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Research Report

Disruption of acute opioid dependence by antisense oligodeoxynucleotides targeting neurogranin

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ABSTRACT

Neurogranin has been suggested to serve as a common regulator synchronizing the activities of PKC and CaMKII in acute opioid tolerance. To examine if a similar mechanism exists in acute opioid dependence, we directly targeted neurogranin using antisense oligodeoxynucleotides. Male ICR mice were pretreated with neurogranin antisense or mismatch oligodeoxynucleotides (2 µg/day, i.c.v.) for 3 consecutive days. On Day 4, morphine (100 mg/kg, s.c.) was used to induce dependence, as revealed by naloxone-precipitated withdrawal in saline or mismatch-pretreated mice. Antisense-pretreated mice showed decreased neurogranin expression, lack of morphine-induced phosphorylation of neurogranin and activation of CaMKII and CREB, and absence of naloxone-induced withdrawal jumping. Taken together, these data suggest that neurogranin plays an essential role in acute opioid dependence, possibly by affecting the CaMKII and CREB signaling pathway.

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1. Introduction

Repeated administration of opioids produces drug tolerance and dependence. Although the exact mechanisms underlying opioid tolerance and dependence remain unclear (Koob and Nestler, 1997; Nestler, 2001), multiple studies have demonstrated that protein kinase (PKC) and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) are critically involved (Wang and Wang, 2006). It is not known if these protein kinases act independently; however, some recent evidence suggests that PKC and CaMKII may have a common regulator in opioid tolerance.

Neurogranin (Ng), previously named RC3 or P17, is a neuron-specific protein that can sequester calmodulin (CaM) by forming CaM/Ng complex (Chakravarthy et al., 1999; Prichard et al., 1999) (Fig. 1). A sufficiently large and sharp increase in intracellular Ca²⁺ dissociates the stable CaM/Ng

complex, prompting the release of CaM (Huang et al., 2004). The latter, in the presence of Ca²⁺, activates Ca²⁺/CaM-dependent enzymes such as CaMKII (Colbran, 2004). Alternatively, Ng can be phosphorylated by PKC, which also dissociates the CaM/Ng complex (Chakravarthy et al., 1999; Huang et al., 2004). Therefore, Ng can serve as a link between PKC and CaMKII. Our previous studies suggest that Ng phosphorylation is up-regulated in acute opioid tolerance (Shukla et al., 2006) and down-regulation of Ng by specific antisense oligodeoxynucleotides attenuates the development of acute opioid tolerance in mice (Tang et al., 2006b). Whether a similar mechanism involving Ng exists in acute opioid dependence is unknown. Intracellular Ca²⁺ levels have been reported to increase in response to the treatment with opioids (Quillan et al., 2002; Spencer et al., 1997). On the other hand, it has been suggested that opioid tolerance and dependence may have different cellular mechanisms (e.g., Aley and Levine,

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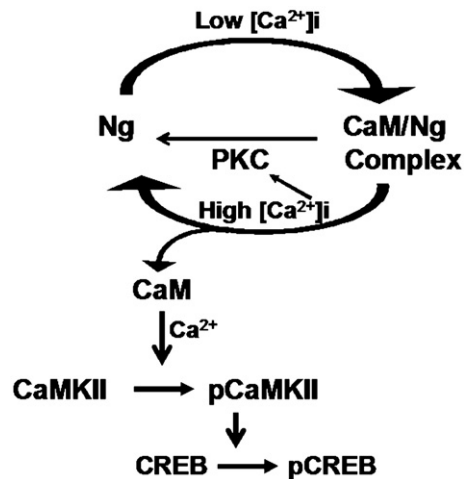


Fig. 1 – A simplified working model illustrating the signaling pathway from protein kinase C (PKC) to neurogranin (Ng) and Ca^{2+} /calmodulin protein kinase II (CaMKII). The equilibrium of intracellular CaM/Ng complex and free CaM regulates the availability of CaM, which in turn regulates the activities of CaM-dependent enzymes such as CaMKII. At low Ca^{2+} levels (e.g., in a resting neuron), the equilibrium favors the CaM/Ng. In contrast, an increase in Ca^{2+} (e.g., in opioid dependence) will shift the balance toward free CaM. The latter, in the presence of Ca^{2+} , activates CaMKII. Alternatively, PKC can phosphorylate Ng, dissociating the CaM/Ng complex. CREB (cAMP response element binding protein) is illustrated as a potential downstream effector of the signaling pathway.

1997a,b; Bohn et al., 2002). In the present study, we investigated whether Ng might play an important role in acute opioid dependence.

2. Results

Mice treated with morphine (100 mg/kg, s.c.) exhibited significant morphine dependence within 2–6 h (Bilsky et al., 1996; Shukla et al., 2006; Tang et al., 2006a). Challenging morphine-treated mice with naloxone (10 mg/kg, i.p., 5 h later) elicited significant withdrawal jumps ($p < 0.001$). Morphine-treated mice recorded 134 ± 24 jumps within 15 min (Fig. 2), which was largely absent in saline-treated control mice (16 ± 11 ; $p < 0.001$). Ng phosphorylation (pNg) was found to be up-regulated in opioid dependence ($p < 0.05$, Fig. 4A). Pretreatment with Ng antisense oligodeoxynucleotides for 3 days significantly decreased Ng expression ($p < 0.05$, Fig. 3A), and reduced the number of withdrawal jumps (7 ± 4 , not significantly different from the saline/saline group), indicating the absence of opioid dependence in Ng antisense-pretreated mice (Fig. 2). In order to control for the specificity of antisense oligodeoxynucleotides, another group of mice were pretreated with mismatch oligodeoxynucleotides which did not alter the expression of Ng (Fig. 3B). Naloxone precipitated a significant number of withdrawal jumps in the mismatch-pretreated mice (105 ± 12 , $p < 0.001$ compared with the saline/saline group), indicative of the presence of opioid dependence.

Morphine-stimulated increase of pNg was completely blocked by Ng-antisense pretreatment ($p < 0.05$, Fig. 4A).

To further elucidate the potential signaling pathway involving Ng, we determined the activity of CaMKII (pCaMKII) in these mice. CaMKII is activated in acute opioid tolerance, and is proposed to be a key factor promoting opioid dependence (Wang and Wang, 2006). Pretreatment with Ng antisense significantly reduced morphine-stimulated pCaMKII ($p < 0.05$, Fig. 4B). One potential downstream effector of CaMKII may be cAMP-response element-binding protein (CREB) (Matthews et al., 1994; Sun et al., 1994). We further tested the phosphorylation of CREB (pCREB) in morphine-treated mice with or without the pretreatment with Ng antisense oligodeoxynucleotides. In Ng antisense-pretreated mice, which showed reduced pNg and pCaMKII, pCREB was also significantly reduced ($p < 0.001$, Fig. 4C).

3. Discussion

Ng is a postsynaptic protein that has been proposed to converge signals from PKC and CaMKII (Chakravarthy et al., 1999; Wu et al., 2002). Both protein kinases have been implicated in the development of opioid tolerance and dependence (Wang and Wang, 2006). In a previous study, we found that phosphorylation of Ng, PKC and CaMKII was similarly regulated in acute opioid tolerance and dependence (Shukla et al., 2006), which led to our hypothesis that Ng may function as a common regulator synchronizing the activities of PKC and CaMKII in opioid dependence. Although several lines of evidence suggest such a role for Ng in other systems, it has never been directly tested in opioid dependence. The

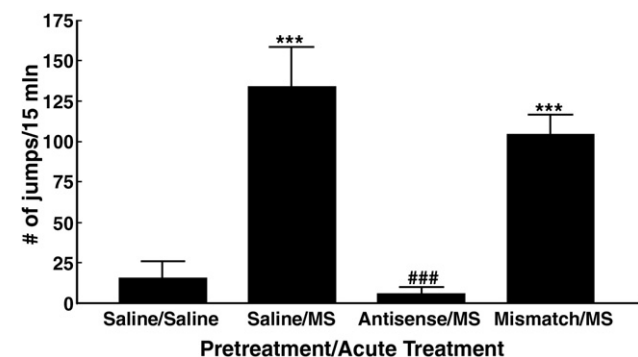


Fig. 2 – Attenuation of acute opioid dependence by antisense oligodeoxynucleotides targeting neurogranin. Separate groups of 6 mice were pretreated with saline, neurogranin antisense, or mismatch oligodeoxynucleotides (2 $\mu\text{g}/\text{day}$, i.c.v.) for 3 consecutive days. To induce dependence, mice were injected with morphine (“MS”, 100 mg/kg, s.c.) on Day 4. Five hours later, naloxone (10 mg/kg, i.p.) was given to determine the presence or absence of morphine dependence. The number of withdrawal jumps was recorded for 15 min. ANOVA: $F = 19.16$, $df = 23$, $p < 0.001$; Dunnett’s t test: *** $p < 0.001$ compared with the control (“Saline/Saline”) group; ### $p < 0.001$ compared with the positive control (“Saline/MS”) group.

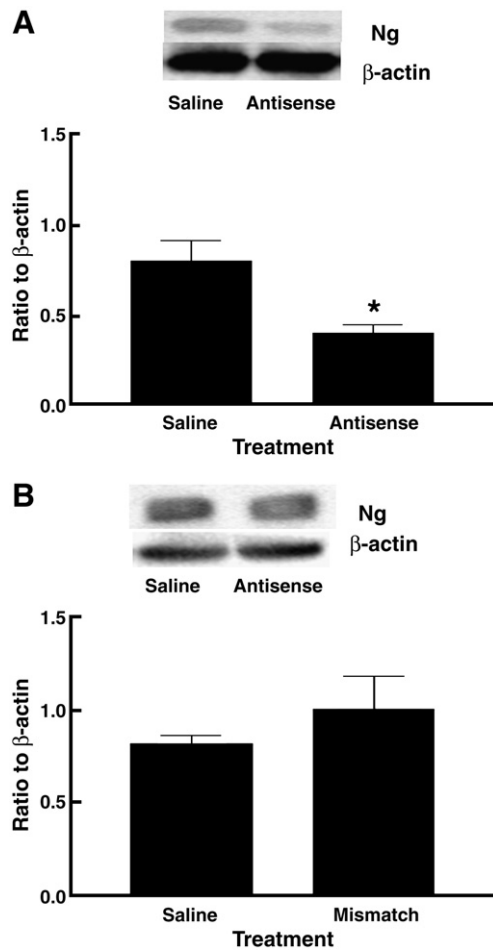


Fig. 3 – Down-regulation of neurogranin by its antisense oligodeoxynucleotides. Mice were treated with saline, neurogranin antisense oligodeoxynucleotides, or mismatch oligodeoxynucleotides (2 μg/day, i.c.v.) for 3 consecutive days. The expression of cortical neurogranin was determined using the Western blotting method on Day 4 to verify the down-regulation of neurogranin by (A) antisense oligodeoxynucleotides, but not by (B) mismatch oligodeoxynucleotides. Student's *t* test: **p*<0.05 compared with the saline treated group.

current study represents our first attempt to test the hypothesis by directly targeting Ng. Since no specific inhibitor or activator is available, antisense oligonucleotides were employed to down-regulate Ng, which decreased the expression of Ng by nearly 50% in our study. This reduction in Ng expression, in turn, suppressed the naloxone-precipitated withdrawal jumping, a key sign of the development of opioid dependence. Biochemical data agreed with the behavioral observation, as morphine-induced phosphorylation of Ng and activation of CaMKII and CREB were absent after the pretreatment with Ng antisense oligonucleotides. Taken together, these results suggest that Ng may be important for the development of acute morphine dependence.

The equilibrium of intracellular CaM/Ng complex and free CaM regulates the availability of CaM, which in turn regulates the activities of CaM-dependent enzymes such as CaMKII (Huang et al., 2004; Huang et al., 2000). At low Ca²⁺ levels (e.g.,

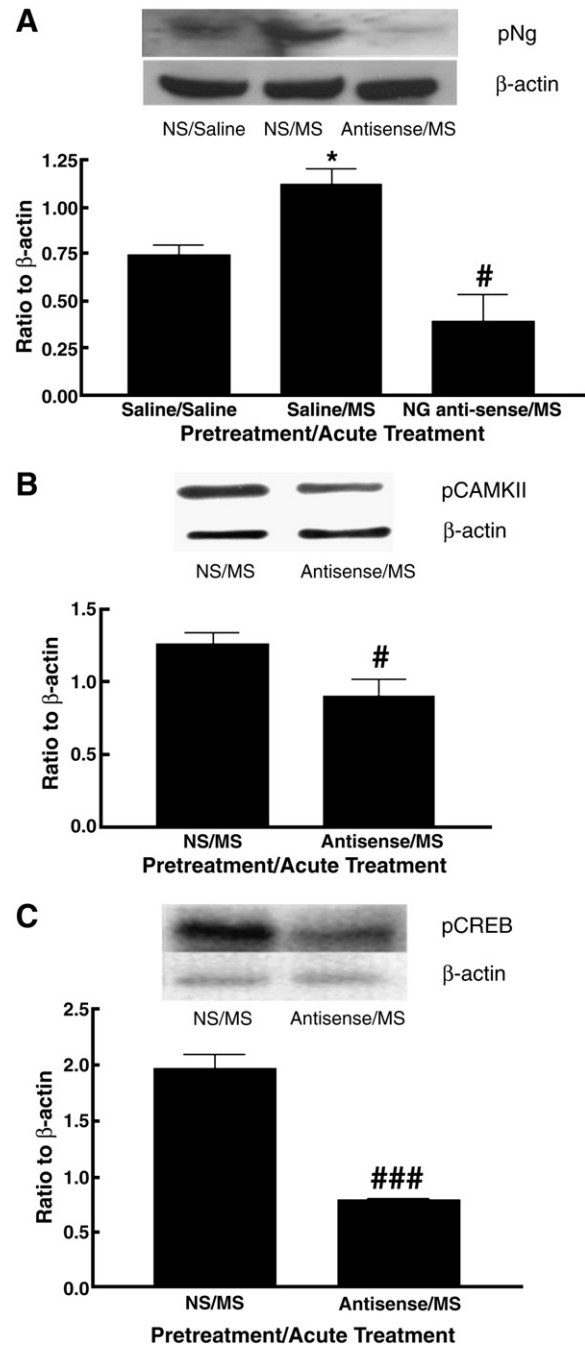


Fig. 4 – Reduction of morphine-induced phosphorylation of (A) neurogranin (Ng), (B) Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), and (C) cAMP response element binding protein (CREB), by antisense oligodeoxynucleotides targeting neurogranin. Groups of 3 mice were treated with neurogranin antisense oligodeoxynucleotides or saline (NS) for 3 consecutive days. On Day 4, supraspinal pNg, pCaMKII, and pCREB were determined by the Western blotting method. (A) ANOVA: *F*=14.48, *df*=8, *p*<0.01; Dunnett's *t* test: **p*<0.05 compared with the "NS/NS" group; #*p*<0.05 compared with the "NS/MS" group. (B, C) Student's *t* test: #*p*<0.05; ###*p*<0.001 compared with the "NS/MS" group.

in a resting neuron), the equilibrium favors the CaM/Ng. In contrast, an increase in Ca²⁺ (e.g., after the exposure to opioids (Quillan et al., 2002; Spencer et al., 1997)) will shift the balance toward free CaM (Huang et al., 2000).

Activities of CaMKII and PKC, and Ng phosphorylation were up-regulated in acute opioid dependence (Shukla et al., 2006). In this study, we found that down-regulation of Ng by antisense oligodeoxynucleotides targeting Ng was able to abolish opioid dependence. Reduced CaMKII activity (pCaMKII) was found in Ng antisense pretreated mice, further supporting the PKC→Ng→CaMKII pathway. Ng-null mice also displayed reduced pCaMKII (Wu et al., 2002).

CREB is a downstream effector of CaMKII in opioid dependence and tolerance (Tang et al., 2006a). CREB has been found to be a key transcriptional factor regulating drug addiction including opioid dependence (Guitart et al., 1992; Lane-Ladd et al., 1997; Maldonado et al., 1996; Nestler, 2001). We found morphine-stimulated pCREB was significantly reduced in mice that have been pretreated with neurogranin antisense. Ng may, therefore, serve as an upstream mechanism that signals to CREB via the PKC→Ng→CaMKII signaling pathway in acute opioid dependence (Fig. 1). It should be noted that additional pathways may exist leading to activation of CREB and other downstream effectors from PKC and CaMKII (e.g., Prichard et al., 1999; Wahlestedt et al., 2000). In this study, we did not systematically examine the importance of anatomical location for the changes of pNG, pCaMKII, and pCREB. It has been shown previously that neurocircuitries involving locus coeruleus and other related regions are critical for opioid dependence (Guitart et al., 1992; Koob and Nestler, 1997; Lane-Ladd et al., 1997). Roles of Ng and CaMKII in these regions need to be examined.

In summary, the present study found that down-regulation of neurogranin was able to abolish morphine-induced phosphorylation of neurogranin and activation of CaMKII and CREB, and block the development of acute opioid dependence. These data suggest that neurogranin may play a critical role in regulating acute opioid dependence. Several chronic models have been used to study chronic opioid dependence, which may or may not share the same mechanisms with the acute opioid dependence used in this study (Tang et al., 2006a; Wang and Wang, 2006). We are in the process of determining if Ng is also important for chronic opioid tolerance. Should the hypothesis be confirmed in other more chronic models of opioid dependence, Ng may become a useful pharmacological intervention site for attenuating opioid dependence.

4. Experimental procedure

4.1. Animals and materials

Male ICR (Institute of Cancer Research) mice (20–25 g, Harlan Industries, Indianapolis, IN) were used in this study and were housed on a 12-h:12-h light/dark cycle (lights on at 6 a.m.–6 p.m.) and provided food and water *ad libitum* before experiments. All experimental procedures were performed in accordance with the NIH guidelines after approval by the Animal Care Committee of the University of Illinois at Chicago. Antisense oligodeoxynucleotides consist of the following sequence for mouse

neurogranin (Tang et al., 2006b; Watson et al., 1990): 5'-CGCTCTCCGTGCAGCAGTC-3'. As a control for antisense activity and specificity, the following mismatch oligonucleotides were applied by introducing four base (inverted sequences) mismatches as described previously (Wahlestedt et al., 2000): 5'-CGCCTTCCGGTCAGCAGTC-3'. Oligodeoxynucleotides were synthesized by Operon (Huntsville, AL). Morphine sulphate was purchased from Abbot Laboratories (North Chicago, IL). NP-40, sodium dodecyl sulphate and other chemicals were purchased from Sigma (St. Louis, MO).

4.2. Behavioral testing

Separate groups of mice received i.c.v. injection (at 10 a.m.) of antisense or mismatch oligodeoxynucleotides (2 µg/5 µl) or saline (5 µl) per day for 3 consecutive days prior to the dependence experiments. On Day 4, mice were randomly selected for behavior testing or Western blot analysis. Groups of 9 mice were made physically dependent on morphine by a single injection of morphine (100 mg/kg, s.c.) in the morning (~10 a.m.) (Bilsky et al., 1996; Tang et al., 2006a). Control mice received (s.c.) equal volume of normal saline. The presence of opioid dependence was revealed by challenging mice with naloxone (10 mg/kg, i.p.) 5 h later as previously described (Tang et al., 2006a). Immediately after the naloxone injection, mice were placed inside glass cylinders, and the number of withdrawal jumps was recorded for 15 min.

4.3. Drug administration

All drugs were dissolved in saline. Intracerebroventricular (i.c.v.) injections were made into the left lateral ventricle as described previously (Bilsky et al., 1996; Tang et al., 2006a). Standard procedures were used for i.p. and s.c. injections.

4.4. Western blot analysis

Western blotting was carried as previously described (Tang et al., 2006a). Brain cortices were immediately dissected out, and homogenized in ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM phenylmethylsulfonyl fluoride, 10 mg aprotinin, 1 mM sodium orthovanadate in PBS buffer, pH 7.4) for the Western blotting analysis. Membrane samples (30 µg) were subjected to 12% (w/v) polyacrylamide gel electrophoresis and subsequently transferred onto PVDF membranes for the analysis of protein expression using the following antibodies: anti-(S36)phospho-neurogranin (1/1000; Upstate Biotechnology, Lake Placid, NY), anti-neurogranin (1/1000; Upstate Biotechnology, Lake Placid, NY), anti-(T286/287)phospho-CaMKII (1/1000; Promega, Madison, WI) and anti-(S133)phospho-CREB (1/1000; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then washed and incubated with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1/1000; Amersham Biosciences, Piscataway, NJ), and developed using an enhanced chemiluminescence detection system (Amersham Biosciences). β-Actin expression was measured in the same blots using a monoclonal antibody (1/10,000; Sigma). Enhanced chemiluminescence signals were captured by a ChemiDoc imaging system and analyzed using QuantityOne

program (BioRad, Hercules, CA). Band densities were quantified with regard to the signals of corresponding β -actin.

4.5. Statistical analysis

All data are expressed as Mean \pm SEM. The differences among multiple groups were first analyzed by ANOVA. When a statistical significance was detected, Dunnett's *t* test was used to determine statistical significance between multiple testing groups and the corresponding control. Student's *t* test was used to evaluate the statistical significance between two groups. These statistical comparisons were analyzed using SPSS software (Chicago, IL). Statistical significance was established at 95%.

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