Effects of Glycogen Synthase Kinase 3β and Cyclin-Dependent Kinase 5 Inhibitors on Morphine-Induced Analgesia and Tolerance in Rats

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ABSTRACT
Repeated administration of morphine is associated with the development of tolerance, yet the mechanism underlying this phenomenon is still poorly understood. Recent evidence implicating glycogen synthase kinase 3 (GSK3) in opioid receptor signaling pathways has prompted us to investigate its role in morphine tolerance. Administration of 10 mg/kg morphine i.p. to Wistar rats twice daily for 8 days resulted in complete tolerance to its analgesic effects as measured by the tail-flick test. When injections of morphine were preceded by intrathecal (i.t.) administration of either an inhibitor of GSK3 [(3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763) or 6-bromoindirubin-3’-oxime] or an inhibitor of cyclin-dependent kinase (Cdk), roscovitine, development of tolerance to morphine analgesia was completely abolished. In addition, a single i.t. injection of either kinase inhibitor was able to restore in a dose-dependent manner the analgesic effect of morphine in morphee-tolerant rats. None of the inhibitors in doses used in the present study had analgesic effects of their own nor an effect on the analgesic potency of morphine. Repeated i.t. administration of either inhibitor had caused an increase in abundance of GSK-3β phosphorylated at Ser9 in the dorsal lumbar part of the spinal cord of rats that were chronically treated with morphine. Furthermore, reversal of morphine tolerance by a single injection of either inhibitor was always associated with increased abundance of phospho-GSK3β. In conclusion, our data indicate that chronic morphine treatment activates a highly efficient pathway by means of which Cdk5 regulates GSK3β activity.

Adaptations in cellular signaling evoked by repeated morphine administration that lead to development of tolerance are still unclear. Binding of morphine to its main target, the μ-opioid receptor, results in the activation of a

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ABBREVIATIONS: GSK, glycogen synthase kinase; Cdk, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; SB216763, 3-[(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione; roscovitine, 2-(R)-(9-(1-methylthethyl)-6-(phenylmethylamino)-9H-purin-2-yl)amino]-1-butanol; BIO, (2’Z,3’E)-6-bromoindirubin-3’-oxime; PP1, protein phosphatase 1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of M, 32,000; NMDA, N-methyl-D-aspartate.
ity, that the glycoen synthase kinase (GSK) 3β may be involved in opioid signaling. GSK3 is one of the primary targets of the Akt kinases, and in vivo induction of Akt phosphorylation by morphine was found (Muller and Unterwald, 2004). Accordingly, it was found that cardioprotection afforded by morphine was indeed associated with GSK3β inhibition (Gross et al., 2004). In addition, lithium salts, which act primarily as GSK3 inhibitors (Gould and Manji, 2005), were found to modulate morphine effects, but depending on dose and administration procedure, both enhancement (You et al., 2001) and reduction (Debouy et al., 1994; Johnston and Westbrook, 2004) of morphine-induced analgesia was reported. Finally, our expression profiling of gene expression in the amygdala after chronic morphine self-administration in rats has implicated possible alterations in the GSK3/Wnt signaling (Rodriguez Parrita et al., 2004). Here we report that spinal administration of GSK3β or Cdk5 inhibitors abolished tolerance to morphine analgesia. Furthermore, reversal of tolerance was always associated with an increase in abundance of GSK3β phosphorylated at Ser9. These findings suggest the involvement of Gsk3 and Cdk5 in opioid antinociception and tolerance and indicate a novel signaling pathway linking Cdk5 to GSK3β in the mechanism of long-term adaptations to morphine action.

Materials and Methods

Behavioral Studies

Animals. Male Wistar rats (250–300 g) were housed in single cages on a sawdust bedding under standard conditions (12 h/12 h light/dark cycle, with lights on at 8:00 AM) with food and water available ad libitum. All experiments were conducted during the light cycle, between 8:00 AM and 1:00 PM. All experiments were performed according to the recommendations of the International Association for the Study of Pain and the Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the local bioethics committee (Krakow, Poland).

Surgical Preparations and Schedule of Drug Administration. Rats were prepared for intrathecal (i.t.) injection of the inhibitors by implanting catheters under pentobarbital anesthesia (60 mg/kg i.p.). The intrathecal catheter consisted of a polyethylene tubing 12 cm long (PE 10, Intramedic; Clay Adams, Parsippany, NJ); O.D. = 0.4 mm, dead space = 10 μl), sterilized by immersion in 70% (v/v) ethanol, and fully flushed with sterile water before insertion. Rats were placed on the stereotaxic table (David Kopf Instruments, Tujunga, CA), and an incision was made in the atlanto-occipital membrane. The catheter (7.8 cm of its length) was carefully introduced into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement (L1–L2) according to Yaksh and Rudy (1976). After the implantation, the first injection of the 10 μl of 0.9% NaCl was performed slowly and the catheter was tightened. One day after the catheter implantation, the rats were monitored for their physical impairments. Those showing motor deficits were excluded from further study. After surgery animals were allowed a minimum 1-week recovery before the experiment. 0.9% (w/v) NaCl, DMSO or respective drugs were delivered slowly (1–2 min) in a volume of 5 μl through the i.t. catheter and followed by 10 μl of 0.9% (w/v) NaCl, which flushed the catheter.

SB216763 was purchased from Torceis Cookson Inc. (Northpoint, UK), (2’Z,3’E)-6-bromindirubin-3’-oxime (BIO) from Calbiochem (Darmstadt, Germany), roscovitine from Sigma Aldrich (St. Louis, MO), morphine hydrochloride from Polfa (Kutno, Poland), SB216763, BIO, and roscovitine were dissolved in 10% (w/v) DMSO and stored at −20°C for up to 2 weeks. The inhibitors were tested at the following concentrations: SB216763 140, 1.4, and 0.14 pmol (0.05, 0.005, 0.0005, and 0.00005 μg), BIO 14, 1.4 and 0.14 pmol (0.005, 0.0005, and 0.00005 μg), and roscovitine 140, 1.4, and 0.14 pmol (0.05, 0.005, 0.0005, and 0.00005 μg). Each dose was administered i.t. in a volume of 5 μl to one experimental group of animals (n = 8–10). Morphine hydrochloride (10 mg/kg) for i.p. injection was dissolved in 0.9% (w/v) NaCl.

Nociceptive Threshold in Naive Rats. The pain threshold to a thermal stimulus in naive rats was assessed by tail-flick latency evoked by noxious thermal stimulation as determined with a tail-flick analgesic meter (Analgiesia Meter; Ugo Basile, Comerio, Italy). The tail-flick test consisted of a beam of light focused on the dorsal tail surface ~2 cm from the tip of the tail. The intensity of the light was adjusted so that the baseline tail-flick latencies were 3 to 3.5 s. The cutoff time for the tail-flick reaction was set to 9 s. Rats were tested for analgesia 15 and 30 min after i.p. morphine administration.

The pain threshold to a mechanical stimulus in naive rats was assessed by the hindlimb withdrawal threshold evoked by noxious mechanical stimulation as determined with a paw pressure analgesic meter (Analgiesia Meter; Ugo Basile). With this device, a mechanical force increasing at a rate of 32 g/s was applied to the hind paw until the rat withdrew its hind limb. The withdrawal threshold of each animal was the average of three separate measurements performed, and the minimum interval between measurements was 2 min. The cutoff force was 400 g. Rats were tested for analgesia 15 and 30 min after i.p. morphine administration.

Nociceptive Threshold in Morphine-Tolerant Rats. The pain threshold during the development of tolerance to the analgesic effect of morphine was assessed using the tail-flick test as described above. Baseline tail-flick measurements were performed every day 15 min before morphine injection.

Experimental Design. Effects of a single administration of GSK3 and/or Cdk5 inhibitors on pain threshold and morphine analgesic effects in naive rats. To determine the effect of single administration of GSK3 and/or Cdk5 inhibitors on pain threshold, a group of naive rats were treated i.t. with: SB216763 (1.4–140 pmol), BIO (1.4–140 pmol), or roscovitine (1.4–140 pmol). Each dose of kinase inhibitors was administered to a separate group of rats (n = 8). Control rats received DMSO (i.t.) instead of inhibitor. Tail-flick latency and paw pressure withdrawal threshold were determined 30 min after i.t. administration of the inhibitors.

To determine the effect of a single administration of GSK3 and/or Cdk5 inhibitors on morphine analgesic effect, groups of naive rats were treated i.t. 15 min before morphine (2, 4, and 8 mg/kg i.p.) with SB216763 (14 pmol), BIO (14 pmol), or roscovitine (14 pmol). Each dose of morphine and kinase inhibitors was administered to separate groups of rats (n = 8). Tail-flick latency and paw pressure withdrawal threshold were determined 30 min after i.p. administration of morphine.

Effects of repeated administration of GSK3 and/or Cdk5 inhibitors on development of morphine tolerance. Rats were injected with morphine (10 mg/kg i.p.) or saline twice daily at 12-h intervals for 8 consecutive days. Influence of GSK3 or Cdk5 inhibitors on development of morphine tolerance was assessed by i.t. injections of: 1.4 to 140 pmol of SB216763 (n = 10 in each group), 1.4 to 14 pmol of BIO (n = 10 in each group), 1.4 to 140 pmol of roscovitine (n = 10 in each group), or DMSO (n = 8) 15 min before the morphine administration (10 mg/kg i.p.) on each testing day (days 1–8, twice daily). Effects of kinase inhibitors per se were tested in additional groups of rats that were injected i.t. with the same doses as above of SB216763, BIO, and roscovitine (n = 8 in each group) 15 min before the saline (i.p.) injection for 8 consecutive days (twice daily). The control groups were tested simultaneously with every experiment with a limited number of animals. Because there were no statistically significant differences between control groups in separate experiments, the measurements.
were pooled, and the final control value was calculated for \( n = 25 \) animals. Tail-flick latency was determined 15 and 30 min after i.p. administration of morphine or saline.

**Effects of a single administration of GSK3 and/or Cdk5 inhibitors on morphine analgesic effects in morphine-tolerant rats.** A paradigm to induce morphine tolerance was identical to that described above. In brief, morphine at a dose of 10 mg/kg (i.p.) was administered twice daily every 12 h. Rats were rendered tolerant to morphine after 8 days. On day 9 of the experiment at the regular morning time, groups of rats tolerant to morphine were treated i.t. 15 min before morphine (10 mg/kg i.p.) with SB216763 (0.14, 1.4, or 14 pmol), BIO (0.14, 1.4, or 14 pmol), or roscovitine (0.14, 1.4, or 14 pmol). Each dose of kinase inhibitors was administered to a separate group of rats (n = 8). Control rats received saline and/or DMSO (i.t.) instead of inhibitor.

**Biochemical Studies**

**Detection of Ser\(^9\) Phospho-GSK3.** Rats were killed by decapitation on the 8th day 4 h after last dose of morphine in experiments with chronic coadministration of morphine and/or a kinase inhibitor. In experiments in which a single dose of each inhibitor was tested in naive and morphine-tolerant rats, animals were sacrificed 30 min and 4 h after the last injection. The spinal cord was rapidly removed, and the lumbar part (L\( _4 \)–L\( _6 \), where the end of the catheter was placed) was dissected. Tissue samples were briefly washed in radioimmunoprecipitation assay buffer [50 mM Tris-Cl, pH 7.4, 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na\(_2\)VO\(_4\), 1 mM NaF, 1 \( \mu \)g/\( \mu \)l aprotinin, 1 \( \mu \)g/\( \mu \)l leupeptin, and 1 \( \mu \)g/\( \mu \)l pepstatin] and then were homogenized in boiling 1% (w/v) SDS, 50 mM NaF with an IKA Ultra Turrax T25 homogenizer. Protein concentration in the supernatant was determined using the BCA Protein Assay Kit (Sigma Aldrich). Samples containing 10 \( \mu \)g of protein were separated in 10% denaturing acrylamide gel and transferred onto a nitrocellulose membrane (Trans-Blot; Bio-Rad, Hercules, CA). After transfer, blots were blocked with 5% (w/v) albumin (Sigma Aldrich) in Tris-buffered saline for 1 h and incubated overnight at 4°C with rabbit anti-phospho GSK3\(^\beta\) polyclonal antibody (dilution 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-GSK3\( \alpha/\beta\) (1:500; Santa Cruz Biotechnology). Immune complexes were revealed by using appropriate peroxidase-conjugated secondary antibodies (mouse anti-rabbit IgG; Vector Laboratories, Burlingame, CA) at a dilution of 1:1000 for 1 h at room temperature. Images were acquired using a Fujifilm Las-1000 fluororaimager system scanner, and densitometry was performed with the Image Gauge (Fuji) software.

**Statistics**

Tail-flick latencies expressed in seconds are presented as the means ± S.E.M. Comparisons between groups were performed using analysis of variance for repeated measurements followed by Bonferroni’s multiple comparison test using InStat (GraphPad Software Inc., San Diego, CA). \( p < 0.05 \) was considered significant. A computerized Litchfield and Wilcoxon method was used to determine the antinociceptive dose necessary to produce a 50% response (ED\(_{50}\)) and 95% confidence limits on quantal data.

**Results**

**Behavioral Studies**

**A Single Administration of GSK3 and/or Cdk5 Inhibitors Has No Effect on Pain Threshold and Morphine Analgesic Potency in Naive Rats.** At all of the doses tested, i.t. administration of GSK3 and/or Cdk5 inhibitors (1.4–14 pmol) had no effects on pain threshold in naive rats in the tail-flick (Fig. 1, left panels) and paw pressure tests (data not shown). In behavioral tests SB216763 and roscovitine were also tested in doses of 140 pmol, but the effect was not different from the result obtained for 14 pmol of the inhibitors (data not shown).

In addition, the dose of 14 pmol of SB216763, BIO, and roscovitine did not affect the analgesic dose-dependent effect of morphine in either the tail-flick (Fig. 1, right panels) or paw pressure test (data not shown). The ED\(_{50}\) values for morphine did not differ from the ED\(_{50}\) values calculated for
coadministration of morphine and GSK3 and/or Cdk5 inhibitors (Fig. 1, right panels).

**Repeated Administration of GSK3 and/or Cdk5 Inhibitors Blocks Development of Tolerance to Analgesic Effects of Morphine.** Eight days of repeated morphine administration (10 mg/kg i.p. twice daily) resulted in development of tolerance to its analgesic effect. On days 7 and 8 tail-flick latency of morphine-injected rats did not differ significantly from latency of saline-treated animals (day 7: 7.07 ± 0.48 s, F_{5,54} = 23.66, p < 0.05; day 8: 8.00 ± 0.14 s versus 3.09 ± 0.32 s, F_{5,54} = 81.07, p < 0.05) (Fig. 2). The analgesic effectiveness of morphine was reduced to 54.5% on day 7 and to 45.4% on day 8 in comparison to the effect of morphine obtained on day 1. DMSO administered i.t. 15 min before morphine (or saline) i.p. administration did not change significantly tail-flick latency (data under Results).

The morphine antinociceptive effectiveness was close to 90% after morphine injection, preceded by administration of kinase inhibitors SB216763, BIO, and roscovitine at a later time point measure (30 min after morphine injection) (Fig. 3, top and bottom). Whereas the lowest dose of BIO (0.14 pmol i.t.) was effective only for SB216763 as well as roscovitine at a later time point measure (30 min after morphine injection) (Fig. 3, top and bottom). Whereas the lowest dose of BIO (0.14 pmol i.t.) did not change the effect of morphine 15 min after its administration in morphine-tolerant rats (Fig. 3, middle), a single i.t. injection of roscovitine (14 pmol i.t.) restored significantly the analgesic effect of morphine in morphine-tolerant rats. As shown in Fig. 3., on the 9th day of morphine treatment a single i.t. injection of two higher doses (1.4 and 14 pmol) of SB216763, BIO, and roscovitine administered 25 min before morphine (10 mg/kg i.p.) were able to restore significantly its analgesic efficiency. The tail-flick latencies measured 15 and 30 min after morphine injection, preceded by administration of kinase inhibitors, were significantly increased compared with morphine alone (3.43 ± 0.17 s), e.g., observed 30 min after morphine injection: SB216763 14 pmol i.t. before morphine 7.04 ± 0.5 s, F_{5,54} = 35.01, p < 0.001; BIO 14 pmol i.t. before morphine 7.88 ± 0.27 s, F_{5,54} = 27.20, p < 0.001; roscovitine 14 pmol i.t. before morphine 6.28 ± 0.40 s, F_{5,54} = 38.27, p < 0.001). A single injection of inhibitors used in the lowest dose (0.14 pmol i.t.) was effective only for SB216763 as well as roscovitine at a later time point measure (30 min after morphine injection) (Fig. 3, top and bottom). Whereas the lowest dose of BIO (0.14 pmol i.t.) did not change the effect of morphine 15 and 30 min after its administration in morphine-tolerant rats (Fig. 3, middle), a single i.t. injection of

![Fig. 2](image-url) Effects of GSK3 and/or Cdk5 inhibitors SB216763, BIO, and roscovitine on the development of morphine tolerance as measured by tail-flick latency 15 (data not shown) and 30 min after morphine administration. Rats were injected twice daily for eight consecutive days with either saline (i.p.), morphine (M, 10 mg/kg i.p.), SB216763 (S, 1.4, 14, and 140 pmol i.t.), BIO (B, 1.4 and 14 pmol i.t.), or roscovitine (R, 1.4, 14, and 140 pmol i.t.) alone or the same doses of SB216763, BIO, and roscovitine administered 15 min before morphine administration. The same morphine-treated control group is presented in all the panels. DMSO administered i.t. 15 min before morphine (or saline) i.p. administration did not change significantly tail-flick latency (data under Results). Values are the means ± S.E.M. *p < 0.05; **p < 0.001 in comparison to saline-treated group on respective day; †, p < 0.05; ‡‡, p < 0.01; ‡‡‡, p < 0.001 in comparison to morphine-treated group on respective day.

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**Results**

The latency of saline-treated animals (day 7: 8.80 ± 0.43 s versus 8.99 ± 0.46 s, respectively, F_{5,54} = 81.07, p < 0.001). In addition, roscovitine (Fig. 2, right), a Cdk inhibitor, completely blocked the development of tolerance to morphine in the three tested doses: 1.4, 14 and 140 pmol. The analgesic effect of roscovitine coadministered with morphine on day 7 and 8 was statistically significant compared with that of morphine injected alone (day 7: 7.63 ± 1.36 s, F_{r,72} = 17.15; day 8: 6.74 ± 1.03 s, p < 0.01, 6.92 ± 1.09 s, p < 0.01, and 8.13 ± 0.60 s, p < 0.001, respectively, F_{r,72} = 11.90). SB216763, BIO, and roscovitine injected alone had no effect on tail-flick latency after repeated (twice daily) administration of all tested doses (Fig. 2) during the tested time period.

**A Single Administration of GSK3 and/or Cdk5 Inhibitors Restores Morphineline Analgesic Effect in Morphine-Tolerant Rats.** All three inhibitors, SB216763, BIO, and roscovitine, restored in a dose-dependent manner the analgesic effect of morphine in morphine-tolerant rats. As shown in Fig. 3, on the 9th day of morphine treatment a single i.t. injection of two higher doses (1.4 and 14 pmol) of SB216763, BIO, and roscovitine administered 15 min before morphine (10 mg/kg i.p.) were able to restore significantly its analgesic efficiency. The tail-flick latencies measured 15 and 30 min after morphine injection, preceded by administration of kinase inhibitors, were significantly increased compared with morphine alone (3.43 ± 0.17 s), e.g., observed 30 min after morphine injection: SB216763 14 pmol i.t. before morphine 7.04 ± 0.5 s, F_{5,54} = 35.01, p < 0.001; BIO 14 pmol i.t. before morphine 7.88 ± 0.27 s, F_{5,54} = 27.20, p < 0.001; roscovitine 14 pmol i.t. before morphine 6.28 ± 0.40 s, F_{5,54} = 38.27, p < 0.001). A single injection of inhibitors used in the lowest dose (0.14 pmol i.t.) was effective only for SB216763 as well as roscovitine at a later time point measure (30 min after morphine injection) (Fig. 3, top and bottom). Whereas the lowest dose of BIO (0.14 pmol i.t.) did not change the effect of morphine 15 and 30 min after its administration in morphine-tolerant rats (Fig. 3, middle), a single i.t. injection of...
SB216763 (14 pmol), BIO (14 pmol), or roscovitine (14 pmol) had no effect on the tail-flick latency in morphine-tolerant rats when administered before saline (Fig. 3, top, middle, and bottom, respectively).

**Biochemical Studies**

**A Single Administration of GSK3 and/or Cdk Inhibitors Had No Effect on the Abundance of Phosphorylated GSK3β in Naive Rats.** A single i.t. injection of morphine (10 mg/kg i.p.) as measured 30 min and 4 h after morphine administration, M1, effect of morphine administration in the 1st day, M9, effect of morphine administration in the 9th day. Each experimental group consisted of eight rats. Values are the means ± S.E.M., *p < 0.05; ***, p < 0.001 compared with saline-treated group on day 9 (M9).

**Repeated Administration of GSK3 and/or Cdk5 Inhibitors Results in Increase of Abundance of Ser9 Phosphorylated GSK3β in the Spinal Cord.** Although chronic morphine treatment had no effect on GSK3β phosphorylation, repeated i.t. administration of either SB216763, BIO, or roscovitine (14 pmol twice daily) caused only a slight, nonsignificant increase in the amount of phospho-GSK3β (Fig. 5, A and B). Chronic treatment with morphine (10 mg/kg i.p.) and i.t. SB216763, BIO, or roscovitine (14 pmol twice daily) causes a profound increase in GSK3β phosphorylation (Fig. 5, A and B). Chronic treatment with kinase inhibitors alone or with morphine had no effect on the total GSK3β protein in the lumbar section of the rat spinal cord. In addition, catheter implantation alone had no effect on total abundance of GSK3β Ser9 phosphorylation (data not shown).

**A Single Administration of GSK3 and/or Cdk5 Inhibitor Increases Abundance of Ser9 Phosphorylated GSK3β in the Spinal Cord of Morphine-Tolerant Rats.** Thus, reversal of morphine tolerance was associated with increases in the abundance of Ser9 phospho-GSK3β. A single i.t. injection of 14 pmol of SB216763, BIO, or roscovitine reversed tolerance and induced phosphorylation of GSK3β (Fig. 6, A and B). Interestingly, SB216763 at doses as low as 0.14 pmol i.t. induced a statistically significant increase in phosphorylation of GSK3β, and the result corresponded to a behavioral effect (compare with Fig. 3A), whereas 0.14 pmol i.t. of BIO or roscovitine caused only a mild, nonsignificant phosphorylation increase and had no significant effect on tolerance. Neither morphine nor inhibitor alone or combined treatment has affected the abundance of total GSKβ protein (Figs. 5A and 6A).

**Discussion**

The main results of this study demonstrate the roles of GSK3β and Cdk5 (the only Cdk present in adult neurons) in development and expression of morphine tolerance. Intrathecal administration of either of GSK3 or Cdk5 inhibitors,
before each morphine injection, abolished development of tolerance to morphine analgesia. A single i.t. injection of either kinase inhibitor was able to restore the analgesic effect of morphine in morphine-tolerant rats. Furthermore, reversal of morphine tolerance by repeated or single injection of either inhibitor was always associated with increased abundance of phospho-GSK3β.

Interestingly, doses as small as 0.14 pmol of SB216763 and 1.4 pmol of BIO and roscovitine were found to reverse tolerance to morphine analgesia. Even if we made the unlikely assumption that after i.t. injection the inhibitors did not diffuse from the site of injection, their effective concentration raises the question that after i.t. injection the inhibitors did not diffuse from the site of injection, their effective concentration would be well below the previously reported IC50 values. This indicates that either the pharmacodynamics of these compounds favors their accumulation in cellular compartments where the target kinases are located or the actual in vivo IC50 values are much smaller than those previously indicated (Meijer et al., 2004).

The ATP-binding sites of GSK3 and Cdks have similar structures, and most small-molecule kinase inhibitors that bind to GSK3 also have considerable affinity for Cdks. To distinguish between those two types of kinase, we have used the three inhibitors that have the best relative selectivity. BIO and SB216763 are several times more selective for GSK3 than for Cdks (Meijer et al., 2004), and roscovitine was not found to inhibit GSK3 (Bain et al., 2003). Furthermore, it was shown that SB216763 has no effect on a large array of other kinases (Coghlan et al., 2000). Thus, SB216763 could be used to rule out the effects from interactions with other kinases.

As noted above, despite SB216763 and BIO being considered selective GSK3 inhibitors, both have similar affinity for Cdk5 (Meijer et al., 1997, 2004). Because all three inhibitors were found to be effective toward Cdk5, we lean toward the conclusion that their effects on Cdk5 in addition to GSK3 were essential for reversal of tolerance. Furthermore, inhibition of Cdk5 by roscovitine leads in our study to inhibitory phosphorylation of GSK3 at Ser9, Cdk5 (Meijer et al., 2004). Because all three inhibitors were effective toward Cdk5, we lean toward the conclusion that their effects on Cdk5 in addition to GSK3 were essential for reversal of tolerance. Furthermore, inhibition of Cdk5 by roscovitine leads in our study to inhibitory phosphorylation of GSK3 at Ser9; thus, Cdk5 inhibitors are indirect GSK3 inhibitors in this case. Still, because roscovitine is not a direct inhibitor of GSK3, it could be hypothesized that Cdk5 inhibition is sufficient to initiate the cascade of events that leads to GSK3 phosphorylation and reversal of tolerance.

Particularly interesting is the relation between inhibition of Cdks and GSK3β phosphorylation. As observed in our experiments, reversal of morphine tolerance was always linked to GSK3β phosphorylation. The anti-phospho GSK3β antibody appears to detect Ser21 phosphorylated GSK3β as well. Therefore it might be suspected, that because no changes were observed in the intensity of the upper band, only the phosphorylation of GSK3β would be significantly...
affected in this manner. The observed induction of GSK3β phosphorylation by Cdk inhibitors is in fact opposite to the previously observed interaction between Cdk5 and GSK3β in isolated squid giant axons (Morfini et al., 2004), in which administration of a single dose of olomoucine, a general Cdk inhibitor, resulted in a decrease in GSK3β phosphorylation. According to Morfini et al. (2004), the decrease in GSK3β phosphorylation was protein phosphatase (PP) 1-dependent and sensitive to okadaic acid inhibition. In our case, single i.t. injections of Cdk5 or GSK3 inhibitor had no effect on GSK3β phosphorylation in naive rats; previous exposure to morphine was necessary for the increased phosphorylation. Thus, i.t. administration of GSK3 or Cdk5 inhibitor resulted in increased abundance of Ser9-phospho-GSK3β only when animals were previously treated with morphine. This result indicates that chronic morphine treatment has caused an adaptation of molecular signaling that enabled regulation of GSK3β phosphorylation by the inhibitors.

It has been recently indicated, that roscovitine was able to block the development of morphine tolerance; however, it displayed analgesic activity when was applied alone (Wang et al., 2004). Moreover, we found no analgesic effects of roscovitine alone as opposed to the previous report, but in the present report we have used doses of roscovitine that were >2 orders of magnitude lower than those used previously. The affinity of roscovitine for extracellular signal-regulated protein kinases is roughly 1 order of magnitude weaker than that for Cdk5 (Meijer et al., 2004); thus, minimizing the dose of inhibitor is essential to avoid unselective effects, especially since extracellular signal-regulated protein kinase were implicated in pain and opioid signaling (Polakiewicz et al., 1998; Bilecki et al., 2005).

A very recent study has explored the role of p35, an activator of the Cdk5 kinase in nociceptive signaling (Pareek et al., 2006). It was found that ablation of p35 in mice resulted in decreased sensitivity to painful thermal stimulation, whereas overexpression of p35 leading to increased Cdk5 activity, had the opposite effect. Based on these observations, the authors have hypothesized that Cdk5 could be an effective target for antinociceptive drugs. Although our results indicate that Cdk5/GSK3 inhibition at the spinal level in rats does not result in analgesia, GSK3/Cdk5 inhibitors clearly influence development of morphine tolerance.

Several potential cellular signaling pathways could account for the observed effects of GSK3/Cdk5 inhibitors. Morphine was found to directly regulate the Akt kinase pathway, presumably through the actions of the βγ subunits of the Gαi protein. GSK3β is one of the major targets for the Akt kinase (Jope and Johnson, 2004). On the other hand, chronic morphine treatment was previously shown to up-regulate the cAMP signaling pathway. Among other effects, this resulted in phosphorylation in the striatum of the DARPP-32 protein, a member of the PP inhibitory subunit family, and affects its interaction with PP1, which in turn was able to dephosphorylate GSK3β. This pathway is the most obvious target for the effects of Cdk5, as it was shown that in striatal neurons this pathway is able to phosphorylate DARPP-32 and change its specificity toward PP1 (Bibb et al., 1999). Although little is known about DARPP-32 function in the spinal neurons, other members of the PP inhibitory subunit family could conceivably perform a similar role. Chronic morphine treatment was also shown to sensitize the NMDA receptors and enhance glutamnergic transmission in the spinal cord (Mayer et al., 1999; Ossipov et al., 2005). This could lead to the up-regulation of the mitogen-activated protein kinase signaling cascade (Xia et al., 1996) and activation of the ribosomal S6 (Rsk/S6K) kinase, which phosphorylate GSK3β. It could be hypothesized, that the reversal of morphine tolerance is achieved by disrupting the interaction between NMDA receptors and Cdk5 and blockade of NMDA receptor currents in a manner similar to that described recently (Li et al., 2001). Thus, the reversal of tolerance would be mediated by the same mechanism as indicated previously for noncompetitive NMDA receptor antagonists (Trujillo and Akil, 1991). It was reported that selective NMDA receptor antagonists attenuated the development of morphine tolerance (Hamdy et al., 2004; Danyasz et al., 2005). Stimulation of NMDA receptors was shown to dis inhibit GSK3β by PPI-mediated dephosphorylation of GSK3β at the Ser9 (Szatmari et al., 2005). Furthermore, memantine administration stimulates GSK3β phosphorylation (De Sarno et al., 2006). Finally, the importance of calcium/calmodulin kinase inhibitors to reverse opioid tolerance and dependence (Wang et al., 2003; Tang et al., 2006).

The involvement of Cdk5 and GSK3β and cross talk between the opioid and glutamate receptors in the development of morphine tolerance are similar to the postulated effects of cocaine on dopamine signaling. It was found in mice lacking a functional dopamine transporter genes that SB216763 reversed the locomotor hyperactivity caused by increased dopamine levels (Beaulieu et al., 2004). This effect was postulated to be dependent on the activity of Akt and GSK3 kinases. Another report indicated that Cdk5 played an important role in dopamine signaling; in particular it was demonstrated that roscovitine was able to attenuate the effects of dopamine denervation (Chergui et al., 2004). This points to the presence of a common mechanism regulating molecular signaling pathways associated with Gαi-protein coupled receptors, which has been substantiated by the recent report on regulation of GSK3β phosphorylation by antidepressant drugs and ligands of the 5-HT1A and 5-HT2 receptors (Li et al., 2004).

In conclusion, our results suggest that chronic morphine treatment activates the GSK3β and Cdk5 pathways. Because GSK3β and Cdk5 seem to be involved in the regulation of several different neuronal signaling systems, there is a theoretical possibility of exploiting this phenomenon in pain management by opioids.

References
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