determine whether AYX1 would block EGR1 function, we performed in vitro experiments examining its effects of AYX1 on molecular and cellular reactions involving EGR1. To determine the functional consequences of EGR1 blockade in vivo, we examined the effect of AYX1 intrathecal (i.t.) administration around the time of injury in several rat models of injury-driven hypersensitivity. The results of these experiments highlight the therapeutic potential of AYX1 for preventing post-surgical acute and chronic pain and for improving recovery from surgery.

2. Methods

2.1. Enzyme-linked immunosorbent assay

Decoy binding activities were measured with an EGR1 enzyme-linked immunosorbent assay (ELISA) kit (Affymetrix, Santa Clara, CA). Briefly, a custom biotin-decoy probe (sense strand: 5′-GTATGGCGTAGATGGGGCGGTATAG-3′, IDTDNA, Coralville, IA) was incubated with TPA-stimulated K-562 cells nuclear extracts (Active Motive, Carlsbad, CA). The protein–decoy mix was loaded on streptavidin-coated, 96-well plates, and the quantity of captured EGR1 measured with an antibody-based colorimetric detection (OD 450 nm). When increasing concentration of competing, non-biotinylated decoys are added to the binding reaction, a reduction of EGR1 binding to the biotinylated probe demonstrates binding specificity. A similar approach was used to detect SP1 and WT1 binding activity. AYX1: sense strand: 5′-GTATGGCGTAGATGGGGCGGTATAG-3′, IDTDNA, Coralville, IA); antisense strand: 5′-GATGCGTTTGTAGATGCTTTCGTTATAG-3′, IDTDNA, Coralville, IA). Decoy transfections were performed with lipofectamine (Invitrogen, Carlsbad, CA) + 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Decoy transfections were performed with lipofectamine (Invitrogen, Carlsbad, CA) + 10% fetal bovine serum (Invitrogen, Carlsbad, CA) + 5% equine serum (Invitrogen, Carlsbad, CA). Cells were transfected with AYX1 (500 nmol/L, lipofectamine) and immediately treated with NGF (100 ng/mL, Sigma Aldrich, St. Louis, MO), an Egr1 inducer, and forskolin (5 μmol/L) that gives competency to Egr1. RNA was collected 24 hours later and processed for RT–PCR detection of the following genes: ion channels (Scn9a, Cacna1b), membrane receptors (Grm5, Bdkrb2, P2rx3, Htr3a), enzyme related (Cdk5r1, Gch1, Pmnt, Nos1) and neurotransmitter (Bdnf). For in vivo trials, rats were anesthetized with 2% isoflurane, i.t. AYX1, or vehicle administered percutaneously. Intrasegmental injection, a plantar incision performed on the left paw 1 hour later after the plantar incision model [4] was made. The left hind paw was clipped and surgically prepared for an incision. A 2-cm incision was made in the plantar surface of the dog's paw just proximal to the metatarsal pad, and the plantaris muscle was lifted and stretched slightly. The incision was sutured with 5-0 Ethicon. Animals remained lightly anesthetized (1%–2% isoflurane in 50% O2/N2O) for 3 hours after the incision. They were then deeply anesthetized, necropsied, and L4 to L6 DRG and spinal segments harvested. L4 to L6 spinal segments and DRG ipsilateral to the incision were harvested in RNAlater (Qiagen, Valencia, CA) for both rat and canine samples, homogenized, and RNA was isolated for semi-quantitative (sq) RT–PCR. Gene expression levels were normalized against Gapdh for PC12, rat, and canine experiments and against Actb for HL60 experiments. For all experiments, RNA was extracted using the RNEAPlus kit (Qiagen, Valencia, CA), and reverse transcription was performed with either the first-strand cdna synthesis kit (GE Lifesciences, NJ) or the Superscript first-strand system (Invitrogen, Carlsbad, CA). PCR was performed in 20 μL using the Promega master mix with the following cycles: 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute (25 cycles for housekeeping genes Actb and Gapdh, 35 cycles for other genes). All PCR primers (IDTDNA, Coralville, IA) have been previously described (Table 1, supplementary material).

2.2. Pharmacodynamics

HL60 cells (CCF; University of California–San Francisco, San Francisco, CA) were grown in RPMI 1640/L-glutamine + 1% antibiotics (Invitrogen, Carlsbad, CA) + 5% fetal bovine serum (Invitrogen, Carlsbad, CA). Decoy transfections were performed with lipofectamine (Invitrogen, Carlsbad, CA) according to the supplier protocol. After transfection, cells were immediately treated with 1 μm/L 1-α, 25-dihydroxyvitamin D3 (Sigma Aldrich, St. Louis, MO). Cells were collected 48 hours after treatment; total RNA was extracted and prepared for reverse transcription–polymerase chain reaction (RT–PCR; Invitrogen, Carlsbad, CA; Promega, Madison, WI). PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/low glucose/1-glutamine/HEPES/NaPyr (Invitrogen, Carlsbad, CA) + 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA) + 10% FBS (Invitrogen, Carlsbad, CA). Cells were transfected with AYX1 (500 nmol/L, lipofectamine) and immediately treated with NGF (100 ng/mL, Sigma Aldrich, St. Louis, MO), an Egr1 inducer, and forskolin (5 μmol/L) that gives competency to Egr1.

2.3. Nomenclature

Gene names are represented using the HUGO Gene Nomenclature Committee recommendation (HGNC, http://www.genenames.org/). Rat gene symbols are italicized, with only the first letter upper case and the remaining letters lower case. Human and canine gene symbols are italicized, with all letters upper case. Protein designations are the same as the gene symbol, but are not italicized. mRNAs and cDNAs use the same formatting conventions as the gene symbol.

2.4. Histology

Rats were sacrificed at day 8 post-SNI surgery, and perfused with 4% paraformaldehyde. L4 to L6 spinal cord segments were extracted, processed, and cut into serial frozen slices (12 μm). Sections were then either processed for Gapm immuno-labeling (1:100, Promega, Madison, WI) or Hoechst–propidium iodide staining (Invitrogen, Carlsbad, CA) according to the suppliers’ recommendations. Images were captured with a fluorescent microscope, and analyses were performed in the dorsal horns ipsilateral and......
contralateral to surgical. The surface of the Gfap labeling was measured and normalized with the total surface of the dorsal horn using ImageJ (National Institutes of Health, Bethesda, MD). Pyknotic nuclei were identified as fragmented nuclei compared to normal nuclei using analysis of variance (ANOVA).

2.7. Behavioral testing

The plantar incisional, CPA, SNI, and knee surgery models were performed as described elsewhere [4,5,10,32]. In each model, rats were habituated in cages with mesh wire floors for at least 1 hour before von Frey testing. von Frey hairs were calibrated at specific levels and applied with the following pseudo-random pattern: 6, 1, 10, 4, 26, 10, and 8 g (except for the CPA testing: 1, 6, and 15 g were tested). For each testing level, the von Frey hair was applied 5 times consecutively around the incision, and the number of paw withdrawals (0 to 5 per level, 0 to 35 total) was recorded as a response. The time interval between each testing level for a given rat was ≥3 minutes. In the SNI and the knee surgery models, incapacitation was measured as the difference in static weight bearing between the ipsilateral versus contralateral hind paw relative to the injury using an incapacitation meter (model 600; IITC Life Science, Woodland Hills, CA). Rats were placed unrestrained on the apparatus, and each hind paw was positioned on a separate platform connected to a dedicated force transducer. Weight borne by each limb within a 10-second period was averaged and displayed on the front indicator. In the knee surgery model, rearing activity was video-recorded and daily analyzed for 30-minute periods. Side and back walls of each cage were covered with white paper, and marker lines were drawn on the frontal wall, consisting of an horizontal line 11 cm above the bottom of the cage and of vertical lines every 5 cm. The number of times the animal crossed any vertical line was used to quantify ambulation, and crossing the horizontal line was used to measure rearing. All studies were conducted in a blinded fashion. All experiments were approved by appropriate animal care and use committees.

2.8. Statistical analysis

A non-parametric Student’s t test, followed by a t Welsh analysis for uneven variance correction, was used to analyze individual conditions and whole data distribution comparisons between experimental conditions. Dose-dependent relationships were analyzed using analysis of variance (ANOVA).

3. Results

3.1. AYX1 binding properties

Composite transcription factor binding sites, which are composed of 2 or more binding sites, naturally occur in genomes. They can induce cooperative protein–protein interactions, thereby influencing transcription factor binding properties as a way to control target selectivity and affinity [27,33]. We hypothesized that the relative positioning of 2 or more transcription factor binding sites would generate decoy compounds with high affinities for their targets (compared to single consensus binding sites). To test this idea, we designed a matrix of DNA sequences for composite EGR1 decoys with multiple binding sites. Decoy molecules with various DNA sequences were generated from the matrix and their binding
activities measured with a transcription factor ELISA, a technology allowing a quantitative and specific screening of affinity and specificity for the human form of EGR1 (Fig. 1a and b). AYX1, a 23-bp, unmodified composite decoy featuring a specific overlap of 2 EGR1 binding sites was identified as an optimal decoy with an affinity for EGR1 superior to its consensus binding site (Fig. 1c). We proposed that the fused nonameres on AYX1 induce interactions between 2 EGR1 proteins that mutually increase their DNA binding affinity.

Fig. 1. (a) Enzyme-linked immunosorbent assay (ELISA) sensitivity. EGR1 binding to a biotin-coupled EGR1 decoy probe in presence of 5 µg, 10 µg, or 15 µg of K-562 cells (TPA stimulated) nuclear extracts was measured. Representative OD450nm values obtained for each protein quantity are shown. A 10-µg quantity of protein extract was selected for binding experiments to produce a strong, non-saturating EGR1 binding signal. (b) ELISA specificity. Non-specific binding was controlled by comparing EGR1 binding activity of the biotinylated EGR1 probe to a biotinylated mutant decoy. Representative OD450nm values obtained for each decoy are shown. (c) AYX1 affinity. Quantitative competition ELISA was performed using a constant concentration of biotinylated-probe (0.125 µmol/L) and quantity of protein extracts (10 µg). The probe–protein mix was incubated with increasing concentrations of non-biotinylated competitors, namely, AYX1 or decoys containing a single or a tandem EGR1 consensus binding site for binding reference. The inhibition of EGR1 probe binding was measured for each competitor and the resulting inhibition curves were fitted to an exponential decay model. Data are given as a percentage of the maximum EGR1 binding obtained with the EGR1 decoy probe in absence of competitor, n = 2 to 4. (d) AYX1 specificity. Relative binding of AYX1 to SP1 and WT1 factors was measured with quantitative ELISA. Binding inhibition curves for each transcription factor are displayed. Mean and SEM are given as a percentage of the maximum binding for each factor obtained with the probe in absence of competitor decoy. ab = antibody. n = 1 to 3. (e) AYX1 specificity illustration. Representative OD450nm values for the binding of the EGR1 probe (0.125 µmol/L) to either SP1 or WT1 compared to EGR1 was detected with specific antibodies for each factor in absence of presence of AYX1 (1 µmol/L). For comparison, the binding of an SP1 decoy probe to SP1 is shown.
Fig. 2. (a) CDK5R1 marker gene inhibition. CDK5R1 mRNA expression level in human HL60 cells was measured by reverse transcription–polymerase chain reaction (RT-PCR) 48 hours after 25-dihydroxyvitamin D3 stimulation (1 μmol/L) and transfection of increasing concentrations of EGR1 decoys (250, 500, 1000, or 2000 nmol/L). CDK5R1 mRNA expression level was normalized against β-Actin (ACTB). The concentrations necessary to obtain 50% inhibition of CDK5R1 expression for the tandem consensus EGR1 decoy, the single consensus EGR1 decoy and AYX1 were, respectively, 443 nmol/L, 502 nmol/L, and 136 nmol/L. *P < .05, different from the single EGR1 consensus decoy, values are given as mean ± SEM; n = 3. (b) AYX1 selectivity in HL60 cells. A 3-fold increase of CDK5R1 mRNA expression was observed after 1-α, 25-dihydroxyvitamin D3 treatment in HL60 cells compared to baseline, consistent with the published model. AYX1 selectivity was controlled by the lack of effect on CDK5R1 expression of a mutant EGR1 decoy versus AYX1 transfected at the same concentration (500 nmol/L). (c) AYX1 efficacy illustration in HL60 cells. Representative detection of CDK5R1 cDNA on agarose gel after either AYX1 or the tandem EGR1 decoy transfection; vitamin = 1-α, 25-dihydroxyvitamin D3 treatment. (d) AYX1 efficacy illustration in PC12 cells. Representative detection of Gch1 cDNA on agarose gel in absence or presence of AYX1 (500 nmol/L) and NGF treatment in PC12 cells. The expression level of 11 selected pain-related genes 24 hours after NGF + forskolin treatment, with and without AYX1 (500 nmol/L) transfection is shown. A portion of the genes was not sensitive to NGF + forskolin nor to AYX1 treatment; expression levels are normalized against GAPDH. *P < .05, different from control; *P < .1 for Bdnf/NGF/forskolin and Bdnf/NGF/forskolin/AYX1 vs Bdnf(control); n = 2 to 4. (e) AYX1 selectivity in PC12 cells. AYX1 specificity was verified by the lack of effect on Gch1 induction after NGF + forskolin of a mutant EGR1 decoy versus AYX1 transfected at the same concentration (500 nmol/L); open bar indicates baseline; filled bar indicates NGF + forskolin; dashed denotes NGF + forskolin + AYX1; and gray denotes NGF + forskolin + mutant decoy; n = 3.
Fig. 3. (a) AYX1 efficacy in the CFA model. Mean ± SEM values of total responses to repetitive von Frey (1.6 and 15 g) stimulations for vehicle (filled bars) or 13 nmol AYX1-treated animals (open bars). Vehicle and AYX1 were administered at −24 hours, −1 hour, +24 hours and +48 hours relative to CFA injection. VF = von Frey hair. n = 6. t test + t-Welsh analysis, different from vehicle at a given time point. *P < .05 (P = .06 at 24 hours). (b) Initial AYX1 efficacy demonstration in the chronic neuropathic pain (SNI) model (Stanford University, CA). Mean ± SEM values of total responses to repetitive von Frey stimulations after vehicle (open triangles) and AYX1 100 nmol (open circles) treatment over a 1-month period. Specificity of AYX1 treatment was shown over a 1-week-long post-surgical period using a mutant decoy version of AYX1 (100 nmol) versus AYX1 (100 nmol) and vehicle; n ≥ 5 rats per condition. t Test followed by a t-Welsh analysis, different from vehicle at a given time point. *P < .05, **P < .0001 (P < .1 at 4 weeks), vehicle versus AYX1 data distribution: P = 1.10. (c) AYX1 efficacy reproducibility and dose response in the SNI model at a separate testing laboratory (University of Arizona, AZ). Mean ± SEM values of total responses to von Frey stimulations after vehicle (open downward triangles) or AYX1 administration at various dose levels: 50 nmol (open circles), 100 nmol (filled upward triangles) or 200 nmol (filled squares) over a 10 post-operative days time course, n ≥ 4. t Test followed by a t-Welsh analysis, different from vehicle at a given time point; **P < .01, ***P < .0001, dose response: vehicle versus 50 nmol versus 100 nmol versus 200 nmol, analysis of variance, P < .0001. (d) AYX1 range of effect in the SNI model (University of Arizona, AZ). Mean ± SEM values of response for each individual von Frey hair out of 5 stimulations over the 10-day testing period (plotted from the experiment displayed in part c above). VF = von Frey hairs. Filled bars denote vehicle; gray bars denote 50 nmol AYX1; dotted bars denote 100 nmol AYX1; open bars denote 200 nmol AYX1. t Test followed by a t-Welsh analysis, different from vehicle. **P < .01, ***P < .0001. (e) AYX1 efficacy reproducibility in the SNI model at a third testing laboratory (University of Kentucky, KY). Median ± 40th and 60th percentile values of total responses to repetitive von Frey stimulations for vehicle-treated (triangle) and 200-nmol AYX1–treated (circle) animals over a 4 week post-surgical period are shown; n = 4. t Test followed by a t-Welsh analysis. Data distribution over the testing period, different from vehicle: P = .008 (only an area-under-the-curve statistical analysis was performed because of the limited number of animals used for this replicative behavioral study).
Further ELISA testing revealed AYX1 was highly specific for EGR1 versus Specificity Protein–1 (SP1) and Wilms Tumor–1 (WT1) (Fig. 1d and e), the 2 other transcription factors known to bind related GC-rich DNA sequences. Those data are consistent with prior independent observations that particular GC-rich binding sequences can efficiently discriminate among EGR1, SP1, and WT1 despite the linear prediction of their respective binding sites [8,12]. Together, those experiments demonstrate that AYX1 binds to EGR1 with high affinity and specificity.

3.2. Pharmacodynamics

The capacity of AYX1 to inhibit EGR1 activity in a human cellular context was measured against the surrogate marker gene “cyclin dependent kinase 5, receptor 1” (CDK5R1). CDK5R1 is an activator of the CDK5 kinase that regulates nociceptive signaling and is over-expressed in nociceptive neurons after peripheral inflammation [28]. In the human promyelocytic leukemia HL60 cell line, EGR1 binds to the CDK5R1 promoter and up-regulates CDK5R1 during cell differentiation by 1,25-dihydroxyvitamin D3 [7]. AYX1 transfection prevented, in a dose-dependent manner, the up-regulation of CDK5R1 in this model (Fig. 2a–c). Importantly, AYX1 gain in affinity for EGR1 translated into a gain of efficacy for inhibiting EGR1 activity in the cellular context compared to other consensus decoys (Fig. 2a–c). These data demonstrate that AYX1 is a potent and selective EGR1 inhibitor.

Complementary pharmacodynamic experiments were pursued to illustrate AYX1 mechanism of action in rat pheochromocytoma PC12 cells, a cell line model extensively used to study pain-signaling pathways. Egr1 drives the differentiation of those cells into a neuronal-like phenotype following nerve growth factor (NGF) stimulation [6,26]. We measured the activity of AYX1 against 11 pain-related genes in this context. These genes were selected because of their published role in 1 or more pain syndromes, their different roles in pain signaling pathways, and/or the parallel between the regulation of their expression between endogenous nociceptive neurons and PC12 cells. AYX1 treatment blocked the regulation of ~70% of those genes after NGF stimulation in this model (Fig. 2d–f). This result illustrates that AYX1 controls the regulation of genes across multiple regions of pain pathways.

3.3. Prevention of mechanical hypersensitivity

The pathogenesis of post-surgical pain is complex, arising from a combination of inflammatory, neuropathic, and incisional mechanisms. Since a role for Egr1 has been previously illustrated in the maintenance of inflammatory pain [21,30], the acute Complete Freund’s Adjuvant (CFA) model of inflammatory pain was first
Fig. 5. (a) Initial demonstration of AYX1 efficacy in the plantar incisional model (Stanford University, CA). Mean ± SEM values of total responses to repetitive von Frey stimulations for vehicle (triangle), 40 nmoles (square), and 80 nmoles (circle) AYX1–treated animals in the incisional model of pain. VF = von Frey hair, n = 6; † Test + † Welsh analysis, different from vehicle at a given time point. **P < .01; ***P < .001 (P < .14 at 4 hours and 72 hours). Dose response for vehicle versus 40 nmoles versus 80 nmoles AYX1: analysis of variance, P < .006. (b) AYX1 range of effect in the plantar incisional model (Stanford University, CA). Mean ± SEM values of responses to each individual von Frey hair out of 5 stimulations 24 hours after surgery for vehicle-treated (filled bars) and AYX1-treated (open bars) animals in the incisional model of pain (plotted from the experiment displayed in part a above). VF = von Frey hair. n = 6; † Test + † Welsh analysis, different from vehicle. **P < .01; ***P < .001. (c) AYX1 efficacy reproducibility in the incisional model at a second testing laboratory (University of Iowa, IA). Mean ± SEM values of total responses to repetitive von Frey stimulations for vehicle and 200 nmoles AYX1–treated animals; n = 5. † Test + † Welsh analysis, different from vehicle at a given time point; *P < .05; **P < .01. (d) Egr1 expression in rat DRG and spinal cord. Representative detection of Egr1 cDNA on agarose gel in basal condition and 3 hours after plantar surgery. (e) AYX1 marker genes in rat DRG: genes regulated by AYX1 out of an 18-gene screening in rat DRG 3 hours after a plantar incision: Htr3a (green), Bdrkb2 (blue), Cebpg (red). A gene not regulated by AYX1 under the study condition is displayed for illustration (Cdk5r1, purple). Animals were treated with vehicle, 100 nmoles AYX1, or 200 nmoles AYX1. † Test followed by † Welsh analysis. A gene not regulated by AYX1 under the study condition is displayed for illustration (Cdk5r1, purple). Animals were treated with vehicle, 100 nmoles AYX1, or 200 nmoles AYX1; n = 3 to 4. † Test followed by † Welsh analysis showed statistical trends only (eg, P = .13 for Sgk/AYX1 200 nmoles). (f) AYX1 marker genes in canine model. Comparison of the parallel inhibition of PSMB9 (spinal cord) and CEBPG (DRG) gene expression by AYX1 in a rat model (open bars, 200 nmoles AYX1) and canine model (filled bars, 7.09 μmoles AYX1) 3 hours after plantar incision. Data are shown as mean ± SEM of the percentage of inhibition in AYX1–treated animals compared to vehicle-treated animals (0% = no inhibition, 100% = complete inhibition with no gene expression observed); canine model: n = 2. No statistical analysis is provided because of the limited number of dogs that were used in the study for ethical reasons.
selected to examine the efficacy of AYX1. The effect of a pilot drug delivery regimen consisting of 4 doses of 13 nmole AYX1 around the time of injury was tested. After CFA injection in the hindpaw, control rats developed a mechanical hypersensitivity at day 1 that was maintained at day 3. With the AYX1 treatment regimen, mechanical sensitization was reduced at day 1 compared to that in controls and was absent by day 3 (Fig. 3a), consistent with the known role of Egr1 in this model.

Neuropathic pain is a common source of chronic pain after surgery, often triggered by direct damage to nerves during the surgical procedure. After the initial demonstration of efficacy in the CFA model, the efficacy of AYX1 for preventing chronic neuropathic pain was tested using the spared nerve injury model (SNI), in which 2 branches of the sciatic nerve are surgically transected [10]. In this trial, the effects of a single i.t. administration of 2 higher dose levels (40 and 100 nmol) compared with the prior multi-delivery study were tested to mimic the anticipated clinical dosing regimen. Vehicle-treated animals developed a long-lasting mechanical hypersensitivity that lasted for ~4 weeks (Fig. 3b). In contrast, a single i.t. dose of 100 nmol AYX1 at the time of surgery suppressed mechanical hypersensitivity in a constant and robust manner throughout the entire testing period (~75% reduction compared to control, area under the curve analysis), until mechanical hypersensitivity started to resolve in control animals (Fig. 3b). A lower AYX1 dose of 40 nmol reduced mechanical hypersensitivity by ~35% over a 1-week period after injury (data not shown). The specificity of the AYX1 effect was verified by the lack of efficacy of a mutant decoy (Fig. 3b). The reproducibility of AYX1 efficacy was tested in follow-up trials at independent laboratories. In 1 laboratory, the effect of increasing dose levels of AYX1 from 50 to 200 nmol was measured for 10 days after SNI surgery, a time point at which mechanical hypersensitivity is established in control animals. AYX1 displayed a dose–response pattern (ANOVA, P < .001) with an increased amplitude and duration of effect as the dose increased from 50 nmol to the highest tested dose of 200 nmol (Fig. 3c). In the same study, the analysis of the number of withdrawal responses per von Frey monofilament stimulus revealed that AYX1 prevented mechanical hypersensitivity across the full range of mechanical stimulus intensities, from light (1 g) to strong noxious stimuli (26 g) that elicit a paw withdrawal response even in absence of an injury (Fig. 3d). In an additional study at a third laboratory designed to confirm the long-term effect of AYX1, a 200-nmol dose of AYX1 produced a ~70%, sustained protection against mechanical hypersensitivity compared to that in the control group throughout the entire 4-week post-SNI testing period (Fig. 3e). Results were consistent across all testing laboratories: a single i.t. dose of AYX1 at the time of surgery suppressed mechanical hypersensitivity in a dose-related manner and lasted throughout the entire course of hypersensitivity in control animals. We conclude that the peri-operative blockade of Egr1 in the DRG–dorsal horn tissue by an intrathecal bolus injection of AYX1 greatly diminished the nociceptive sequelae that follow nerve injury in rats.

Following nerve injury, the remodeling of the spinal cord dorsal horn contributes to the perpetuation of chronic pain. Neuronal cell death and glial activation are landmark features of this phenomenon that are developed and quantifiable after 1 week following injury [24,31]. At day 8 after SNI, Hoechst and glial fibrillary acidic protein (GFap) staining of dorsal horn histological sections confirmed an increase in those 2 landmark features in control animals (Fig. 4a and b). In contrast, a single AYX1 administration at the time of surgery prevented neuronal cell death and glial activation from developing (Fig. 4a and b). Likely, the sustained protection that AYX1 provides against neuronal hypersensitivity prevents the glial activation and excitotoxicity known to arise from the hyperactive DRG–dorsal horn synapse. This neuroprotective effect may contribute to long-lasting effect of AYX1 in reducing post-injury pain.

Tissue incision is a common denominator to surgical procedures. The acute plantar incisional model mimics such post-surgical pain lasting for ~72 hours [4]. Experiments at 1 laboratory demonstrated that a single injection of 80 nmol i.t. AYX1 before surgery provided robust and sustained prevention of mechanical hypersensitivity compared to that in vehicle-treated animals. The effect was apparent at 4 hours (~35% protection), fully developed at 24 hours (~85% protection), and maintained until pain resolved in control animals at 72 hours post-incision (Fig. 5a). The effect was dose dependent (ANOVA, P < .006): a 40-nmol dose exhibited a shorter and reduced efficacy (~30%–35% protection at 4 hours and 24 hours). Similar to results observed in the SNI model, AYX1 maintained von Frey responses across all mechanical forces tested to near baseline (Fig. 5b). AYX1 efficacy profile in the plantar incisional model was confirmed at a separate laboratory (Fig. 5c). A study was then undertaken in this model to identify molecular biomarkers that could be used to assess AYX1 pharmacological activity across animal species. To do so, the AYX1 effect on the expression level of a selected set of spinal and DRG genes was measured 3 hours after plantar incision. Eighteen genes were selected to conduct the assay (Egr1, Cebpg, Sgk, Psmnb, Htr3a, Syn1, Syn2, Sstm, Cacna1a, Cacna1b, Scn8a, Nrk1, Dyn, Bdkrb2, Pges2, Tap, Cdk5r1, and Nos1) based on their known expression in DRG and/or spinal cord, their induction in tissues after a noxious stimulation, their regulation by Egr1, and/or their participation in pain signaling. The assay was designed to provide a selective but informative snapshot of AYX1 activity shortly after surgery; the 3-hour post-surgical time point of the assay was selected based on practical animal care and use considerations for later testing in higher species. In rats, RT–PCR gene profiling confirmed Egr1 induction in both the spinal cord and the DRG after plantar surgery (mean values of Egr1 transcript expression level normalized on Gapdh: 1.3 vs 2.3 in the spinal cord and 1.3 vs 2.6 in the DRG, arbitrary units) (Fig. 5d). In this assay, AYX1 treatment blocked, in a dose-dependent fashion, the expression of Cebpg, Htr3a, and Bdkrb2 genes in the DRG (Fig. 5e). While the in vivo and PC12 (see Pharmacodynamics section) marker assays were not designed to be replicative, it was noted that AYX1 treatment did not alter

Table 2

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<th>Test</th>
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<th>SEM</th>
<th>P Value</th>
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Effect of vehicle or 200 nmol AYX1 (higest tested dose) on 18 genes was measured in rat L4–L6 dorsal root ganglia (DRG) ipsilateral to injury 3 hours after a plantar incision. Assay was designed to identify DRG marker genes of AYX1 activity from a small number of animals in light of minimizing the use of rats and dogs. A gene was identified as a marker to further test in dogs if its inhibition by AYX1 compared to vehicle was statistically significant or showed a statistical/numerical trend of inhibition after AYX1 treatment of at least ~30% reduction with a maximum variability of ~30%. Gene expression levels were normalized against Gapdh (n = 3–6).
Gene expression levels were normalized against Expression level of AYX1 biomarker genes after incision in vehicle- and AYX1-treated dogs.

Expression level of 18 spinal genes after incision in vehicle- and AYX1-treated rats.

Htra3a expression levels were normalized against incision. Selection criteria for spinal marker genes of AYX1 activity to further test in ensured in rat L4–L6 spinal cord segments ipsilateral to injury 3 hours after a plantar incision. Effect of vehicle or 200 nmoles AYX1 (highest tested dose) on 18 genes was measured in L4–L6 spinal cord segments and DRG (Fig. 5g and Table 4). A potential effect on genes expression in the spinal cord and the PSMB9

Effect of vehicle or 200 nmoles AYX1 (highest tested dose) on 18 genes was measured in rat L4–L6 spinal cord segments ipsilateral to injury 3 hours after a plantar incision. Selection criteria for spinal marker genes of AYX1 activity to further test in dogs were the same as for dorsal root ganglia (DRG) genes (see Table 2). Gene expression levels were normalized against Gapdh (n = 2–4).

**Htra3a and Bdkrb2 gene regulation in the PC12 assay.** This could be explained by differences in experimental conditions between the 2 assays, such as the systems in use and their specificities, the scope and nature of the noxious inputs, the differences in AYX1 treatment paradigms and doses, or the difference in the timing of gene expression measurement. In the spinal cord, a numerical inhibition of Psmb9, Sgk, and Cebpg genes was observed after AYX1 treatment (t test P values were, respectively, .13/.18/.3 for Sgk/Psmb9/Cebpg following AYX1 200 nmoles vs vehicle treatments) (Fig. 5f). Although the inhibition of those genes was not statistically significant, it met the pre-specified selection criteria for markers to be tested in a small number of higher-species animals (see Tables 2–4 legends for selection criteria). No effect was observed on the other tested genes under the assay conditions (Tables 2 and 3). To evaluate the effect of AYX1 in higher animals, the same experiment (unilateral intraplantar incision) was performed in beagle dogs prepared with lumbar intrathecal catheters and the effect of AYX1 on the marker genes identified in rats was tested. A 7.09 µmol dose of AYX1 (ie, corresponding to ~150 nmoles dose in rats corrected for a 1-mL volume of i.t. injection in dogs) was administered to 2 dogs and results were compared to those obtained with 2 vehicle-treated animals. Consistent with results from the rat, EGR1 expression was observed (data not shown) in the dog and AYX1 treatment inhibited CEbpG and Psmb9 genes expression in the spinal cord and the CEBPG gene in DRG (Fig. 5g and Table 4). A potential effect on Bdkrb2 expression was noticed in the DRG at this early time point after surgery, although in the opposite direction compared to the rat. No regulation trend was observed for the other tested markers in the dog (Table 4). Collectively, the spinal cord and DRG biomarker studies in rats and dogs indicate that AYX1 is pharmacologically active across species.

Taken together, these data demonstrate that the focal and temporal blockade of Egr1 function in the DRG–dorsal horn around the time of an injury with a single i.t. administration of AYX1 efficiently prevents behavioral and molecular signs of acute and chronic post-surgical pain. These preventive effects are dose dependent, consistent with the typical pharmacology of a competitive antagonist compound.

**3.4. Functional recovery after surgery**

AYX1 effect on pain-related functional behaviors was evaluated in a rat model of knee surgery that was developed to mimic total knee arthroplasty in patients. In this model, the knee skin is incised, the patellar tendon reflected and 0.5 mm deep holes are drilled in the femur and tibia bones above and below the knee joint. Holes are then filled with cyanoacrylate, the patellar tendon is released and returned to the midline, and the skin is closed with 2 horizontal mattress sutures [5]. After this surgery, the rats’ locomotion is impaired and quantifiable for 72 to 96 hours [5]. Accordingly, vehicle-treated rats distributed considerably more weight to their non-injured limb compared to the injured one (~80-g difference compared to baseline), reflecting a debilitating physical incapacitation. In comparison, a single AYX1 dose of 150 nmoles (a dose level range with robust efficacy in other tested models and studies) before surgery allowed animals to distribute ~50% more weight to their injured limb over the 72 h testing period compared to vehicle controls, demonstrating a robust and sustained prevention of physical disability (Fig. 6a). Rearing and ambulation counts were also measured to assess the general spontaneous activity and locomotion of the animals after surgery. AYX1-treated rats demonstrated substantially greater capacity for functional activity compared to vehicle-treated animals with a ~260% significant increase (Fig. 6b) in rearing and ~135% numerical increase in ambulation. In addition to examining these effects after knee injury, the effect of AYX1 on functional incapacitation was also assessed after spared nerve injury (initial SNI trial); a 100-nmole AYX1 dose had a significant preventive effect (~50%) on weight-bearing incapacitation compared to controls after the first week following surgery (Fig. 6c). These data provide evidence that, beyond its pain prevention feature, the blockade of Egr1 by a single i.t. AYX1 dose improves functional recovery from multiple etiologies.

**4. Discussion**

Persistent post-surgical pain remains a critical, high incidence public health issue. In response to the limitation of standard of care for managing pain, the Food and Drug Administration developed a
task force to facilitate the development of novel pain therapeutics, particularly for preventing chronic post-surgical pain, called ACTTION (Analgesic Clinical Trial Translations Innovations, Opportunities, and Networks) [29]. The design of AYX1 is consistent with the ACTTION goal to prevent perioperative and chronic post-surgical pain. AYX1 works by inhibiting the surgical trauma–induced activity of the transcription factor EGR1 in the DRG and spinal cord dorsal horn. Functioning as a transcription factor decoy, AYX1 binds and masks the EGR1 DNA-binding site with high affinity and specificity, preventing the regulation of EGR1 target genes. A direct translation of high EGR1 affinity and selectivity into a dose-dependent efficacy pattern for altering a pharmacodynamic marker of EGR1 activity in human cells was observed. AYX1 efficacy for inhibiting EGR1 activity was also superior compared to consensus decoys, confirming its potency. There are 2 pivotal components to AYX1 mechanism of action: the prevention of the delayed genomic regulations necessary for pain signaling maintenance in the DRG and spinal cord and the capacity to concurrently affect a large number of pain-related genes. An illustration of these 2 effects was established at the molecular level as AYX1 blocked the delayed regulation of multiple complementary pain genes in PC12 cells following NGF stimulation and in vivo in the DRG and spinal cord tissue after surgery. Consistent with this mechanism of action, a single administration of AYX1 around the time of injury produced a large and sustained prevention of mechanical hypersensitivity (~60%–70% compared to controls) regardless of whether the tested pain model was acute or chronic in nature. This effect was also similar regardless of model modality, including incisional, bone

![Graphs showing AYX1 effect on weight-bearing incapacitance after knee surgery and spared nerve injury.](image)

**Fig. 6.** AYX1 effect on weight-bearing incapacitance after knee surgery (Stanford University, CA). Mean ± SEM of incapacitance during the 72-hour testing period after knee surgery for vehicle-treated (filled bar) or 150-nmoles AYX1–treated (open bar) animals. Incapacitance was calculated as the difference of weight the animals distributed between its hindlimbs (weight from non-injured limb - weight from injured limb); data were corrected for baseline for each animal, n = 10. t Test followed by a t Welsh analysis for data distributions over the 72-hour testing period, AYX1 versus vehicle: P = .0005. (b) AYX1 effects on spontaneous mobility after knee surgery. Mean ± SEM of rearing counts during the 72-hour testing period after knee surgery for vehicle-treated (filled bar) or 150 nmoles AYX1-treated (open bar) animals; rearing counts were measured each day over a 30 min period; data were normalized on baseline for each animal (arbitrary units), n = 8 or 9. t Test followed by a t Welsh analysis for data distributions over the 72-hour testing period; AYX1 versus vehicle: P = .05. (c) AYX1 effect on weight bearing incapacitance after spared nerve injury. Mean ± SEM of incapacitance for vehicle-treated (filled bar) or 100-nmoles AYX1–treated (open bar) animals (measured during the initial chronic neuropathic pain (SNI) trial depicted in Fig. 3A). Results are presented as the average incapacitance during the first week after SNI (where no AYX1 effect was observed on functional recovery) and the remaining testing weeks 2 to 4 after SNI, n = 5 rats per condition. t test followed by a t Welsh analysis for data distributions during weeks 2 to 4 after SNI. AYX1 versus vehicle: P = .02.
injury (knee), inflammatory, and neuropathic pain models that mimic the complementary etiologies of acute and chronic post-surgical pain. These results are consistent with the development of pain that is associated with EGR1 induction in response to numerous types of injurious and noxious stimuli.

The AYX1 preventive effect was maintained for the duration of mechanical hypersensitivity in control animals, suggesting the feasibility of an effective pain prevention treatment in patients. The effect on mechanical hypersensitivity, a surrogate preclinical endpoint for movement-associated pain in patients, was significant for both light and robust mechanical stimuli already painful without injury, suggesting that AYX1 is active against both allodynia and hyperalgesia associated with post-traumatic movement. Furthermore, AYX1 injection reduced static lower extremity incapacitation after knee surgery or nerve injury and allowed greater spontaneous activity compared to that in control animals. These results demonstrate that the capacity of AYX1 to suppress pain translates efficiently into an improved functional status after surgery compared to that in control animals. The reproducibility of AYX1 efficacy by several independent laboratories demonstrates the robustness of its effect. The observed variations in the dose levels that produced a similar efficacy across laboratories were attributed to technical differences in the model performance and testing, as illustrated by the differences in the absolute von Frey response levels in control groups. Regardless, the same 3 components of AYX1 dose-response were clearly identified across studies: the intensity of the prevention effect, its duration, and time to effect. The greater the inhibition of EGR1 activity around the time of injury, the faster and the more robust the attenuation of post-surgical pain sequelae observed.

The reproducibility of AYX1 activity on EGR1 biomarkers in rat and canine DRG and spinal cord after surgery suggests that AYX1 pharmacology is conserved across species. These results are consistent with the independent body of literature showing the conservation of EGR1 structure (90% identity between the rat and human protein, and 100% identity for its DNA-binding site as per NCBI Blast alignment analyses) and biology across several species [20]. The current work suggests that the “pain switch” feature of EGR1 is conserved across mammalian species, and highlights the clinical therapeutic potential of AYX1 for preventing pain and facilitating physical rehabilitation after surgery.

Conflict of interest statement

J.M., D.M. and S.H. are Adynxx employees and shareholders. T.Y., B.T., W.S., and D.Y. are Adynxx consultants and/or shareholders.

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