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The BDNF Protein and its Cognate mRNAs in the Rat Spinal Cord during Amylin-induced Reversal of Morphine Tolerance

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Abstract—The pancreatic peptide, Amylin (AMY), reportedly affects nociception in rodents. Here, we investigated the potential effect of AMY on the tolerance to morphine and on the expression of BDNF at both levels of protein and RNA in the lumbar spinal cord of morphine tolerant rats. Animals in both groups of control and test received a single daily dose of intrathecal (i.t.) morphine for 10 days. Rats in the test group received AMY (1, 10 and 60 pmoles) in addition to morphine from days 6 to10. Morphine tolerance was established at day 5. AMY alone showed enduring antinociceptive effects for 10 days. Real-Time PCR, western blotting and ELISA were used respectively to assess levels of BDNF transcripts and their encoded proteins. Rats tolerant to i.t. morphine showed increased expression of exons I, IV, and IX of the BDNF gene, and had elevated levels of pro-BDNF and BDNF protein in their lumbar spinal cord. AMY, when co-administered with morphine from days 6 to 10, reversed morphine tolerance and adversely affected the morphine-induced expression of the BDNF gene at both levels of protein and mRNAs containing exons I, IV and IX. AMY alone increased levels of exons I and IV transcripts. Levels of pro-BDNF and BDNF proteins remained unchanged in the lumbar spinal cord of rats treated by AMY alone. These results suggest that i.t. AMY not only abolished morphine tolerance, but also reduced the morphine induced increase in the expression of both BDNF transcripts and protein in the lumbar spinal cord.

Key words: morphine tolerance, amylin, brain derived neurotrophic factor.

INTRODUCTION

The pancreatic hormone, Islet amyloid polypeptide or Amylin (AMY), works with insulin in glucose regulation (Young, 2005) and energy balance (Boyle et al., 2011). AMY belongs to the calcitonin family of peptides that also includes two calcitonin gene related peptides (αCGRP and βCGRP) and Adrenomedullin (Muff et al., 2004; Russell et al., 2014). These peptides share some components in their multimeric receptors (Barwell et al., 2012) which are widely distributed in various peripheral tissues and in the central nervous system (CNS), where they mediate a wide variety of biological functions (Lee et al., 2016). Specifically, Both CGRP and Adrenomedullin are localized in neurons in dorsal root ganglion (DRG) (Gibson et al., 1984; Ma et al., 2006; Staton et al., 2007) and show pronociceptive (Brain and Grant, 2004; Ma et al., 2006) and proinflammatory properties (Gibson et al., 1984; Hokfelt et al., 1992; Wang et al., 2014). We have also reported that both CGRP and Adrenomedullin are stimulatory to spinal neurons in terms of increased cAMP accumulation (Takhshid et al., 2006) and induced c-Fos expression (Takhshid et al., 2004). CGRP (Menard et al., 1996) and Adrenomedullin (Wang et al., 2016) have also been suggested to contribute to the development of tolerance to opioids, as their receptor antagonists prevented the development of tolerance to morphine (Powell et al., 2000; Wang et al., 2011). In situ hybridization results show the expression of the mRNA for AMY in the DRG of rats (Mulder et al., 1995). Although the presence of AMY transcripts in the DRG of rats was also demonstrated in other works (Ferrier et al., 1989; Nicholl et al., 1992; Mulder et al., 1995), but the mRNA expression of this transcript in the spinal cord is not demon-

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Abbreviations: AMY, islet amyloid polypeptide or Amylin; BDNF, Brain-derived neurotrophic factor; CNS, central nervous system; DRG, dorsal root ganglion.

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strated yet. Nonetheless, AMY-like immunoreactivity exists in motor neurons and deep lamina of spinal cord (Skofitsch et al., 1995; Huang et al., 2010). AMY like immunoreactivity has also been observed in the neurons from DRG of rats (Ferrier et al., 1989; Nicholl et al., 1992; Mulder et al., 1995) in CGRP and/or substance P containing neurons (Mulder et al., 1995). Finally, it is noteworthy that, AMY has also shown to pass the blood-brain barrier (Banks et al., 1995). To unravel the role of AMY in sensory neurons, the expression of AMY in DRG and its distribution in the dorsal horn were investigated after unilateral adjuvant-induced inflammation in the rat paw. Results of this study suggested a role for AMY in the initial phase of localized inflammation (Mulder et al., 1995). Our previous results in the behavioral and c-Fos studies revealed an antinociceptive effect for AMY in the formalin model of inflammatory pain in rats (Khoshdel et al., 2014; 2016). No studies to date have examined the role of AMY in morphine tolerance. Therefore, as the first goal of this study, we sought to investigate the role of AMY in opioid tolerance, and the potential of this peptide to reverse the established tolerance to morphine. Brain-derived neurotrophic factor (BDNF) is a small secreted protein that belongs to the neurotrophin family of peptides. In addition to its canonical neurotrophic role, BDNF is shown to control neuronal activity as a neuromodulator (Mattson, 2008; Merighi et al., 2008; Santos et al., 2010). Thus, results of a number of studies suggest BDNF as a negative modulator of morphine action in the ventral tegmental area (Koo et al., 2012). Published data also suggest a likely role for BDNF in the transmission of pain (Kerr et al., 1999; Malcangio and Lessmann, 2003) and development of morphine tolerance (Matsushita and Ueda, 2009). Interestingly, a recent study has shown attenuation of opioid analgesic tolerance in BDNF receptor (p75NTR) null mice (Trang et al., 2009). Data presented in this study revealed the potency of i.t AMY to reverse morphine tolerance. Hence, the second aim of this study was to investigate possible implications of BDNF in the anti-tolerance effects of AMY. Accordingly, we investigated the effects of i.t. AMY on the expression of the BDNF gene. The rat BDNF gene contains eight 5′ noncoding exons (I–VIII), which each of them can splice to one 3′ coding exon (IX) that encodes the mature BDNF protein. This gene is transcribed to eleven distinct mRNAs, each of which is characterized by one 5′UTR exon linked by alternative splicing to the common coding exon IX (Liu et al., 2006; Aid et al., 2007). The different BDNF splice variants responds differently to various types of treatments (Dias et al., 2003; Calabrese et al., 2007). As an example, ethanol induced differential expressions of exons I, III and IV in the brain has been reported (Schmidt et al., 2012; Shojaei et al., 2015). However, BDNF mRNAs containing exon 1, 2 and 4 are transcriptionally more important (Timmusk et al., 1993), although the definite function of each transcript is not known. Also, differential expression of BDNF exon 4 is observed in the spinal cord following epigenetically prolonged opioid induced hyperalgesia (Liang et al., 2014). All eleven distinctive BDNF mRNAs are translated into the same pro-BDNF protein (Aid et al., 2007). Pro-BDNF protein is a precursor for BDNF that also acts as a signaling molecule through distinct receptors. Several roles have been proposed for various 5′UTRs of the BDNF gene, including regulation of transcription, modulation of mRNA stability and translation initiation, and differential expression in different parts of the CNS during lifetime (Wilkie et al., 2003). Alterations in the expression levels of exon transcripts in the BDNF mRNA and its encoded protein have been detected in addiction and other nervous system disorders (Akbarian, 2002; Cattaneo et al., 2016). Here, we evaluated levels of transcripts containing the non-coding exons I, IV and the coding exon IX of the BDNF gene, as well as, on the levels of BDNF and pro-BDNF protein in the lumbar spinal cord of morphine tolerant rats.

EXPERIMENTAL PROCEDURES

Chemicals

Rat AMY was obtained from Bachem Americas, Inc. (Torrance, CA). Morphine sulfate was purchased from Temad Company. All chemicals and western blot materials used in this study were obtained from Sigma-Aldrich, Santa Cruz, and Abcam, respectively. ELISA kit was obtained from Promega.

Animal treatment and surgery

Adult Male Sprague – Dawley rats weighing 250 ± 20 g were provided by the laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. Rats were anesthetized with ketamine (50 mg/kg) – xylazine (5 mg/kg) and i.t. catheterization was performed as described by Yaksh and Rudy (Yaksh et al., 1977). Briefly, a slit was made on cisternal membrane and 7.5 cm length of a polyethylene catheter (PE-10, Betcton Dickenson, San Jose, CA) was inserted into the subarachnoid space of lumbar enlargement (L3- L4). The rostral part was sutured to the adjacent muscles to immobilize the catheter and the wound was closed in two layers with 4–0 silk. The position of the caudal tip was always confirmed after the animals were sacrificed. Rats with paralysis or motor weakness during the recovery period of 7 days were excluded from the study. Drugs and vehicle were administered i.t. in volume of 10 μL, followed by10 μL flushes of normal saline to clear the catheter. Final doses of morphine (22 nmoles/rat) and AMY (1–60 pmoles/rat) were prepared in 10 μL of sterile saline for i.t injection. AMY was injected 10 min prior to morphine. All experimental protocols were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Medical and Research Ethics Committee of the Shiraz University of Medical Sciences, Shiraz, Iran.

Behavioral test

The antinociceptive potential of morphine and AMY was tested using a nociceptive spinal reflex test; the tail flick test. The tail flick test (D'Amour and Smith, 1941) assessed the animal's response to a thermal stimulus. In brief, radiant heat was applied to the bottom section of the animal's tail using an analgesia meter (Tajhizgostar,

Iran) and the response latency required for the animal to remove its tail was recorded (Owen, et al. 1981; Yang, et al. 2011). The intensity of the heat source was adjusted to yield a base line response of 2 ± 3 s and a cut - off latency of 10 s was implemented to minimize tissue damage. Results at each time point were expressed as the percentage of maximal possible anti-nociceptive effect (MPE) according to the following formula:

$$\mathrm{MPE} = \frac{postdrug\,latency - baseline\,latency}{Cut - off\,value - baseline\,latency} \times 100$$

Induction of morphine tolerance

To induce a state of morphine tolerance, animals were injected once daily for 10 days with i.t. morphine (22 nmoles or 15 μg/10 μl). Injections were made each day between 9 and 11 AM. Control rats received saline. Nociceptive testing was performed both before and 30 min after drug administration to determine baseline and drug-induced antinociceptive effects, respectively. Cumulative dose – response curves were generated to determine the potency of acute morphine on the 11th day. To prepare these curves, rats were given four ascending doses of morphine every 30 min until a maximal level of antinociception was reached in the tail flick test. Nociceptive responses were evaluated 25 min. after post injection. The morphine ED_{50} value that is an index of morphine potency, were derived from the dose–response curve. A state of tolerance was verified by a progressive decrease in the daily antinociceptive response to morphine, manifested as a rightward shift in the acute morphine dose response curve, namely, an increase in the ED_{50} value for morphine.

Chronic effects of AMY on nociception and on established morphine tolerance

We examined the persistence of the effects of AMY on nociception by i.t. injections of different doses (1, 10 and 60 pmoles) of the peptide to rats in the AMY group once daily. Animals in the control group received only i.t. saline once a day. Rats in the above two groups were subjected to nociceptive testing daily. In order to examine the ability of AMY to reverse morphine tolerance, animals in the morphine-AMY group received injections of i.t. morphine (22 nmoles) once daily for 5 days. On days 6–10, animals were co-treated i.t. with single doses of 1, 10 or 60 pmoles of AMY (based on our previous studies; (Khoshdel et al., 2014, 2016) and 22 nmol of morphine, once daily. Nociceptive testing was performed daily, and cumulative dose response curves for acute morphine were generated on the 11th day, and the ED_{50} values calculated for these curves. The ability of AMY treatments to reverse tolerance was indicated by (a) a recovery of morphine induced antinociception during chronic treatment and (b) a recovery of morphine potency (ED_{50}).

Tissue preparation

Two hours after last administration of the drugs or saline, animals were decapitated after CO_2 inhalation and lumbar spinal cord were isolated from the animals. The tissues were, placed in aluminum foil, frozen in liquid nitrogen and stored at −80 °C until used for mRNA analyses.

Real time quantitative PCR

We employed qRT-PCR to assess transcripts of BDNF exons during the development of morphine tolerance and reversal of spinal morphine tolerance by AMY treatment. Total RNA from the lumbar spinal cord (L4-L5) was extracted using the Tripure isolation reagent (Roche Co., Germany), according to the manufacturer's instruction, and quantified by Nano-drop (Thermo Scientific) to define their concentration and purity. The denaturing gel electrophoresis method was used to check the integrity of RNAs (Fleige and Pfaffl, 2006). The RNA extracted prior to use cDNA synthesis was treated with DNase I (EN0521, Fermentas, Germany) to remove any DNA contamination prior to use for cDNA synthesis. cDNAs were synthesized using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Eu). All procedures were based on the manufacturer's instruction. q RT-PCR was performed, using a 7500 real-time PCR system (Applied Biosystem, Foster City, CA, USA). The sequence of primers used for PCR of BDNF exons and reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin, were reported in our previous study (Shojaei, 2017). All genes were amplified under the following conditions: initial denaturing at 95 °C for 30 s, 40 cycles of 95 °C for 5 s and annealing and extension at 60 °C for 30 s. Each PCR reaction was run in duplicate. The ΔΔCT method was used to calculate the relative expression level of BDNF exons in the lumbar spinal cord of the animals (Pfaffl and International University Line: La Jolla 2004). Amplification efficiencies were calculated from a relative standard curve derived from tenfold serial dilutions of pooled cDNA. The quality and accuracy of the PCR products were checked using electrophoresis on 2% agarose gels.

Western blotting

Tissue samples were homogenized in an extraction buffer containing a protease inhibitor cocktail (P8340, Sigma-Aldrich) as proposed by the manufacturer and NP40 lysate buffer. This was followed by 3 replicates of sonication for 5 s. After centrifugation at 10,000 × g at 4 °C for 10 min, the supernatant was collected. The Protein concentration was determined using the Bradford method. Western blot was performed with 50 μg of protein extracts (per lane) and protein from each sample was separated using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein ladder (Fermentase, USA) was used to define the weight of separated bands. Separated samples were transferred to nitrocellulose membrane at 100 V for 120 min after electrophoresis. Then membranes were blocked by 5% skimmed milk in TBS buffer containing

0.2% tween 20 (TBST) at room temperature for 1 h to avoid non-specific binding sites. The membranes were probed at 4 °C overnight with either at 1:250 dilution of polyclonal rabbit anti–Pro-BDNF (sc-546, Santa Cruz, USA) or a 1:500 titer of the anti β-actin antibody (ab1801, Abcam, USA) was used as an internal control. The membranes were then washed 3 times for 20 min each using 0.2% TBST and incubated with the secondary antibody: 1: 7500 dilution of goat anti-rabbit conjugated with HRP (Aviva System Biology USA) at room temperature for 1 h. After rinsing membranes in TBST 0.2% three times, the blots were visualized using a home-made enhanced chemiluminescence (ECL). Band intensities were quantified by Image J software. The level of pro-BDNF protein of each sample was normalized against β-actin and fold change of Pro - BDNF in the treatment groups were calculated relative to the control group.

Enzyme-linked immunosorbent assay

BDNF protein was quantified from the tissue extracts using an ELISA kit (Promega, USA). Results were normalized to the total protein concentration and expressed as picograms of BDNF per microgram of total protein. Protein was measured by the Bradford method.

Data analysis

Graph pad Prism program (Version 6, GraphPad Software, San Diego, CA, USA) was used for statistical analysis of data presented as mean ± SEM. Numbers of independent measurements are denoted in the corresponding figure legends. Differences between groups were assessed by nonparametric analysis of variance (Kruskal-Wallis test) followed by Dunn's post hoc analysis. Statistical analysis between two samples was performed using Mann Whitney U test with p value of $\leq$0.05 being set as the level of significance.

RESULTS

The effects of AMY on nociception and on morphine tolerance

Fig. 1A represents the time course of the antinociceptive effects of daily i.t. administration of morphine (22.0 nmoles) in the tail flick test, and the development of tolerance to morphine. As can be seen in the figure, morphine produced maximal anti-nociceptive response on day 1. After that, a progressive decline in the analgesic response to morphine was observed, reflecting the development of tolerance to morphine. Morphine tolerance was established on day 5 and thereafter morphine analgesia dropped to baseline levels (hatched area). The effect of AMY (1, 10 and 60 pmoles) on the established morphine tolerance in the tail flick test is presented in the Fig. 1B–D. Co-administration of AMY (1, 10 and 60 pmoles) with morphine (22.00 nmoles) on days 6–10 restored the morphine anti-nociceptive response gradually and (at the dose of 60 pmoles) reached to approximately 70% of the original value on day 10. For further comparison, the area under the curve responses of morphine antinociceptive effects in days 6–10 (hatched areas in the Fig. 1A–D) were calculated. As illustrated in the Fig. 1E a significant increase in the areas under the curve responses of morphine antinociceptive effects was observed in rats co-treated with morphine and AMY compared to the corresponding area in the rats treated with morphine alone ($p < 0.05$, Kruskal-Wallis analysis followed by Dunn's test).

As shown in Fig. 1B, C and D, daily i.t. injection of AMY (10 and 60 pmoles) caused dose dependent and durable antinociceptive effects as assessed by daily tail flick tests ($p < 0.05$, Kruskal-Wallis analysis followed by Dunn's test).

To further investigate the potency of AMY in reversing established morphine tolerance, the animals in the morphine and morphine plus AMY groups were treated with increasing doses of morphine on the eleventh day. As demonstrated in Fig. 2, AMY combined with morphine shifted the morphine dose–response curve to the left and restored morphine potency in tolerant rats. The mean ± SEM of ED_{50} values for morphine in the various groups are shown in Table 1. As can be seen in the table, ED_{50} for morphine (111.50 ± 4.12) in the morphine tolerant rats were significantly higher than those obtained for the animals treated with AMY (60 pmole) and morphine concomitantly (17.96 ± 2.56 nmoles). The dose response curve for antinociceptive effects of acute morphine in rats that received AMY alone for 10 days and in those treated with saline showed overlapping confidence intervals. The difference between ED_{50}s obtained from these two curves was not statistically significant (Table 1).

Expression pattern of exons I, IV and common exon IX of BDNF gene in the spinal cord of morphine tolerant rats

The results of RT-PCR confirmed the presence of PCR products with predicted lengths of about 156 bp (exon I), 148 bp (exon IV) and 212 bp (exon IX) in the cDNA library prepared from the spinal cord of rats (data not shown). qRT- PCR method was used to evaluate the effects of morphine tolerance and AMY on the expression of BDNF transcripts containing exons I, IV and IX in the spinal cord of rats. As shown in the Fig. 3, morphine tolerance significantly increased the level of BDNF transcripts containing exons I, IV and IX ($p < 0.01$, Kruskal–Wallis, Dunn's post hoc tests) compared to those of the saline-treated control group. The i.t. administration of AMY alone (1, 10 and 60 pmoles) for 10 days increased levels of BDNF transcripts containing exons I and IV, but the increase was only significant at the dose of 10 pmoles of AMY ($p \leq 0.05$, Kruskal–Wallis, Dunn's post hoc tests). However, i.t. AMY (60 pmoles) decreased the expression level of exon IX compared with saline-treated control rats ($p < 0.01$, Kruskal–Wallis, Dunn's post hoc tests). Co-administration of morphine and various doses of AMY to morphine tolerant animals

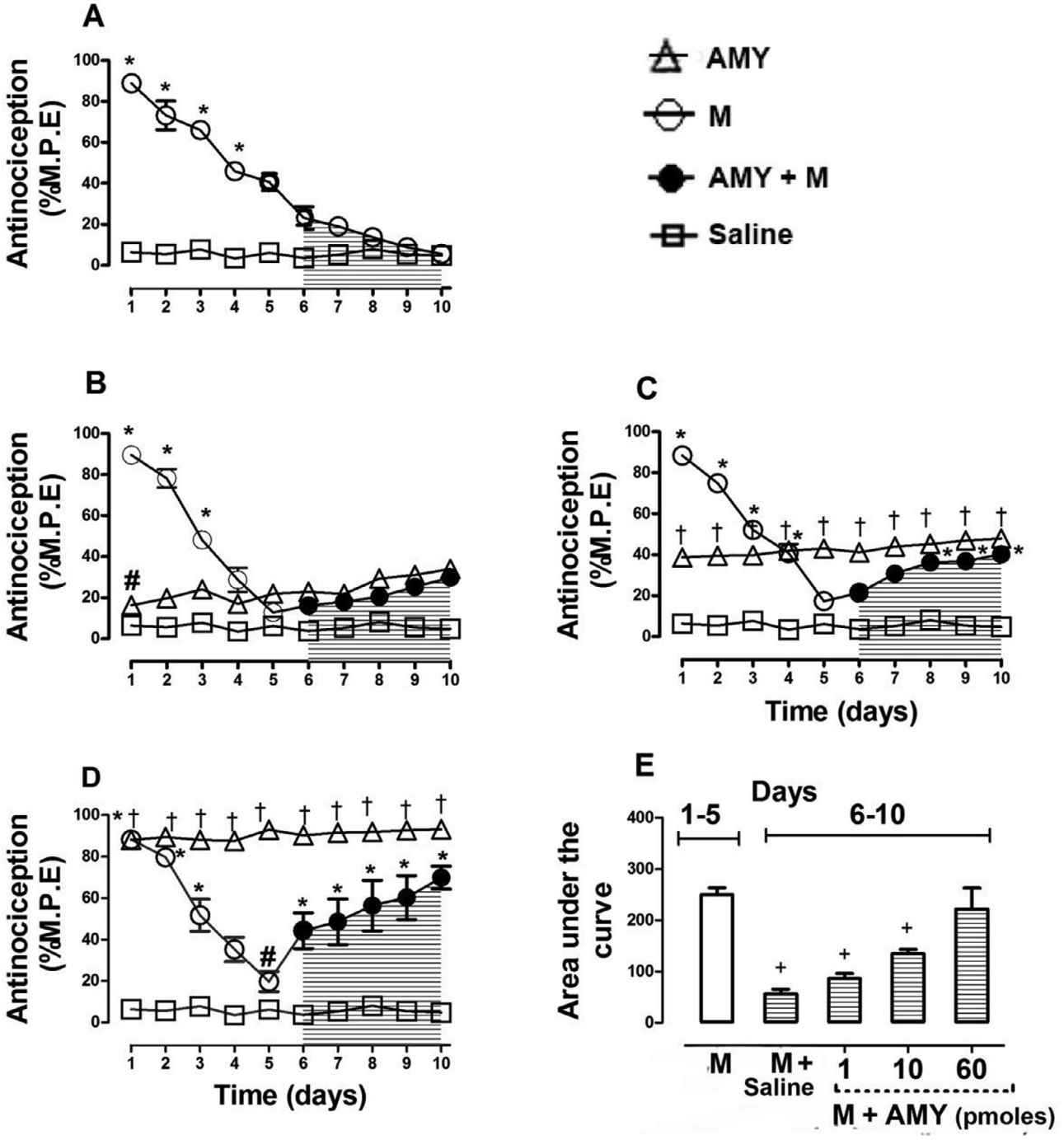

Fig. 1. Reversal of the established tolerance to morphine by AMY. A) Induction of established tolerance to morphine. Tolerance was induced by the i.t. administration of a single dose of morphine (22.00 nmoles) from days 1–5. From days 6–10, rats received either morphine or morphine combined with AMY in doses of 1 pmole (B), 10 pmoles (C) and 60 pmoles (D). Tolerance to morphine tended to decrease in animals co-treated by morphine and AMY 1 pmole and 10 pmoles, and reversed in those by AMY 60 pmoles (E). Effects of AMY alone at i.t doses of 1, 10 and 60 pmoles are shown at figures B, C and D respectively. Data are expressed as mean ± S.E.M for 6–7 animals. Asterisks indicate significant differences between the saline and the morphine (and/or morphine + AMY) treated groups ($p < 0.05$, Mann Whitney U test). Crosses show significance between the antinociception caused by i.t. AMY alone and/or saline ($p < 0.05$, Mann Whitney U test). Number signs show significance between the groups treated by morphine and/or by AMY alone ($p < 0.05$, Mann Whitney U test). Plus signs show significant differences ($p < 0.05$, Kruskal-wallis analysis followed by Dunn's test) between the areas under the curve responses of morphine antinociceptive effects in days 1–5 (blank area in A) and in days 6–10 (Hatched areas). AMY = amylin, M = morphine.

led to a decrease in levels of BDNF exons I, IV and the common exon IX expression compared to those of the morphine tolerant group. This decrease was statistically significant with respect to exons I, IV and the common exon IX ($p < 0.01$, Kruskal–Wallis, Dunn's post hoc tests),

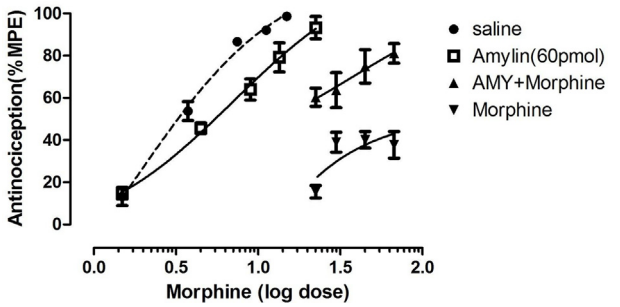

Fig. 2. Dose response curves for the effect of AMY (60 pmoles) on the acute antinociceptive potency of morphine. Following 10-days of morphine treatment, cumulative dose response curves for the acute antinociceptive potency of morphine were prepared on day 11. On the eleventh day, rats in all groups were given ascending doses of morphine every 30 min until a maximal level of antinociception was reached in the tail flick test. The dose response curve for acute morphine showed a shift to the right in animals that received morphine for 10 days, when compared to those treated by saline. This shift was smaller when AMY (60pmoles) was added to morphine from days 6–10 under the same experimental conditions. Therefore AMY can cause the morphine dose response curve to shift to the left and restore morphine potency in tolerant rats. Antinociceptive potency of acute morphine in rats injected by AMY alone for 10 days was close to that in saline treated animals. Data are mean ± SEM of the percentage of maximum possible effect produced by the acute injection of ascending doses of morphine every 30 min. Five to six rats were tested at each dose.

Table 1. The effect of AMY on morphine tolerant rats.

Treatment (n = 5)	Morphine ED50 (nmoles)
Saline	2.381 ± 0.13
Morphine (22nmoles)	111.50 ± 4.12**
AMY (60pmoles)	7.79 ± 1.39 ††
AMY + Morphine (60 pmoles + 22 nmoles)	17.96 ± 3.11††

Data are mean ± SEM of ED_{50} values (nmoles/10 μL) of morphine in various groups. Following 10-days of i.t treatment of rats with saline, morphine, AMY, and AMY + Morphine, cumulative dose response curves for the acute antinociceptive potency of morphine were prepared on day 11. ED_{50} values were derived from dose response curves using nonlinear regression analysis. Statistical significance of differences between each group and saline (*) and or morphine treated groups (†) were determined using Kruskal-Wallis analysis followed by Dunn's post hoc test. $P < 0.05$ was considered as a significant difference.

AMY abolished morphine tolerance-induced production of pro-BDNF protein in the lumbar spinal cord of rats

Western blotting was performed to analyze the levels of pro-BDNF protein expression in the lumbar spinal cord of rats before and after the development of morphine tolerance (Fig. 4). As shown in the Fig. 4 chronic morphine (22.0 nmoles) injection caused a significant increase in the pro-BDNF level compared to the normal saline group. Co-administration of morphine and AMY at dose of 60 pmoles (Fig. 4C) for 5 days after established morphine tolerance, led to significant decrease in levels of pro-BDNF as compared to the morphine group. Injection of AMY 60 pmoles alone caused an increase, albeit not significant, in the pro–BDNF level in comparison to saline treatment. As depicted in Fig. 4 the level of pro-BDNF remained unchanged in the animals co–administered with morphine and AMY in doses of 1 and 10 pmoles. The administration of AMY (1 and 10 pmoles) alone from days 1–10, had no significant effects on the expression of pro-BDNF compared to saline injection to rats.

AMY reduced morphine tolerance-induced BDNF protein in the lumbar rat spinal cord

ELISA assay (Fig. 5) showed a significant increase in the levels of BDNF protein in lumbar spinal cord of morphine tolerant rats compared to control saline treated group ($P < 0.05$, Kruskal-Wallis test followed by Dunn's test). Co-administration of AMY in doses of 60 and 10 pmoles with morphine reduced the levels of BDNF protein compared to morphine tolerant group in a significant and dose dependent manner ($p < 0.05$, Kruskal-Wallis test followed by Dunn's test). Co-administration of AMY in a dose of 1 pmole also caused a decrease in the level of BDNF protein; however this effect was not statistically significant. Administration of AMY (1–60 pmoles/10 μL) alone from days 1–10 had no significant effects on the level of BDNF compared to control specimens (Fig. 5).

DISCUSSION

Tolerance to and physical dependence on morphine, the most effective pain-reliever, has restricted the therapeutic use of this drug (Chu et al., 2012). In the current study, we employed a widely used experimental model to investigate effects of i.t. administration of AMY against morphine tolerance (Powell et al., 2000). Our findings revealed that antinociceptive effects of AMY did not undergo tolerance after ten days of i.t. injection. This peptide could also restore both acute and chronic antinociceptive effects of morphine in morphine tolerant rats. We further demonstrated that co-administration of AMY with morphine could reduce morphine-induced BDNF expression at both mRNA and protein level. To the best of our knowledge, this is the first study which demonstrates a novel function for AMY as an effective sensitizer for morphine effects on pain relief. This effect of morphine is accompanied by the inhibition of BDNF expression. Both CGRP and Adrenomedullin (Maggi, 1995; McCoy et al., 2012; Wang et al., 2014) have shown pronociceptive properties and their peptide antagonists are reported to reverse morphine tolerance when injected intrathecally to rats (Powell et al., 2000; Wang et al., 2011). In contrast to these peptides, AMY has previously been reported to have antinociceptive effects (Khoshdel et al., 2014; Khoshdel et al., 2016). Our data from cumulative dose response curves for acute morphine also revealed that AMY restored the potency of morphine in animals already tolerant to this opiate. Accordingly, AMY demonstrates the capacity to reverse the tolerance to morphine. Further, our results also show the dose dependent antinociceptic effects of AMY. This effect remained unchanged when rats were exposed daily to an i.t. dose of the peptide for 10 days, implying that repeated exposure to i.t. AMY may not lead to a tolerance to its antinociceptive proper-

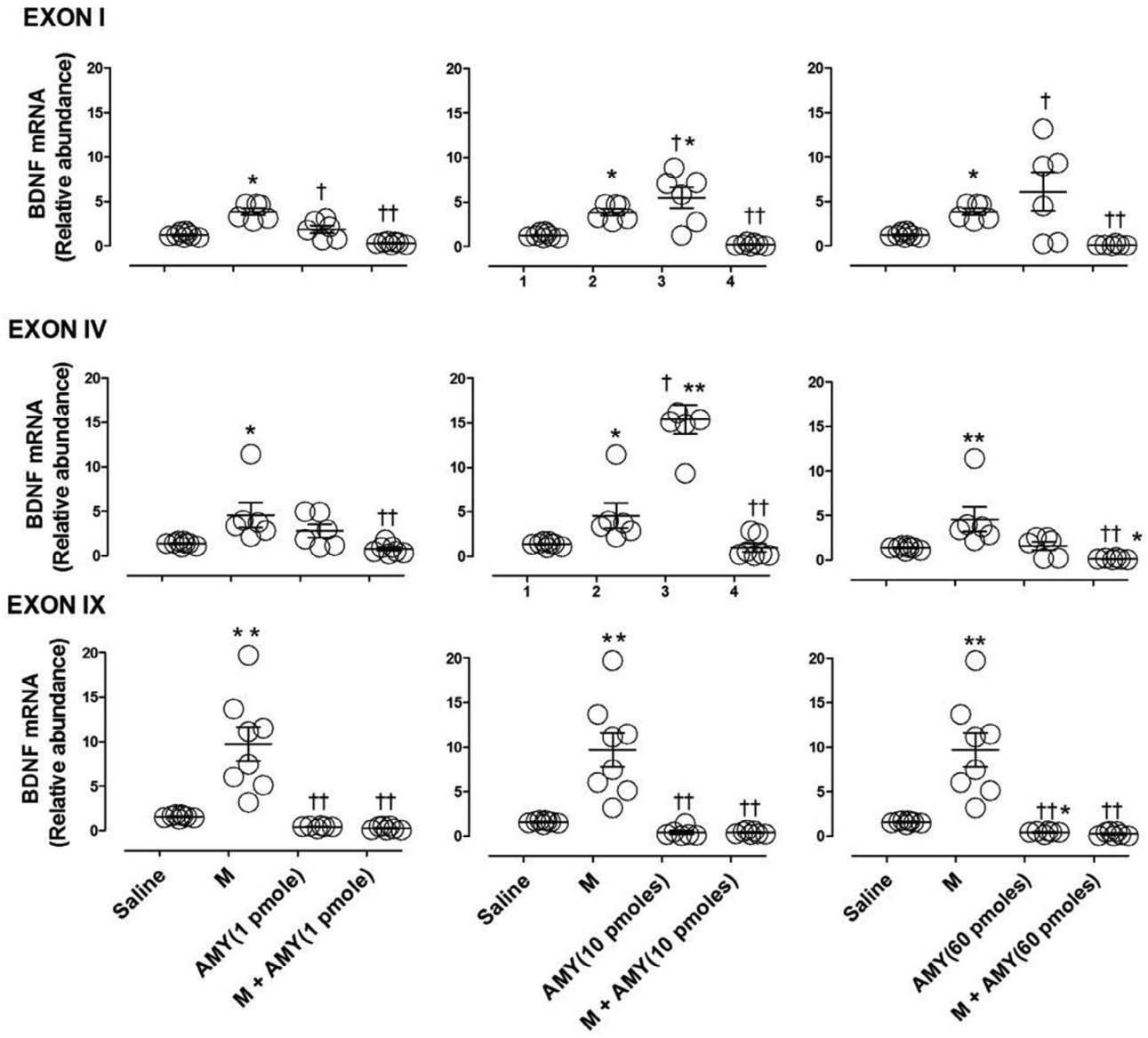

Fig. 3. qRT-PCR data related to the expression of BDNF transcripts containing exons I, IV and IX in the spinal cord of rats before and after development of morphine tolerance. Tolerance was induced by the administration of a single i.t morphine (22.0 nmoles) from days 1–10. Control rats were treated chronically with saline for 10 days. Amylin (1, 10 and 60 pmoles) was injected i.t from days 6–10. The lumbar spinal cord region was dissected from rats 2 h after the last injection on day 10. β-actin and GAPDH were used as housekeeping genes . Expression levels of BDNF exons were normalized with β-actin and analyzed by ΔΔCT method. Data (Means ± SEM of 6–8 rats) are the fold change of BDNF exons expression in the treatment groups compared to the control group. Differences between groups were assessed by nonparametric analysis of variance (Kruskal–Wallis, Dunn's post hoc tests). Significant differences between the relative expression of exons in the saline-treated rats and the morphine-treated animals are respectively shown by asterisks (* $p < 0.05$, ** $p < 0.01$) and crosses († $p < 0.05$, †† $p < 0.01$). AMY = Amylin, M = Morphine.

ties. Whether AMY has the potential to hinder the development of morphine tolerance is a subject of further investigation. The present data and those reported earlier (Khoshdel et al., 2014; 2016) show that spinal cord of rats are sites of AMY action. The functions of AMY are mediated through specific receptors that are composed of a combination of calcitonin receptor (CTR) and one of the three receptor activity modifying proteins (RAMPs) (D.L. Hay Oct 26, 2004). The expression of RAMPs (oliver, 2001) and CTR has been demonstrated in both DRG and dorsal horn of the rat spinal cord (Pondel, 2000). However, the exact molecular structure of AMY receptors in the spinal cord is not verified yet.

The mechanisms underlying development of tolerance to morphine has been under intense investigation (Wang et al., 2017; Martyn, et al. 2019). Neurotrophins like BDNF have been proposed as candidates implicated in this process (Akbarian S 2002). BDNF has been shown to participate in the transmission of pain (Fukuoka, 2001; Malcangio and Lessmann, 2003). It has also been reported that morphine induces BDNF expression in cultured microglia (Small, 1998) and that central

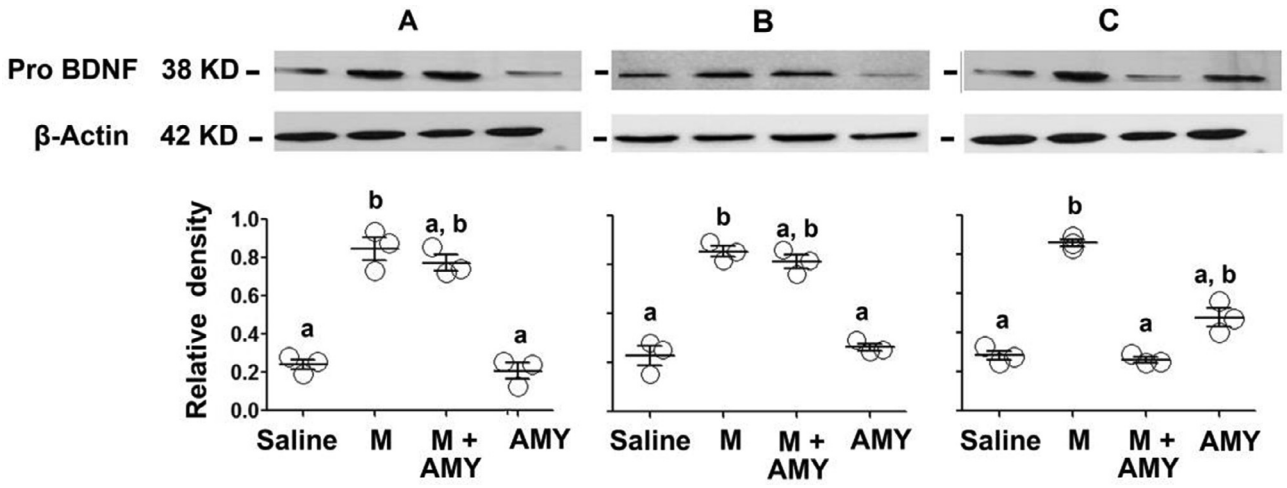

Fig. 4. Western blot analysis of pro-BDNF protein in the rat spinal cord. Rats received a daily i.t. dose of either saline (control) or M (22.0 nmoles) on days 1 to10. AMY was injected i.t. at doses of 1pmole (A), 10 pmoles (B) and 60 pmoles (C) from day 6 to 10. Pro-BDNF was up-regulated in rats injected by morphine compared to those treated by saline. Intrathecal co-administration of morphine and a 60 pmoles dose of AMY led to a significant decrease in the levels of pro-BDNF as compared to the morphine group. The level of pro-BDNF protein in each sample was normalized with β-actin. Antibodies against β-actin and BDNF recognized protein bands of approximately 38 and 42KDa for pro-BDNF and β-actin, respectively. Relative density of each band was calculated by dividing the pixel density of bands related to pro-BDNF by those to β-actin. Small letters show groups (n = 3 each) with significant differences at $p < 0.05$ (Kruskal–Wallis, Dunn's post hoc tests). Groups with the same letters are not significantly different at $p < 0.05$. M = Morphine, AMY = Amylin.

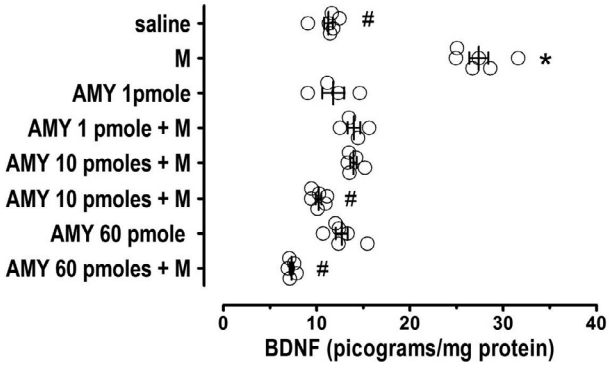

Fig. 5. Scatter plot of various intrathecal treatments versus levels of BDNF in the lumbar spinal cord. Rats were treated by morphine (22 nmoles/10 μL) from day 1–10 and by AMY (1, 10 and 60 pmoles/10 μL) from day 6 to 10. The Mean and SEM of data are shown by vertical (|) and horizontal (⊢) markers. Significance of differences between each group was analyzed by Kruskal-Wallis test followed by Dunn's test with $P < 0.05$ as the level of significance. The number of rats in each group was 4–6. (*) significance relative to the saline group, (#) significant in comparison to the morphine group. M = morphine, AMY = amylin.

injection of anti-BDNF antibody reduced morphine tolerance (Matsushita and Ueda, 2009). In agreement with these studies (Ueda and Ueda, 2009), our present findings clearly demonstrate that following morphine tolerance, there is an increase in the BDNF expression at both levels of mRNA and protein. This notion is evidenced by qRT-PCR for BDNF transcripts as well as by western blot analyses for pro-BDNF and ELISA assay for BDNF concentration. These data suggest a role for BDNF in the development of morphine tolerance. The rat BDNF gene has eight 5′ noncoding exons (I–VIII), which each of them can splice to a one 3′ coding exon (IX) that encodes the mature BDNF protein. Results of the present study revealed that BDNF transcripts containing exons I and IV, as well as the coding exon IX are increased following morphine tolerance in the spinal cord. This data is in line with research showing that chronic morphine tolerance significantly increased the expression of BDNF transcripts of I and IV in the periaqueductal gray matter (PAG) of mice. This area is the major brain region involved in morphine analgesia (Matsushita and Ueda, 2009). The morphine-induced mRNA expression of BDNF exons was completely reversed upon injection of AMY at all doses we tested. AMY, when injected alone, did not show a uniform effect on the expression levels of BDNF exons tested in this study. Thus, AMY tended to decrease levels of transcripts containing the common exon IX, while increasing the mRNA expression of the exon I and IV, although this increase achieved significance at 10 pmoles of the peptide. An explanation for this dichotomy would be that the decreased levels of the common exon IX transcripts by AMY alone may be due to the declined levels of transcripts that contain exons other than exons I and IV. The decreasing effect of AMY alone on levels of transcripts containing the coding exon IX is not consistent with the fact that levels of the pro-BDNF and BDNF proteins remained unchanged under the same experimental conditions. This lack of correlation between levels of BDNF mRNA and its encoded protein implies that post-transcriptional events including mRNA transcript–specific regulation of translation and protein degradation may play a prominent role in controlling the levels of BDNF in spinal cells. The finding that intrathecal co-administration of AMY with morphine from days 6–10 completely suppressed morphine induced BDNF expression along with restoration of morphine sensitivity suggests a possible role for BDNF in the observed anti-tolerance effects of AMY. The main source of the morphine induced increase

in the BDNF level observed in this study remains to be investigated. However, chronic exposure to opioids is shown to increase the secretion of BDNF from DRG neurons into the dorsal horn (Garraway et al., 2005) and to induce the synthesis of BDNF by microglia (Takayama and Ueda, 2009; Ferrini et al., 2013). It is hypothesized that BDNF induces synaptic facilitation in lamina II neurons. This action of BDNF may contribute to central sensitization and exaggerated pain responses (Garraway et al., 2005). Given that AMY has been shown to be expressed in the neurons from DRG of rats (Ferrier et al., 1989; Nicholl et al., 1992; Mulder et al., 1995), it would be interesting to analyze the expression of AMY and BDNF in this tissue during the course of morphine tolerance. Experimental evidence also suggests that the BDNF released following microglia activation is a key regulator in morphine tolerance mechanisms (Takayama and Ueda, 2009; Ferrini et al., 2013). Thus, the inhibitory effect of AMY on morphine induced expression of BDNF in microglial cells, if demonstrated, can explain its contradictory effects on morphine tolerance. To what extent AMY affects the expression and or release of BDNF from microglial cells needs further investigation. A vast number of reports relate that over expression of BDNF has been associated with neuropathic pain induced by tissue, nerve and or spinal cord injury (Yajima et al., 2002; Ramer et al., 2007; RWang et al., 2009; Khan and Smith, 2015). Published results also suggest that activation of BDNF-TrkB receptor signaling causes a down regulation of the potassium chloride co-transporter 2 (KCC2). The outcome is reduced Cl^- extrusion capacity of spinal neurons leading to a shift in the GABA A receptor signaling from inhibitory to excitatory. The resulting enhanced nociceptive processes may be a major factor in the development of chronic inflammatory and neuropathic pain that is induced by tissue and nerve injury (Lee-Hotta et al., 2019). Whether or not AMY could also affect KCC2 levels is another subject for further research.

Up-regulation of pronociceptive mediators in the spinal cord such as CGRP (Menard et al., 1996), substance P (Powell et al., 2000), NO (Kielstein et al., 2007) and TRPV1 (Chen et al., 2008) are other proposed mechanisms underlying the development of morphine tolerance (King et al., 2005). The inhibitory actions of NMDA receptor antagonists (Dunbar, 1996) and or inhibitors of cyclooxygenase activity (Powell et al., 1999) on the development of tolerance to morphine have also been indicated. Whether these mechanisms are also involved in the reverse effects of AMY on the established tolerance to morphine needs more investigation.

These results suggest that i.t. administration of AMY alone produces enduring antinociceptive effects. This peptide reverses established tolerance to morphine when co-injected with the drug. This effect is accompanied by an inhibition in the morphine-induced expression of BDNF in the lumbar spinal cord.

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AUTHORS CONTRIBUTIONS

Zahra Khoshdel and Somayeh Ahmadpour Jirandeh performed the experiments. Farideh Jalali Mashayekhi shared in doing the Real-Time PCR experiment. Shahla Shojaei shared in doing the ELISA assay. Ali Akbar Owji and Mohammad Ali Takhshid proposed and developed the idea and designed the study. Ali Akbar Owji and Zahra Khoshdel wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res 85:525–535.

Akbarian S, Rios M, Liu R-J, Gold SJ, Fong H-F, Zeiler S, et al. (2002) Brain-derived neurotrophic factor is essential for opiate-induced plasticity of noradrenergic neurons. J Neurosci 22 (10):4153–4162.

Banks WA, Kastin AJ, Maness LM, Huang W, Jaspan JB (1995) Permeability of the blood-brain barrier to amylin. Life Sci 57:1993–2001.

Barwell J, Gingell JJ, Watkins HA, Archbold JK, Poyner DR, Hay DL (2012) Calcitonin and calcitonin receptor-like receptors: common themes with family B GPCRs? Br J Pharmacol 166:51–65.

Boyle CN, Rossier MM, Lutz TA (2011) Influence of high-fat feeding, diet-induced obesity, and hyperamylinemia on the sensitivity to acute amylin. Physiol Behav 104:20–28.

Brain SD, Grant AD (2004) Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev 84:903–934.

Calabrese F, Molteni R, Maj PF, Cattaneo A, Gennarelli M, Racagni G, Riva MA (2007) Chronic duloxetine treatment induces specific changes in the expression of BDNF transcripts and in the subcellular localization of the neurotrophin protein. Neuropsychopharmacology 32:2351–2359.

Cattaneo A, Cattane N, Begni V, Pariante CM, Riva MA (2016) The human BDNF gene: peripheral gene expression and protein levels as biomarkers for psychiatric disorders. Transl Psychiatry 6 e958.

Chen Y, Geis C, Sommer C (2008) Activation of TRPV1 contributes to morphine tolerance: involvement of the mitogen-activated protein kinase signaling pathway. J Neurosci 28:5836–5845.

Chu LF, D'Arcy N, Brady C, Zamora AK, Young CA, Kim JE, Clemenson AM, Angst MS, Clark JD (2012) Analgesic tolerance without demonstrable opioid-induced hyperalgesia: a double-blinded, randomized, placebo-controlled trial of sustained-release morphine for treatment of chronic nonradicular low-back pain. Pain 153:1583–1592.

D'Amour FS, Smith DL (1941) A method for determining loss of pain sensation. J Pharmacol Exp Ther 72(1):74–78.

Dias BG, Banerjee SB, Duman RS, Vaidya VA (2003) Differential regulation of brain derived neurotrophic factor transcripts by antidepressant treatments in the adult rat brain. Neuropharmacology 45:553–563.

Dunbar S, Yaksh T (1996) Concurrent spinal infusion of MK801 blocks spinal tolerance and dependence induced by chronic intrathecal morphine in the rat. J Am Soc Anesthesiol 84 (5):1177–1188.

Ferrier GJ, Pierson AM, Jones PM, Bloom SR, Girgis SI, Legon S (1989) Expression of the rat amylin (IAPP/DAP) gene. J Mol Endocrinol 3:R1–R4.
Ferrini F, Trang T, Mattioli TA, Laffray S, Del'Guidice T, Lorenzo LE, Castonguay A, Doyon N, Zhang W, Godin AG, Mohr D, Beggs S, Vandal K, Beaulieu JM, Cahill CM, Salter MW, De Koninck Y (2013) Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. Nat Neurosci 16:183–192.
Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27:126–139.
Fukuoka T, Kondo E, Dai Y, Hashimoto N, Noguchi K (2001) Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. J Neurosci 21(13):4891–4900.
Garraway SM, Anderson AJ, Mendell LM (2005) BDNF-induced facilitation of afferent-evoked responses in lamina II neurons is reduced after neonatal spinal cord contusion injury. J Neurophysiol 94:1798–1804.
Gibson SJ, Polak JM, Bloom SR, Sabate IM, Mulderry PM, Ghatei MA, McGregor GP, Morrison JF, Kelly JS, Evans RM, et al. (1984) Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. J Neurosci 4:3101–3111.
Hay DL, Christopoulos G, Christopoulos A, Sexton PM (2004) Amylin receptors: molecular composition and pharmacology. Biochem Soc Trans 32:865–867.
Hokfelt T, Arvidsson U, Ceccatelli S, Cortes R, Cullheim S, Dagerlind A, Johnson H, Orazzo C, Piehl F, Pieribone V, et al. (1992) Calcitonin gene-related peptide in the brain, spinal cord, and some peripheral systems. Ann N Y Acad Sci 657:119–134.
Huang X, Yang J, Chang JK, Dun NJ (2010) Amylin suppresses acetic acid-induced visceral pain and spinal c-fos expression in the mouse. Neuroscience 165:1429–1438.
Kerr BJ, Bradbury EJ, Bennett DL, Trivedi PM, Dassan P, French J, Shelton DB, McMahon SB, Thompson SW (1999) Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. J Neurosci 19:5138–5148.
Khan N, Smith MT (2015) Neurotrophins and neuropathic pain: role in pathobiology. Molecules (Basel, Switzerland) 20:10657–10688.
Khoshdel Z, Takhshid MA, Owji AA (2014) Intrathecal amylin and salmon calcitonin affect formalin induced c-fos expression in the spinal cord of rats. Iran J Med Sci 39:543.
Khoshdel Z, Takhshid MA, Owji AA (2016) Effects of intrathecal amylin on formalin-induced nociception and on cAMP accumulation in the rat embryonic spinal cells. Neuropeptides 57:95–100.
Kielstein A, Tsikas D, Galloway GP, Mendelson JE (2007) Asymmetric dimethylarginine (ADMA)–a modulator of nociception in opiate tolerance and addiction? Nitric oxide: Biol Chem 17:55–59.
King T, Ossipov MH, Vanderah TW, Porreca F, Lai J (2005) Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance? Neuro-Signals 14:194–205.
Koo JW, Mazei-Robison MS, Chaudhury D, Juarez B, LaPlant Q, Ferguson D, Feng J, Sun H, Scobie KN, Damez-Werno D, Crumiller M, Ohnishi YN, Ohnishi YH, Mouzon E, Dietz DM, Lobo MK, Neve RL, Russo SJ, Han MH, Nestler EJ (2012) BDNF is a negative modulator of morphine action. Science 338:124–128.
Lee-Hotta S, Uchiyama Y, Kametaka S (2019) Role of the BDNF-TrkB pathway in KCC2 regulation and rehabilitation following neuronal injury: a mini review. Neurochem Int 128:32–38.
Lee SM, Hay DL, Pioszak AA (2016) Calcitonin and amylin receptor peptide interaction mechanisms: insights into peptide-binding modes and allosteric modulation of the calcitonin receptor by receptor activity-modifying proteins. J Biol Chem 291:8686–8700.
Liang DY, Sun Y, Shi XY, Sahbaie P, Clark JD (2014) Epigenetic regulation of spinal cord gene expression controls opioid-induced hyperalgesia. Mol Pain 10:59.
Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR (2006) Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. Brain Res 1067:1–12.
Ma W, Chabot JG, Quirion R (2006) A role for adrenomedullin as a pain-related peptide in the rat. PNAS 103:16027–16032.
Maggi CA (1995) Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. Prog Neurobiol 45:1–98.
Malcangio M, Lessmann V (2003) A common thread for pain and memory synapses? Brain-derived neurotrophic factor and trkB receptors. Trends Pharmacol Sci 24:116–121.
Martyn JAJ, Mao JR, Bittner EA (2019) Opioid tolerance in critical illness. New Engl J Med 380:365–378.
Matsushita Y, Ueda H (2009) Curcumin blocks chronic morphine analgesic tolerance and brain-derived neurotrophic factor upregulation. NeuroReport 20:63–68.
Mattson MP (2008) Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann N Y Acad Sci 1144:97–112.
McCoy ES, Taylor-Blake B, Zylka MJ (2012) CGRPalpha-expressing sensory neurons respond to stimuli that evoke sensations of pain and itch. PLoS One 7 e36355.
Menard DP, Van Rossum D, Kar S, St Pierre S, Sutak M, Jhamandas K, Quirion R (1996) A calcitonin gene-related peptide receptor antagonist prevents the development of tolerance to spinal morphine analgesia. J Neurosci 16:2342–2351.
Merighi A, Salio C, Ghirri A, Lossi L, Ferrini F, Betelli C, Bardoni R (2008) BDNF as a pain modulator. Prog Neurobiol 85:297–317.
Muff R, Born W, Lutz TA, Fischer JA (2004) Biological importance of the peptides of the calcitonin family as revealed by disruption and transfer of corresponding genes. Peptides 25:2027–2038.
Mulder H, Leckstrom A, Uddman R, Ekblad E, Westermark P, Sundler F (1995) Islet amyloid polypeptide (amylin) is expressed in sensory neurons. J Neurosci 15:7625–7632.
Nicholl CG, Bhatavdekar JM, Mak J, Girgis SI, Legon S (1992) Extra-pancreatic expression of the rat islet amyloid polypeptide (amylin) gene. J Mol Endocrinol 9:157–163.
Oliver KR, Kane S, Salvatore CA (2001) Cloning characterization and central nervous system distribution of receptor activity modifying proteins in the rat. Eur J Neurosci 14:618–628.
Owen JA, Milne B, Jhamandas K, Nakatsu K (1981) Assembly of an inexpensive tail flick analgesia meter. J Pharmacol Methods 6:33–37.
Pfaffl, M.W.B., S.A., Ed.;, International University Line: La Jolla, C., USA, 2004. Quantification Strategies in Real-Time PCR. In a–z of Quantitative PCR. pp. 87–112.
Pondel M (2000) Calcitonin and calcitonin receptors: bone and beyond. Int J Exp Pathol 81:405–422.
Powell KJ, Hosokawa A, Bell A, Sutak M, Milne B, Quirion R, Jhamandas K (1999) Comparative effects of cyclo-oxygenase and nitric oxide synthase inhibition on the development and reversal of spinal opioid tolerance. Br J Pharmacol 127:631–644.
Powell KJ, Ma W, Sutak M, Doods H, Quirion R, Jhamandas K (2000) Blockade and reversal of spinal morphine tolerance by peptide and non-peptide calcitonin gene-related peptide receptor antagonists. Br J Pharmacol 131:875–884.
Ramer LM, McPhail LT, Borisoff JF, Soril LJ, Kaan TK, Lee JH, Saunders JW, Hwi LP, Ramer MS (2007) Endogenous TrkB ligands suppress functional mechanosensory plasticity in the deafferented spinal cord. J Neurosci 27:5812–5822.
Russell FA, King R, Smillie SJ, Kodji X, Brain SD (2014) Calcitonin gene-related peptide: physiology and pathophysiology. Physiol Rev 94:1099–1142.
Wang X, Ratnam J, Zou B, England PM, Basbaum AI (2009) TrkB signaling is required for both the induction and maintenance of tissue and nerve injury-induced persistent pain. J Neurosci 29:5508–5515.
Santos AR, Comprido D, Duarte CB (2010) Regulation of local translation at the synapse by BDNF. Prog Neurobiol 92:505–516.
Schmidt HD, Sangrey GR, Darnell SB, Schassburger RL, Cha JH, Pierce RC, Sadri-Vakili G (2012) Increased brain-derived neurotrophic factor (BDNF) expression in the ventral tegmental

area during cocaine abstinence is associated with increased histone acetylation at BDNF exon I-containing promoters. J Neurochem 120:202–209.
Shojaei S, Panjehshahin M, Shafiee SM, Khoshdel Z, Borji M, Ghasempour G, Owji AA (2017) Differential effects of resveratrol on the expression of brain-derived neurotrophic factor transcripts and protein in the hippocampus of rat brain. Iran J Med Sci 42:32–39.
Shojaei S, Ghavami S, Panjehshahin MR, Owji AA (2015) Effects of ethanol on the expression level of various BDNF mRNA isoforms and their encoded protein in the hippocampus of adult and embryonic rats. Int J Mol Sci 16:30422–30437.
Skofitsch G, Wimalawansa SJ, Jacobowitz DM, Gubisch W (1995) Comparative immunohistochemical distribution of amylin-like and calcitonin gene related peptide like immunoreactivity in the rat central nervous system. Can J Physiol Pharmacol 73:945–956.
Small DL, Murray C, Mealing GA, Poulter MO, Buchan AM, Morley P (1998) Brain derived neurotrophic factor induction of N-methyl-D-aspartate receptor subunit NR2A expression in cultured rat cortical neurons. Neurosci Lett 252(3):211–214.
Staton PC, Wilson AW, Bountra C, Chessell IP, Day NC (2007) Changes in dorsal root ganglion CGRP expression in a chronic inflammatory model of the rat knee joint: differential modulation by rofecoxib and paracetamol. Eur J Pain (London, England) 11:283–289.
Takayama N (2005) Morphine-induced chemotaxis and brain-derived neurotrophic factor expression in microglia. J Neurosci 25 (2):430–435. https://doi.org/10.1523/JNEUROSCI.3170-04.2005.
Takhshid MA, Owji AA, Vasei M, Panjehshahin MR, Tabei SM, Tabatabaee HR, Ay J (2004) Expression of spinal cord Fos protein in response to intrathecal adrenomedullin and CGRP in conscious rats. Brain Res 1020:30–36.
Takhshid MA, Poyner DR, Chabot JG, Fournier A, Ma W, Zheng WH, Owji AA, Quirion R (2006) Characterization and effects on cAMP accumulation of adrenomedullin and calcitonin gene-related peptide (CGRP) receptors in dissociated rat spinal cord cell culture. Br J Pharmacol 148:459–468.
Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10:475–489.
Trang T, Koblic P, Kawaja M, Jhamandas K (2009) Attenuation of opioid analgesic tolerance in p75 neurotrophin receptor null mutant mice. Neurosci Lett 451:69–73.
Ueda H, Ueda M (2009) Mechanisms underlying morphine analgesic tolerance and dependence. Front Biosci 14:5260–5272.
Wang D, Chen P, Li Q, Quirion R, Hong Y (2011) Blockade of adrenomedullin receptors reverses morphine tolerance and its neurochemical mechanisms. Behav Brain Res 221:83–90.
Wang D, Li J, Chen P, Hong Y (2014) Upregulation of pronociceptive mediators and downregulation of opioid peptide by adrenomedullin following chronic exposure to morphine in rats. Neuroscience 280:31–39.
Wang D, Zeng J, Li Q, Huang J, Couture R, Hong Y (2016) Contribution of adrenomedullin to the switch of G protein-coupled mu-opioid receptors from Gi to Gs in the spinal dorsal horn following chronic morphine exposure in rats. Br J Pharmacol 173:1196–1207.
Wang J, Xu W, Shao J, He Z, Ding Z, Huang J, Guo Q, Zou W (2017) miR-219-5p targets CaMKIIgamma to attenuate morphine tolerance in rats. Oncotarget 8:28203–28214.
Wilkie GS, Dickson KS, Gray NK (2003) Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. Trends Biochem Sci 28:182–188.
Yajima Y, Narita M, Narita M, Matsumoto N, Suzuki T (2002) Involvement of a spinal brain-derived neurotrophic factor/full-length TrkB pathway in the development of nerve injury-induced thermal hyperalgesia in mice. Brain Res 958:338–346.
Yaksh TL, Kohl RL, Rudy TA (1977) Induction of tolerance and withdrawal in rats receiving morphine in the spinal subarachnoid space. Eur J Pharmacol 42:275–284.
Yang CH, Huang HW, Chen KH, Chen YS, Sheen-Chen SM, Lin CR (2011) Antinociceptive potentiation and attenuation of tolerance by intrathecal beta-arrestin 2 small interfering RNA in rats. Br J Anaesth 107:774–781.
Young A (2005) Tissue expression and secretion of amylin. Adv Pharmacol 52:19–45.

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