

Rapid communication

# Attenuation of opioid tolerance by antisense oligodeoxynucleotides targeting neurogranin

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## Abstract

Neurogranin is capable of regulating protein kinase C and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II. In this study, we examined the role of neurogranin in opioid tolerance. Increased phosphorylation of neurogranin was found in opioid tolerance. Opioid tolerance was absent in morphine (100 mg/kg)-treated mice that were also pretreated with neurogranin antisense oligodeoxynucleotides (2 µg/day, i.c.v. for 3 days). The behavioral effect correlated with the decreased expression of neurogranin. These data suggest that neurogranin may be critical in the development of opioid tolerance.

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Prolonged use of opioid leads to the development of tolerance. Several serine/threonine protein kinases such as protein kinase C (PKC) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) have been demonstrated to be important for opioid tolerance (e.g., Tang et al., 2006a,b; Zeitz et al., 2001). Activities of PKC and CaMKII are up-regulated in opioid tolerance (Tang et al., 2006a,b; Zeitz et al., 2001). While chemical inhibition or genetic deletion of these kinases disrupted the development of opioid tolerance (Tang et al., 2006a,b; Zeitz et al., 2001), it is not clear if these two kinases are regulated by a common mechanism in opioid tolerance.

Neurogranin is a neuronal PKC substrate and Ca<sup>2+</sup>/calmodulin binding protein that regulates the availability of free calmodulin in the cytoplasm, thus affecting the activity of calmodulin-dependent enzymes such as CaMKII (Huang et al., 2004). At low levels of Ca<sup>2+</sup>, neurogranin binds and sequesters calmodulin. An increase in intracellular Ca<sup>2+</sup> dissociates the stable calmodulin–neurogranin complex, releasing free calmodulin to activate CaMKII and other enzymes. As a phosphoprotein, neurogranin phosphorylation by PKC is another mechanism to dissociate the calmodulin–neurogranin complex, releasing calmodulin and in-

creasing activity of calmodulin-dependent enzymes such as CaMKII (Huang et al., 2004). Recent studies suggest that neurogranin can also function as an upstream modulator and regulate the activity of PKC. Mice lacking neurogranin exhibited decreased phorbol ester-activation of PKC and decreased agonist-stimulated autophosphorylation of CaMKII (Huang et al., 2004).

To test the hypothesis that neurogranin can serve as a common regulator of PKC and CaMKII, thus affecting opioid tolerance, we directly targeted neurogranin using antisense oligodeoxynucleotides in this study. All experimental procedures were performed in accordance with the National Institutes of Health guidelines after approval by the Animal Care Committee of the University of Illinois. Male ICR mice (20–25 g) were obtained from Harlan Industries (Indianapolis, IN) and housed on a 12/12 h light/dark cycle and provided with food and water *ad libitum* before experiments. Antisense oligodeoxynucleotides consisting of the following sequence for mouse neurogranin, 5'-CGCTCTCCGTGCAG-CAGTC-3' (Watson et al., 1990), was synthesized by Operon (Huntsville, AL). Mice received i.c.v. injection of 2 µg of antisense oligodeoxynucleotides per day for 3 consecutive days prior to the tolerance experiments. Tolerance in mice was produced by morphine (100 mg/kg, *s.c.*, Abbot Laboratories, Chicago, IL) and tested using a tail-flick test as previously described (Tang et al., 2006a,b). Latencies to a rapid tail-flick were recorded before and 30 min after the injection of a test dose of morphine (10 mg/kg, *s.*

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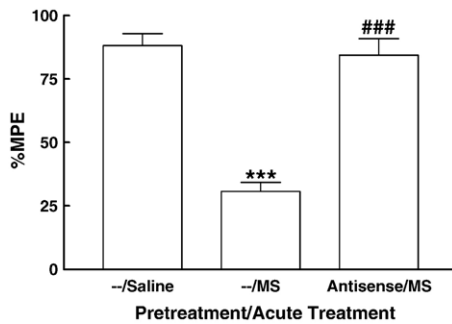


Fig. 1. Blockade of opioid tolerance by antisense oligodeoxynucleotides targeting neurogranin. Separate groups of 6 mice were pretreated with saline or neurogranin antisense oligodeoxynucleotides (2  $\mu$ g/d, i.c.v.) for 3 days (pretreatment). To induce tolerance, saline or antisense-treated mice were injected with morphine (100 mg/kg, *s.c.*, “MS”). A test dose morphine (10 mg/kg, *s.c.*) was given 4 h later to determine the antinociception and presence of antinociceptive tolerance using a 52 °C warm water tail-flick test (Tang et al., 2006a,b). Data are expressed as mean  $\pm$  S.E.M. ( $N=6$ ). \*\*\*  $P<0.001$  compared with the control (“--Saline”) group; ###  $P<0.001$  compared with the morphine tolerant (“--MS”) group.

*c.*) Results are presented in “%MPE” (maximal possible effect) as defined by the formula: %MPE =  $100 \times (\text{test} - \text{control}) / (\text{cut-off} - \text{control})$ . A cut-off of 12 s was applied to prevent tissue damage. All data are expressed as the mean  $\pm$  S.E.M. Differences in response between the experimental groups were determined using the analysis of variance (ANOVA) followed by Dunnett’s *t* test (for multiple groups).

In naive mice, morphine (10 mg/kg, *s.c.*) produced  $88.2 \pm 4.7\%$ MPE antinociception (Fig. 1). Following morphine treatment (100 mg/kg, *s.c.*, 4 h), the test dose of morphine produced only  $30.2 \pm 7.0\%$ MPE ( $P<0.001$ ), indicative of the development of opioid antinociceptive tolerance. Paralleling the development of tolerance, phosphorylation of neurogranin in the brain and spinal cord was significantly increased, as determined by the western blotting method using an anti-phospho-neurogranin antibody (1/1000, Upstate, Lake Placid, NY) (data not shown). In mice pretreated with antisense, neurogranin expression was significantly decreased in 3 days (data not shown). Strikingly, morphine (100 mg/kg, *s.c.*, 4 h), which induced opioid tolerance in the control group and in other studies (Tang et al., 2006a,b), failed to produce antinociceptive tolerance in neurogranin antisense-pretreated mice (Fig. 1). These data suggested that inhibiting neurogranin by down-regulating its expression could block the development of opioid tolerance.

Our study for the first time revealed a critical role of neurogranin in opioid tolerance. Earlier studies suggest that neurogranin interacts with and activates PKC and CaMKII. Mice lacking neurogranin exhibited decreased activation of PKC by phorbol 12-myristate 13-acetate (PMA), decreased forskolin-stimulated activation of cAMP dependent protein kinase (PKA), decreased phosphorylation of cAMP response element-binding protein (CREB), and decreased phosphorylation of mitogen-activated protein (MAP) kinase (Wu et al., 2002). In addition to PKC, PKA, MAP kinase and CREB have also been implicated in opioid tolerance (Nestler, 2001; Trapaidze et al., 2000). These studies support the hypothesis that neurogranin can serve as a common regulator in the neurocircuitry leading to opioid tolerance. However, future studies will be needed to elucidate how neurogranin regulates these different intracellular signaling molecules in opioid tolerance and possibly opioid dependence.

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