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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.

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1. Introduction

With 30 million surgical procedures performed annually in the United States, post-surgical pain constitutes a public health issue [11]. Its most debilitating component is the mechanical hypersensitivity associated with movement-evoked pain, which is poorly treated by current therapeutics [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 [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 [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 [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,

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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 AYY1, 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 AYY1 would block EGR1 function, we performed in vitro experiments examining its effects of AYY1 on molecular and cellular reactions involving EGR1. To determine the functional consequences of EGR1 blockade in vivo, we examined the effect of AYY1 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 AYY1 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'-GTTATGCGTGGCGGTAGATGCGGGCGGTATAG-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, not 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. AYY1: sense strand: 5'-GTATGCGTGGCGGTGGGCGTAG-3', EGR1 single consensus decoy: sense strand: 5'-GATGCGTGGGCGTAGG-3', EGR1 tandem consensus decoy: sense strand: 5'-GTTATGCGGGGCGGGCGGGCGGGCGGTTTAC-3', SP1 decoy: sense strand: 5'-GTTATGCGTTTGTAGATGCTTTCGTTATAG-3', EGR1 mutant probe: sense strand: 5'-GTTATGCGTTTGTAGATGCTTTCGTTATAG-3', antisense strand: 5'-CTATTTGAAACGATCTACATTGGCATAAC-3'.

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) + 10% 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 µmol/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/l-glutamine/HEPES/NaPyr (Invitrogen, Carlsbad, CA) + 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA) + 10% FBS (Invitrogen, Carlsbad, CA) + 5% equine serum (Invitrogen, Carlsbad, CA). Cells were transfected with AYY1 (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*, *Pnmt*, *Nos1*) and neurotransmitter (*Bdnf*). For in vivo trials, rats were anesthetized with 2% isoflurane, i.t. AYY1, or vehicle administered via a percutaneous lumbar intrathecal injection, a plantar incision performed on the left paw 1 hour later after the plantar incisional model. Animals maintained on anesthesia for 3 additional hours and sacrificed for L4 to L6 DRG and spinal cord collection. Dogs were sedated (atropine 0.04 mg/kg, i.m.; xylazine (0.4 mg/mL, 0.04 mg/kg), with anesthesia masked-induced; animals were intubated, anesthesia and maintained with 1% to 2% isoflurane in O₂/N₂O at 40%/60%. i.t. AYY1 or vehicle were administered via a lumbar intrathecal catheters [1] and after 1 hour, a plantar incision similar to the rat plantar incisional 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 RNEasyplus 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).

2.3. 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 Gfap 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

Table 1
Polymerase chain reaction primers.

Primer	5'–3' Orientation
ACTB AS	TACATGGCTGGGGTGTGAA
ACTB S	AAGAGAGGCATCCTCACCCT
Bdkrb2 AS	CCGTCTGGACCTCCTTGAAAC
Bdkrb2 S	GAACATCTTTGTCTCAGC
Bdnf AS	GTAGGCCAAGTTGCCTTGTCCT
Bdnf S	GGCTTTGATGAGACCGGGTTCCT
Cacna1a AS	CATGGGCTTTGGGCGCTCT
Cacna1a S	CACCAACCTGGTCCCGCT
Cacna1b AS	TTGTCCATGATCAGCAAC
Cacna1b S	ATGCTGTTCTTCATCTACGC
Cdk5r1 AS	CTTCTGTCTCTCTGACACTC
Cdk5r1 S	GCTCTGACGGGATGTTATCTCC
CDK5R1 AS	GTCCGGCATTATCTGCAGCA
CDK5R1 S	GCCGTACAGAACAGCAAGAA
Cebpg AS	ACGTTGTCTGCGAGGCTGTGCCGATGC
Cebpg S	GGCCGCTCGGAGTGAGGCGCTGTGG
Dyn AS	TGTGTGGAGAGGACATCTCA
Dyn S	GGGTCGCTGGATTCAATA
Egr1 AS	AGTAAATGGGACTGTCTGCG
Egr1 S	AGATGATGTCTGAGCAAC
Gapdh AS	GGCATGTGATCCACACCGATAC
Gapdh S	CCGCTGATGCCCCATGTTTGAT
Gch1 AS	AGGCTGCAAGGCTTCTGTGATGGC
Gch1 S	CCACGCCATGCAAGTCTTACCA
Grm5 AS	GTGGCCGCGGTGGACAACAT
Grm5 S	GTGGCCGAGGAGAGGAGAGC
Htr3a AS	GAGGACAGCTCTTGCAAGAGGC
Htr3a S	AATCAGGGCGAGTGGGAGC
Nos1 AS	TCCTCCAGGAGGCTGTCACCGCA
Nos1 S	GAATACCAGCCTGATCCATGGAAC
Nr1 AS	GTCATGTGATGCCCGTGATGC
Nr1 S	GTCCTCTGCCATGTGGTTT
P2rx3 AS	CAGTGGCCTGGTACTGGCGA
P2rx3 S	TGGCGTTCTGGGTATTAAGATCGG
Pge2s AS	CCGCTTCCACAGGATCT
Pge2s S	GGCCTCTTTCGACTACATCG
Pnmt AS	TTATTAGGTGCCACTTCGGGTG
Pnmt S	CAGACTTCTGGAGGTCAACCTG
Psmb9 AS	CCAGAGTGATGGCGTCTGTGGTGAA
Psmb9 S	AGTCCACACCGGACAACCATCATG
Scn8a AS	TTGTTGACAATATCGATTTCGAACCG
Scn8a S	AAGTGGACAGCTACGGC
Scn9a AS	TCTCTCGAGTCTCTTCCTG
Scn9a S	TTCATGACCTTGAGCAACCC
Sgk AS	AGGAGCGAGATACTCAGGCGTGCCACA
Sgk S	AACCCCTCACCTCTCCAAGTCCCTCTCA
Stx4 AS	GGTGATGCCAATGATGACAGCCA
Stx4 S	CGACAGGACCCATGAGTTGAGG
Syn1 AS	GTTGACCACAAGTCCACGAT
Syn1 S	CCCTTCATTGATGCTAAATACG
Syn2 AS	CCGGTTCCTTAAGTGTCCA
Syn2 S	ATGACATCCGGGTTTCTGAGG
Tap1 AS	CAGTTTCACTACCTCTGTGTATAGCCCT
Tap1 S	TGGACCATGAGTGTCTCGGAATGC

contralateral to surgery. 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 that appear homogenous after Hoechst staining. Analysis of histological sections was conducted in a blinded fashion.

2.5. Animals

Sprague-Dawley rats (Harlan Industries, Livermore, CA), male, 250 to 300 g were used. In addition, beagle dogs (Marshall Farms, North Rose, NY), male, 10 to 15 kg, aged 12 to 24 months, were used.

2.6. Control and test article administration

Either vehicle, AYX1 or mutant decoy (sense: 5'-GTTATGCC TTTGTAGATGCTTTCGTTATAG-3', antisense: 5'-CTATTTGAAACGA

TCTA CATTGGCATAAC-3') were delivered via a single bolus, percutaneous 20 µL i.t. injection as previously described [25]. Rats were anesthetized with 2% isoflurane, and the lumbar region of the back and lateral surface of the left thigh shaved. A 50-µL Hamilton syringe with an attached 27-gauge needle was inserted between L5 and L6 until the intrathecal space was reached as indicated by tail twitch, and then articles were injected. The i.t. injections were performed once at 1 hour before surgery for the incisional and knee surgery models, once at the time of surgery for the SNI model and at –24 hours, –1 hour, +24 hours, and +72 hours relative to injury in the CFA model.

2.7. Behavioral testing

The plantar incisional, CFA, 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 CFA 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, incapacitance was measured as the difference in static weight bearing between the ipsilateral versus contralateral hind paw relative to the injury using an incapacitance 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 period. 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 *t* test, followed by a *t* Welch 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 OD_{450nm} 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 OD_{450nm} 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 OD_{450nm} 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 μ mol/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. * $P \leq .05$, different from the single EGR1 consensus decoy, values are given as mean $\pm$ SEM; $n \geq 3$. (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. (e) Inhibition of pain-related gene regulation 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*. * $P \leq .05$, different from control ($P \leq 0.1$ for *Bdnf*/NGF/forskolin and *Bdnf*/NGF/forskolin/AYX1 vs *Bdnf*/control); $n = 2$ to 4. (f) 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; $n \geq 3$.

Fig. 3. (a) AYX1 efficacy in the CFA model. Mean $\pm$ SEM values of total responses to repetitive von Frey (1.6 and 15 g) stimulations for vehicle (filled bars) or 13 nmoles 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. $^{*}P \leq .05$ ($P = .06$ at 24 hours). (b) Initial AYX1 efficacy demonstration in the chronic neuropathic pain (SNI) model (Stanford University, CA). Mean $\pm$ SEM values of total responses to repetitive von Frey stimulations after vehicle (open triangles) and AYX1 100 nmoles (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 nmoles) versus AYX1 (100 nmoles) and vehicle; $n \geq 5$ rats per condition. t Test followed by a t Welch analysis, different from vehicle at a given time point. $^{*}P \leq .05$, $^{****}P \leq 0.0001$ ($P \leq 0.1$ at 4 weeks), vehicle versus AYX1 data distribution: $P = 1.10^{-4}$. (c) AYX1 efficacy reproducibility and dose response in the SNI model at a separate testing laboratory (University of Arizona, AZ). Mean $\pm$ SEM values of total responses to von Frey stimulations after vehicle (open downward triangles) or AYX1 administration at various dose levels: 50 nmoles (open circles), 100 nmoles (filled upward triangles) or 200 nmoles (filled squares) over a 10 post-operative days time course, $n \geq 4$. t Test followed by a t Welch analysis, different from vehicle at a given time point; $^{**}P \leq .01$, $^{****}P \leq 0.0001$, dose response: vehicle versus 50 nmoles versus 100 nmoles versus 200 nmoles, analysis of variance, $P \leq .0001$. (d) AYX1 range of effect in the SNI model (University of Arizona, AZ). Mean $\pm$ 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 nmoles AYX1; dotted bars denote 100 nmoles AYX1; open bars denote 200 nmoles AYX1. t Test followed by a t Welch analysis, different from vehicle. $^{**}P \leq .01$, $^{****}P \leq .0001$. (e) AYX1 efficacy reproducibility in the SNI model at a third testing laboratory (University of Kentucky, KY). Median $\pm$ 40th and 60th percentile values of total responses to repetitive von Frey stimulations for vehicle-treated (triangle) and 200-nmoles AYX1-treated (circle) animals over a 4 week post-surgical period are shown; $n = 4$. t Test followed by a t Welch 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 AXX1 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 AXX1 binds to EGR1 with high affinity and specificity.

3.2. Pharmacodynamics

The capacity of AXX1 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 D381 [7]. AXX1 transfection prevented, in a dose-dependent manner, the up-regulation of *CDK5R1* in this model (Fig. 2a–c). Importantly, AXX1 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 AXX1 is a potent and selective EGR1 inhibitor.

Complementary pharmacodynamic experiments were pursued to illustrate AXX1 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 AXX1 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. AXX1 treatment blocked the regulation of ~70% of those genes after NGF stimulation in this model (Fig. 2d–f). This result illustrates that AXX1 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. 4. (a) Spinal cell death. Cell death was measured in the dorsal horn (L4–L6 spinal cord segment), ipsilateral and contralateral to injury by the density of pyknotic cell nuclei (Hoechst staining) 8 days after spared nerve injury (SNI) surgery; pyknotic nuclei appear condensed, non-homogenous, and bright. (b) Spinal neuro-inflammation. Neuroinflammation was measured by the quantitation of Gfap immunostaining (red) surface in the ipsilateral and contralateral L4–L6 dorsal horns from the same cohorts of animals. Rats were treated with either a single administration of 100 nmoles AXX1 or inactive EGR1 mutant decoy just before surgery. Data were compared to normal cell death level measured in naive rats. Open bars denote baseline; filled bars denote AXX1, shaded (gray) bars denote SNI/mutant decoy; ipsi/contra denotes dorsal horn ipsilateral or contralateral to surgery. Left panel: $n = 3$ rats per condition, mean $\pm$ SEM; right panel: $n = 2$ to 3 per condition, mean $\pm$ SEM, different from AXX1 treatment, * $P \leq .05$ ($P = .1$ for contralateral mutant decoy/pyknotic nuclei).

Fig. 5. (a) Initial demonstration of AYX1 efficacy in the plantar incisional model (Stanford University, CA). Mean $\pm$ 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, $n \geq 6$. t Test + t Welch analysis, different from vehicle at a given time point. $***P \leq .001$; $****P \leq .0001$ ($P \leq .14$ at 4 hours and 72 hours). Dose response for vehicle versus 40 nmoles versus 80 nmoles AYX1: analysis of variance, $P \leq .006$. (b) AYX1 range of effect in the plantar incisional model (Stanford University, CA). Mean $\pm$ 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, $n \geq 6$; t Test + t Welch analysis, different from vehicle. $**P \leq .01$; $***P \leq .001$. (c) AYX1 efficacy reproducibility in the incisional model at a second testing laboratory (University of Iowa, IA). Mean $\pm$ SEM values of total responses to repetitive von Frey stimulations for vehicle and 200 nmoles AYX1-treated animals; $n \geq 5$. t Test + t Welch analysis, different from vehicle at a given time point; $*P \leq .05$; $**P \leq .01$. (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. t Test followed by a t Welch analysis. $*P \leq .05$ ($P \leq .1$ for *Htr3a*/AYX1 100 nmoles and *Bdrkb2*/AYX1 100 nmoles); $n = 3$ to 5. (f) AYX1 marker genes in rat spinal cord. Gene regulation by AYX1 in rat spinal cord 3 hours after a plantar incision in the same screening assay as above: *Sgk* (blue); *Psmb9* (red); *Cebpg* (green). Animals were treated with vehicle, 100 nmoles AYX1, or 200 nmoles AYX1; $n = 3$ to 4. t Test followed by a t Welch analysis showed statistical trends only (eg, $P = .13$ for *Sgk*/AYX1 200 nmoles). (g) AYX1 marker genes in canine model. Comparison of the parallel inhibition of *PSMB9* (spinal cord) and *CEBPG* (DRG) genes expression by AYX1 in a rat model (open bars, 200 nmoles AYX1) and canine model (filled bars, 7.09 μ moles AYX1) 3 hours after plantar incision. Data are shown as mean $\pm$ 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 nmoles 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 nmoles) 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 nmoles 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 nmoles 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 nmoles 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, $P \leq .001$) with an increased amplitude and duration of effect as the dose increased from 50 nmoles to the highest tested dose of 200 nmoles (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 nociceptive 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-nmole 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 local 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 nmoles 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, $P \leq .006$): a 40-nmole 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*, *Psm9*, *Htr3a*, *Syn1*, *Syn2*, *Stx4*, *Cacna1a*, *Cacna1b*, *Scn8a*, *Nr1*, *Dyn*, *Bdkrb2*, *Pges2*, *Tap*, *Cdk5r1*, and *Nos1*) based on their known expression in DRG and/or spinal cord, their induction in those 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

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

	Vehicle			AYX1 200 nmoles			t Test
	Mean	SD	SEM	Mean	SD	SEM	Vehicle vs AYX1
<i>Bdkrb2</i>	0.93	0.37	0.18	0.10	0.07	0.04	0.01
<i>Cacna1a</i>	0.94	0.72	0.41	1.83	1.56	0.78	0.18
<i>Cacna1b</i>	0.65	0.61	0.35	1.56	1.23	0.62	0.13
<i>Cdk5r1</i>	0.82	0.60	0.27	0.67	0.12	0.05	0.27
<i>Cebpg</i>	1.69	0.91	0.41	0.34	0.42	0.24	0.01
<i>Dyn</i>	0.66	0.42	0.24	1.73	1.73	1.00	0.20
<i>Egr1</i>	2.53	0.42	0.24	2.29	0.57	0.33	0.33
<i>Htr3a</i>	0.52	0.44	0.18	0.05	0.07	0.04	0.02
<i>Nos1</i>	0.29	0.26	0.15	0.86	1.25	0.72	0.26
<i>Nr1</i>	2.17	0.32	0.18	2.47	2.47	1.23	0.41
<i>Pges2</i>	0.23	0.22	0.13	0.51	0.88	0.51	0.32
<i>Psm9</i>	0.42	0.39	0.22	1.24	1.05	0.53	0.11
<i>Scn8a</i>	0.81	0.71	0.35	1.41	1.26	0.63	0.23
<i>Sgk</i>	0.59	0.56	0.32	1.13	0.98	0.49	0.20
<i>Stx4</i>	0.36	0.31	0.18	0.22	0.31	0.22	0.33
<i>Syn1</i>	2.27	2.01	1.16	3.38	3.82	1.91	0.32
<i>Syn2</i>	2.24	1.55	0.89	3.95	3.11	1.55	0.19
<i>Tap1</i>	1.01	1.29	0.74	1.35	1.04	0.60	0.37

Effect of vehicle or 200 nmoles AYX1 (highest 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* ($n = 3–6$).

Table 3

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

	Vehicle			AYX1 200 nmoles			<i>t</i> Test Vehicle vs AYX1
	Mean	SD	SEM	Mean	SD	SEM	
<i>Bdkrb2</i>	0.56	0.41	0.24	0.75	0.09	0.07	0.26
<i>Cacna1a</i>	0.97	0.43	0.25	1.29	0.33	0.23	0.21
<i>Cacna1b</i>	0.66	0.68	0.40	0.76	0.35	0.25	0.43
<i>Cdk5r1</i>	1.74	1.49	0.75	1.41	0.54	0.38	0.26
<i>Cebpg</i>	0.59	0.41	0.20	0.45	0.26	0.15	0.30
<i>Dyn</i>	0.52	0.63	0.36	0.45	0.29	0.21	0.43
<i>Egr1</i>	2.08	0.35	0.09	1.95	1.29	0.75	0.47
<i>Htr3a</i>	0.17	0.30	0.15	0.21	0.01	0.01	0.41
<i>Nos1</i>	0.15	0.18	0.10	0.12	0.15	0.11	0.31
<i>Nr1</i>	1.59	0.31	0.18	1.43	0.19	0.13	0.26
<i>Pges2</i>	0.02	0.01	0.00	0.01	0.01	0.01	0.31
<i>Psm9</i>	0.22	0.20	0.10	0.12	0.05	0.03	0.18
<i>Scn8a</i>	0.46	0.77	0.40	0.61	0.84	0.60	0.43
<i>Sgk</i>	0.61	0.67	0.33	0.14	0.21	0.12	0.13
<i>Stx4</i>	0.62	0.39	0.23	1.00	0.10	0.07	0.12
<i>Syn1</i>	1.82	0.62	0.36	1.92	0.74	0.52	0.45
<i>Syn2</i>	1.34	0.79	0.46	1.91	0.77	0.55	0.25
<i>Tap1</i>	0.17	0.05	0.03	0.23	0.14	0.10	0.32

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* ($n = 2-4$).

Htr3a 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 *Psm9*, *Sgk*, and *Cebpg* genes was observed after AYX1 treatment (*t* test *P* values were, respectively, .13/.18/.3 for *Sgk/Psm9/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 μ mol 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

Table 4

Expression level of AYX1 biomarker genes after incision in vehicle- and AYX1-treated dogs.

	DRG						Spinal cord					
	Vehicle			AYX1			Vehicle			AYX1		
	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM	Mean	SD	SEM
<i>BDKRB2</i>	0.15	0.10	0.05	0.86	0.77	0.55	—	—	—	—	—	—
<i>CDK5R1</i>	1.00	0.51	0.26	0.70	0.11	0.07	—	—	—	—	—	—
<i>CEBPG</i>	0.53	0.45	0.22	0.08	0.12	0.08	1.53	2.15	1.07	0.00	0.00	0.00
<i>PSMB9</i>	—	—	—	—	—	—	0.11	0.14	0.07	0.00	0.00	0.00
<i>SGK</i>	—	—	—	—	—	—	0.61	0.39	0.19	0.52	0.73	0.52

Effect of vehicle or 7.09 μ moles AYX1 on the DRG and spinal marker genes identified in rats (see Tables 2 and 3) was measured in L4–L6 spinal cord segments and DRG ipsilateral to injury 3 hours after a plantar incision. No DRG *HTR3A* transcript was detected under our experimental conditions, neither in vehicle nor in AYX1-treated dogs. Gene expression levels were normalized against *GAPDH* ($n = 2$).

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 AYY1 is consistent with the ACTTION goal to prevent perioperative and chronic post-surgical pain.

AYY1 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, AYY1 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. AYY1 efficacy for inhibiting EGR1 activity was also superior compared to consensus decoys, confirm-

ing its potency. There are 2 pivotal components to AYY1 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 AYY1 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 AYY1 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

Fig. 6. AYY1 effect on weight-bearing incapacitation after knee surgery (Stanford University, CA). Mean $\pm$ SEM of incapacitation during the 72-hour testing period after knee surgery for vehicle-treated (filled bar) or 150-nmoles AYY1-treated (open bar) animals. Incapacitation 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 Welch analysis for data distributions over the 72-hour testing period, AYY1 versus vehicle: $P = .0005$. (b) AYY1 effects on spontaneous mobility after knee surgery. Mean $\pm$ SEM of rearing counts during the 72-hour testing period after knee surgery for vehicle-treated (filled bar) or 150 nmoles AYY1-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 Welch analysis for data distributions over the 72-hour testing period; AYY1 versus vehicle: $P = .05$. (c) AYY1 effect on weight bearing incapacitation after spared nerve injury (SNI) trial depicted in Fig. 3A). Results are presented as the average incapacitation during the first week after SNI (where no AYY1 effect was observed on functional recovery) and the remaining testing weeks 2 to 4 after SNI, $n \geq 5$ rats per condition. t test followed by a t Welch analysis for data distributions during weeks 2 to 4 after SNI. AYY1 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 AXY1 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 AXY1 is active against both allodynia and hyperalgesia associated with post-traumatic movement. Furthermore, AXY1 injection reduced static lower extremity incapacity after knee surgery or nerve injury and allowed greater spontaneous activity compared to that in control animals. These results demonstrate that the capacity of AXY1 to suppress pain translates efficiently into an improved functional status after surgery compared to that in control animals. The reproducibility of AXY1 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 AXY1 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 AXY1 activity on EGR1 biomarkers in rat and canine DRG and spinal cord after surgery suggests that AXY1 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 AXY1 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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References

- [1] Allen JW, Mantyh PW, Horais K, Tozier N, Rogers SD, Ghilardi JR, Cizkova D, Grafe MR, Richter P, Lappi DA, Yaksh TL. Safety evaluation of intrathecal substance P-saporin, a targeted neurotoxin, in dogs. *Toxicol Sci* 2006;91:286–98.
- [2] Apfelbaum JL, Chen C, Mehta SS, Gan TJ. *Anesth Analg* 2003;97:534–40.
- [3] Beckmann AM, Wilce PA. Egr transcription factors in the nervous system. *Neurochem Int* 1997;31:477–510 [discussion 517–476].
- [4] Brennan TJ, Vandermeulen EP, Gebhart GF. Characterization of a rat model of incisional pain. *PAIN®* 1996;64:493–501.
- [5] Buvaendran A, Kroin JS, Kari MR, Tuman KJ. A new knee surgery model in rats to evaluate functional measures of postoperative pain. *Anesth Analg* 2008;107:300–8.
- [6] Cao XM, Koski RA, Gashler A, McKiernan M, Morris CF, Gaffney R, Hay RV, Sukhatme VP. Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. *Mol Cell Biol* 1990;10:1931–9.
- [7] Chen F, Wang Q, Wang X, Studzinski GP. Up-regulation of Egr1 by 1,25-dihydroxyvitamin D3 contributes to increased expression of p35 activator of cyclin-dependent kinase 5 and consequent onset of the terminal phase of HL60 cell differentiation. *Cancer Res* 2004;64:5425–33.
- [8] Christy B, Nathans D. DNA binding site of the growth factor-inducible protein Zif268. *Proc Natl Acad Sci USA* 1989;86:8737–41.
- [9] Costigan M, No Woolf CJ. No DREAM, no pain. Closing the spinal gate. *Cell* 2002;108:297–300.
- [10] Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *PAIN®* 2000;87:149–58.
- [11] DeFrances CJ, Lucas CA, Buie VC, Golosinskiy A. National hospital discharge survey. *Natl Health Stat Report* 2008 2006;5:1–20.
- [12] Hamilton TB, Borel F, Romaniuk PJ. Comparison of the DNA binding characteristics of the related zinc finger proteins WT1 and EGR1. *Biochemistry* 1998;37:2051–8.
- [13] Herdegen T, Kovary K, Leah J, Bravo R. Specific temporal and spatial distribution of JUN, FOS, and KROX-24 proteins in spinal neurons following noxious transsynaptic stimulation. *J Comp Neuro* 1991;313:178–91.
- [14] Herdegen T, Leah JD. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res Brain Res Rev* 1998;28:370–490.
- [15] Hucho T, Levine JD. Signaling pathways in sensitization: toward a nociceptor cell biology. *Neuron* 2007;55:365–76.
- [16] James AB, Conway AM, Morris BJ. Genomic profiling of the neuronal target genes of the plasticity-related transcription factor—Zif268. *J Neurochem* 2005;95:796–810.
- [17] Johansen A, Romundstad L, Nielsen CS, Schirmer H, Stubhaug A. Persistent postsurgical pain in a general population: prevalence and predictors in the Tromsø study. *PAIN®* 2012;153:1390–6.
- [18] Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, Charnay P, Bozon B, Laroche S, Davis S. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci* 2001;4:289–96.
- [19] Kehlet H, Jensen TS, Woolf CJ. Persistent postsurgical pain: risk factors and prevention. *Lancet* 2006;367:1618–25.
- [20] Khachigian LM. Early growth response-1 in cardiovascular pathobiology. *Circ Res* 2006;98:186–91.
- [21] Ko SW, Vadakkan KI, Ao H, Gallitano-Mendel A, Wei F, Milbrandt J, Zhuo M. Selective contribution of Egr1 (zif/268) to persistent inflammatory pain. *J Pain* 2005;6:12–20.
- [22] Lacroix-Fralich ML, Tawfik VL, Tanga FY, Spratt KF, DeLeo JA. Differential spinal cord gene expression in rodent models of radicular and neuropathic pain. *Anesthesiology* 2006;104:1283–92.
- [23] Lavand'homme P. The progression from acute to chronic pain. *Curr Opin Anaesthesiol* 2011;24:545–50.
- [24] Lee JW, Siegel SM, Oaklander AL. Effects of distal nerve injuries on dorsal-horn neurons and glia: relationships between lesion size and mechanical hyperalgesia. *Neuroscience* 2009;158:904–14.
- [25] Mestre C, Pellissier T, Fialip J, Wilcox G, Eschaliere A. A method to perform direct transcutaneous intrathecal injection in rats. *J Pharmacol Toxicol Methods* 1994;32:197–200.
- [26] Milbrandt J. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* 1987;238:797–9.
- [27] Miner JN, Diamond MI, Yamamoto KR. Joints in the regulatory lattice: composite regulation by steroid receptor-AP1 complexes. *Cell Growth Differ* 1991;2:525–30.
- [28] Pareek TK, Keller J, Kesavapany S, Pant HC, Iadarola MJ, Brady RO, Kulkarni AB. Cyclin-dependent kinase 5 activity regulates pain signaling. *Proc Natl Acad Sci USA* 2006;103:791–6.
- [29] Rappaport BA, Cerny I, Sanhai WR. ACTION on the prevention of chronic pain after surgery: public-private partnerships, the future of analgesic drug development. *Anesthesiology* 2010;112:509–10.
- [30] Rygh LJ, Suzuki R, Rahman W, Vonsy JL, Sandhu H, Webber M, Hunt S, Dickenson AH. Local and descending circuits regulate long-term potentiation and zif268 expression in spinal neurons. *Eur J Neurosci* 2006;24:761–72.
- [31] Scholz J, Broom DC, Youn DH, Mills CD, Kohno T, Suter MR, Moore KA, Decosterd I, Coggeshall RE, Woolf CJ. Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. *J Neurosci* 2005;25:7317–23.
- [32] Stein C, Millan MJ, Herz A. Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. *Pharmacol Biochem Behav* 1988;31:445–51.
- [33] Tverberg LA, Russo AF. Regulation of the calcitonin/calcitonin gene-related peptide gene by cell-specific synergy between helix-loop-helix and octamer-binding transcription factors. *J Biol Chem* 1993;268:15965–73.
- [34] Tverskoy M, Oren M, Dashkovsky I, Kissin I. Alfentanil dose-response relationships for relief of postoperative pain. *Anesth Analg* 1996;83:387–93.
- [35] Wilder-Smith CH, Hill L, Wilkins J, Denny L. Effects of morphine and tramadol on somatic and visceral sensory function and gastrointestinal motility after abdominal surgery. *Anesthesiology* 1999;91:639–47.
- [36] Woolf CJ, Costigan M. Transcriptional and posttranslational plasticity and the generation of inflammatory pain. *Proc Natl Acad Sci USA* 1999;96:7723–30.