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Dual κ -agonist / μ -antagonist opioid receptor modulation reduces L-dopa induced dyskinesia and corrects dysregulated striatal changes in the non-human primate model of Parkinson's disease

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Abstract

Objective: Effective medical management of L-dopa induced dyskinesia (LID) remains an unmet need for patients with Parkinson's disease (PD). Changes in opioid transmission in the basal ganglia associated with LID suggest a therapeutic opportunity. Here we determined the impact of modulating both mu and kappa opioid receptor signaling using the mixed antagonist/agonist analgesic nalbuphine in reducing LID and its molecular markers in the non-human primate model.

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Authorship

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Potential Conflicts of Interest

S.P.B. and M.V. own equity in MentiNova, Inc., which is developing nalbuphine for the treatment of L-dopa induced dyskinesia. S.M.P. has received research support from NIH, Michael J. Fox Foundation, Pfizer, Inc., EnVivo Pharmaceuticals, Inc., Forum Pharmaceuticals, Inc., GeneGraft, Ltd, and Key Neurosciences. She is a consultant for Teva Neuroscience. M.M.M. has received research support from NIH, Michael J. Fox Foundation, American Parkinson Disease Association, Eisai, and Signum Biosciences. She has served as a consultant for Abbvie and Teva, owns equity in MentiNova, Inc., and is compensated as Editor-in-Chief of *Neurotherapeutics*.

Methods: MPTP-treated macaques with advanced parkinsonism and reproducible LID received a range of nalbuphine doses or saline s.c. as: 1) monotherapy, 2) acute co-administration with L-dopa, and 3) chronic co-administration for a month. Animals were assessed by blinded examiners for motor disability and LID severity using standardized rating scales. Plasma L-dopa levels were determined with and without nalbuphine, and postmortem brain samples were subjected to Western blot analyses.

Results: Nalbuphine reduced LID in a dose-dependent manner by 48% ($p < 0.001$) without compromising the anti-parkinson effect of L-dopa or changing plasma L-dopa levels. There was no tolerance to the anti-LID effect of nalbuphine given chronically. Nalbuphine co-administered with L-dopa was well tolerated and did not cause sedation. Nalbuphine monotherapy had no effect on motor disability. Striatal tissue analyses showed that nalbuphine co-therapy blocks several molecular correlates of LID including over-expression of FosB, prodynorphin, dynorphin A, cyclin-dependent kinase 5 (Cdk5), and increased phosphorylation of DARPP-32 at Threonine34.

Interpretation: Nalbuphine reverses the molecular milieu in the striatum associated with LID and is a safe and effective anti-LID agent in the primate model of PD. These findings support repurposing this analgesic for the treatment of LID.

Introduction

Five decades since its discovery, L-dopa is still the gold standard therapy for Parkinson's disease (PD). Despite being the most effective agent to control the motor symptoms of the disease, its chronic use is complicated by motor response alterations, the most troubling of which is L-dopa-induced dyskinesia (LID). These abnormal, involuntary movements can be disabling and interfere with activities of daily living. On average, about half the patients treated with L-dopa for 5 years develop LID and the majority do so by ten years¹. Currently, amantadine is the only available agent that can ameliorate LID but does so only moderately, not in all patients, and is often associated with adverse effects that can necessitate its discontinuation²⁻⁴. Therefore, the development of novel treatment strategies to reduce dyskinesia is critical for improving the quality of life for PD patients.

Central to the development of LID are changes in neuronal networks that are modulated by various neurotransmitter systems^{5,6}. Of these, opioid receptor neurotransmission is of particular interest as opioids are co-transmitters that modulate basal ganglia function, and changes in opioid transmission occur in dyskinetic PD patients and animal models. For example, in LID, precursors of endogenous opioid receptor ligands are significantly up-regulated including preproenkephalin A, which is the precursor of Met- and Leu-enkephalins, and preproenkephalin B, which produces dynorphin A and B, and beta-neoendorphin⁷ (for review see Huot *et al*)⁸. Preproenkephalin mRNA and protein levels are increased in the striatum in animal models of PD and in human post-mortem brain tissue^{9,10}. Additionally, these opioid peptides and their mRNAs are significantly elevated in the dyskinetic state, but not in normal or nondyskinetic parkinsonian state¹¹⁻¹⁵. In living PD patients with LID compared with nondyskinetic PD patients, position emission tomography using a nonselective opioid receptor ligand shows decreased binding in the striatum and other brain regions suggesting increased endogenous opioid transmission¹⁶. Given these

elevated levels of ligand, opioid receptor antagonism may be of benefit in diminishing LID. However, there are several intricacies of the response to opioids that must be considered.

Three major classes of opioid receptors exist with different distributions and functions in the basal ganglia, namely mu (μ), kappa (κ) and delta (δ) receptors. Furthermore, expression of opioid receptors changes in the parkinsonian state, likely secondary to up-regulation of opioid ligand expression^{16–19}. This complexity in distribution and function is probably the reason why non-selective antagonists have shown extremely varied efficacy in LID, worsening²⁰, not affecting²¹, or ameliorating symptoms²² in animal models, and have not been effective in small clinical trials²³. Therefore, a level of specificity is believed to be required, but the precise nature of this specificity appears to be complex.

Various opioid compounds have been evaluated in LID, and the totality of the data indicates that mu-antagonism has the most benefit, with kappa-agonism also having desirable effects. For example, selective mu receptor antagonists reduce LID in primates^{24, 25}. Kappa agonists are also efficacious in rodent^{26–28} and primate models²⁷, while the kappa-selective antagonist *nor*-BNI had no effect on primate LID²⁴. Nonselective stimulation of opioid receptors with morphine has been shown to reduce dyskinetic movements in parkinsonian primates and patients perhaps related to kappa receptor mediated neurotransmission^{29, 30}. Nonselective opioid receptor antagonists, such as naloxone and naltrexone, have had variable effects in primates and humans, with no change, increased or decreased LID reported^{20–23}.

Nalbuphine is a synthetic opioid analgesic with mixed activity as a mu-opioid receptor antagonist and kappa-opioid receptor agonist and a weak affinity to delta-opioid receptors (K_i of 0.5, 29 and 60 nM, respectively)³¹. With this specific pharmacological profile, the present study was designed to test its anti-LID activity and safety, as well as its ability to reverse the biochemical changes in the striatum that occur with LID in the advanced primate model of PD.

Methods

Non-Human Primate Model

Six adult monkeys (*Macaca fascicularis*, 2 female and 4 male) were rendered parkinsonian by repeated systemic MPTP administration as previously described³². After stabilization of parkinsonian motor disability, all monkeys received oral L-dopa [carbidopa/levodopa (Sinemet® 25/100)] 1–4 times daily as maintenance treatment and gradually developed dyskinesia. Oral L-dopa doses were determined individually according to the animal's sensitivity to produce an acceptable “on” state regarding mobility and function. After LID was fully developed and stabilized with chronic oral Sinemet administration, the optimal subcutaneous (s.c.) dose of L-dopa methyl ester plus benserazide (1/4 of L-dopa dose; both from Sigma-Aldrich, St. Louis, MO) was determined for each animal based on their response to repeated tests of various doses. This optimal injectable L-dopa dose, which was defined as the lowest dose that induced a satisfactory “on” response (> 50% improvement) with clear dyskinesias, was used in acute tests of nalbuphine (Diamondback Drugs, Scottsdale, AZ).

At the end of behavioral studies, three monkeys selected at random were euthanized for analysis of brain tissue. To complete postmortem molecular studies, brains obtained previously from four adult monkeys (*Macaca fascicularis* and *mulatta*, 2 female and 2 male) prepared with the same model of advanced parkinsonism and LID, and exposed only to L-dopa, were also analyzed. The characteristics of each animal are described in Table 1. Animal maintenance and procedures were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. In keeping with these guidelines, three of the six animals used for behavioral tests were kept alive and transferred to other research studies in order to minimize the number of primates used in scientific research.

Nalbuphine Tests

On test days, nalbuphine and/or L-dopa methyl ester were administered s.c. to six animals followed by behavioral evaluations as described below. All tests were performed after overnight fast and at least 48 hours apart to ensure adequate washout of nalbuphine. Sinemet maintenance treatment was withheld on all test days. During co-administration tests, nalbuphine was injected immediately before L-dopa methyl ester. In all acute studies, nalbuphine doses were tested in random order, and each dose was repeated three times. Data were averaged to yield a mean from three values for each dose in each monkey. Nalbuphine was tested in three forms of treatment: monotherapy, acute co-administration and chronic co-administration with L-dopa.

Monotherapy.—Nalbuphine was tested alone at 0 (control saline injection), 0.016, 0.05, 0.16, and 0.5 mg/kg s.c. (nalbuphine HCl, dissolved in saline) for evaluating safety and effects on parkinsonian motor disability. Animals were assessed before and following nalbuphine administration for 2 to 3 hours depending on the observed response and until the effect wore off.

Acute Nalbuphine-Ldopa Co-administration.—Nalbuphine was administered at 0, 0.03, 0.06, 0.13, and 0.25 mg/kg s.c. just before L-dopa methyl ester at the preselected optimal doses s.c. as acute single injections to determine the efficacy of nalbuphine in reducing LID and any possible effects on the antiparkinsonian action of L-dopa.

Chronic Nalbuphine Treatment.—Nalbuphine (0.13 mg/kg s.c.) was injected together with the animals' morning oral Sinemet® dose daily for one month to determine the development of tolerance or the appearance of adverse effects with chronic treatment. On evaluation days, the morning Sinemet dose was withheld, and nalbuphine was given immediately before L-dopa methyl ester both s.c. L-dopa doses for these tests were the same optimal doses used in acute tests. Assessments were made at baseline (day 0: L-dopa plus nalbuphine 0 mg/kg), midway through the chronic administration schedule (days 14 and 15: L-dopa plus nalbuphine 0.13 mg/kg), at the end of the month-long treatment (days 30 and 31: L-dopa plus nalbuphine 0.13 mg/kg), and again one week after discontinuation of daily nalbuphine administration (days 37 and 38: L-dopa plus nalbuphine 0 mg/kg). The repetition of each test on two consecutive days provided duplicate data that was averaged for analysis for consistency of results.

Behavioral Evaluations

Motor evaluations were performed live by trained examiners using a standardized primate motor scale (PMS) for MPTP-treated monkeys³³. The PMS has two parts: motor disability is rated in Part I (which is similar to Part III of the UPDRS used for PD patients), and dyskinesias are rated in Part II as described previously (Cao 2007). In addition, other common neurologic effects of drugs such as sedation were assessed with the “Drug Effects on the Nervous System (DENS) scale (Uthayathas et al. 2013). This scale serves to assess rapid changes in cognitive, extrapyramidal and autonomic functions after acute drug administration. Motor Disability Scores (MDS), LID and DENS scores were obtained prior to drug treatment (time = 0, “off” state), 30 minutes after injection(s) and again every 20 minutes thereafter for 180 minutes or until dyskinesias were no longer present. Examiners were blinded to the treatment and nalbuphine dose during both mono- and co-therapy testing.

Pharmacokinetics

Pharmacokinetic studies were performed in two awake animals under chair restraint following appropriate chair training. No sedation was used for this procedure. Blood samples were obtained via a vascular access port in the saphenous vein immediately prior to drug injection and again at 60, 120, and 180 minutes post-injection(s) for all experiments. Samples were collected following: 1) L-dopa methyl ester 25 mg plus saline, and 2) L-dopa methyl ester 25 mg plus nalbuphine 0.13 mg/kg, each tested twice. Samples were processed in duplicates according to standard laboratory procedures and plasma stored at –80°C until processed for measurements of L-dopa concentrations using liquid chromatography/mass spectrometry^{34, 35}.

Western Blot Analysis

Three animals were euthanized for analysis of brain tissue at the completion of behavioral studies. These animals received daily injections of nalbuphine (0.13 mg/kg s.c.) every morning for a week prior to euthanasia while their regular L-dopa dose was kept constant. One hour before euthanasia, they received nalbuphine (0.13 mg/kg) and L-dopa methyl ester at the optimal dose used in acute tests (both s.c.). Animals were sedated with ketamine and euthanized with a combination of lethal drugs including overdoses of barbiturates. Immediately after obtaining the brain, the striatum was dissected fresh and rapidly stored at –80°C until processing. Four additional brains were obtained from previously euthanized animals that had the same advanced parkinsonism, chronic exposure to L-dopa and stable LID as the animals used in the nalbuphine behavioral experiments. The method of euthanasia and brain processing were also identical, but these four animals did not receive nalbuphine and were only treated with L-dopa until euthanasia, thus served as controls for tissue analysis (total n=7; 3 nalbuphine treated, and 4 controls)

For Western blots, striatal tissue was homogenized in UREA-TEAB lysis buffer (8 M urea, 100 mM Triethylammonium bicarbonate) containing phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA) and protease inhibitor cocktail set V (Calbiochem). Lysates (25 µg) were mixed with LDS (Lithium dodecyl sulfate)-sample loading buffer (Life technologies, Grand Island, NY), separated in 4–20% gradient gel (GenScript, Piscataway,

NJ), and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA) followed by blocking with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20. Primary antibodies used were FosB (Cell Signaling, Danvers, MA), Dynorphin A (Abcam, Cambridge, MA), Prodynorphin (Abcam), p-DARPP-32 (Thr34) (Cell Signaling), p-DARPP-32 (Thr75) (Abcam), DARPP-32 (Santa Cruz Biotechnology, Dallas, TX), Cdk5 (Santa Cruz Biotechnology), p-ERK (Cell Signaling) and β -actin (Sigma-Aldrich, St. Louis, MO). Immunoblots were developed using ECL plus (PerkinElmer, Boston, MA), and band intensity was quantified by Image J.

Statistical Analysis

Behavioral effects of nalbuphine were analyzed with one or two-way ANOVA for repeated measures with Dunnett's post-hoc tests. All data were normalized to control values for analysis. In monotherapy tests, time course of MDS, total MDS (sum of MDS obtained at all post-injection time points) and DENS scores were analyzed. In acute and chronic co-administration tests, time course of LID, peak-dose LID (50 minutes post-injection), total LID (sum of dyskinesia scores obtained at all post-injection time points), MDS and DENS scores were analyzed. Densitometric quantification of Western blot data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple range test. Data are presented as means $\pm$ SEM. Significance was determined at $p < 0.05$.

Results

Nalbuphine reduces L-dopa induced dyskinesia.

When administered in conjunction with a dyskinesia-inducing dose of L-dopa, nalbuphine significantly reduced LID at doses ranging between 0.06 mg/kg and 0.25 mg/kg, with the most effective dose being 0.13 mg/kg ($p < 0.001$) (Fig 1). The effect size for 0.13 mg/kg nalbuphine was not significantly different than that for 0.25 mg/kg. When considering specific dyskinesia parameters, both peak and total dyskinesia scores showed significant reduction by 43% and 48%, respectively, at the most effective dose (see videos in supplementary materials). Dyskinesia duration, on the other hand, was not altered by nalbuphine co-administration. LID duration with L-dopa alone was 100 ± 13 minutes, and with L-dopa plus nalbuphine ranged between 90 ± 14 and 101 ± 9 minutes with various doses, none being significantly different from L-dopa alone.

The intensity of dyskinesias in the tested monkeys with profound motor impairment and prolonged exposure to L-dopa replacement therapy was considered moderate to severe with mean peak-dose LID scores between 8 and 15.7 in four animals (Table 1). Commonly, LID in these monkeys combine choreodystonic movements of the limbs and orofacial dyskinesias with tongue dystonia. Notably, nalbuphine was effective in every animal in the group.

The anti-dyskinetic effect of nalbuphine is specific.

The therapeutic anti-parkinson action of L-dopa was not affected by nalbuphine at any of the doses tested in any animal (Fig 2A–B). Peak, total and duration of the “on” response to L-dopa were the same with or without nalbuphine co-administration. Additionally, none of the nalbuphine doses (0.03–0.25 mg/kg) co-administered with L-dopa produced any sedative

effect, as animals were normally active and did not have measurable scores on the DENS scale, indicating that the LID reduction was not caused by sedation. Importantly, plasma L-dopa levels and clearance were unaffected when L-dopa was given alone or co-administered with nalbuphine (Fig 2C). Both peak L-dopa levels and the rate of clearance were unchanged by nalbuphine co-administration in both monkeys tested. This indicates that the anti-dyskinetic effect of nalbuphine is due to its central activity at its target receptors and not confounded by altering L-dopa pharmacokinetics. Based on all these reasons, nalbuphine appears to act specifically on mechanisms of LID.

The anti-LID effect of nalbuphine is sustained with its chronic administration.

To determine if the anti-LID activity of nalbuphine is maintained with long term repeated administration, animals received daily injections (0.13 mg/kg) for one month. Efficacy was tested at 2 weeks and one month during chronic nalbuphine injections, and again after one week of washout. These responses were compared to baseline dyskinesia scores with L-dopa alone tested immediately prior to initiation of chronic nalbuphine injections. Testing at both two weeks and one month of daily administrations showed that the anti-LID activity of nalbuphine is maintained at significant levels (Fig 3). There was no significant difference in the effect size at two weeks compared to one month. Both peak and total dyskinesia scores showed the same profile. As expected, after one week of washout from nalbuphine, LID scores returned to prenalbuphine baseline levels. Consistent with the findings from the acute co-administration phase of the study, the anti-parkinsonian activity of L-dopa was not affected by nalbuphine co-therapy, nor was the chronic daily nalbuphine treatment given with oral Sinemet associated with any sedation. These results indicate that nalbuphine is effective as an anti-LID agent for chronic therapy without impacting the antiparkinson action of L-dopa. In addition, chronic administration of nalbuphine is well tolerated and does not produce adverse effects in parkinsonian primates.

Nalbuphine reverses the molecular changes in the striatum associated with LID.

Comparison of putamenal tissue samples obtained from four animals with LID not treated with Nalbuphine and three animals that received L-dopa and nalbuphine one hour prior to euthanasia showed significant differences in a number of indices associated with LID. First,

FosB expression, which is markedly up-regulated in LID^{36, 37}, was ~50% lower in nalbuphine treated animals compared to those that received only L-dopa prior to euthanasia (Fig 4A, B). Similarly, both dynorphin A and prodynorphin, which are known to be up-regulated in LID in animal models and postmortem brains of patients with LID^{11–14, 36, 38}, had significantly lower expression in nalbuphine treated animals by 80% and 51%, respectively (Fig 4A, C). Additionally, phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32kDa (DARPP-32) at threonine 34, which is increased in LID³⁹, was reduced by 45% in animals treated with both nalbuphine and L-dopa prior to euthanasia compared to animals that were treated with L-dopa alone (Fig 4D, E). No significant differences were detected in DARPP-32 phosphorylated at threonine 75 or total DARPP-32 (Fig 4D, F). Expression of cyclin-dependent kinase 5 (Cdk5), which is reportedly increased in LID^{40, 41}, was also lower in nalbuphine treated animals by 52% (Fig 4D, G). Another kinase, Extracellular Signal-Regulated Kinase (ERK), which is activated during priming rather than maintenance of dyskinesia⁴², was not different between the two

groups of animals with chronic LID (Fig 4D, H). These findings correlate with the behavioral data and demonstrate objective molecular changes in the striatum produced by nalbuphine indicative of target engagement.

Nalbuphine monotherapy has no motor effects.

Nalbuphine was also tested as monotherapy to determine any potential intrinsic motor effects in the parkinsonian setting. In the absence of L-dopa, nalbuphine had no motor effects in parkinsonian monkeys at any of the doses tested (0.16 – 0.5 mg/kg). Baseline MDS in the “off” state did not change at any time point during the post-injection period or after computing total scores (Table 2). Nalbuphine monotherapy demonstrated no adverse effects except at the highest dose tested (0.5 mg/kg), which induced sedation manifested mainly by reduced reactivity (Table 2).

Discussion

The present findings demonstrate that the dual pharmacology mu antagonist and kappa agonist opioid agent nalbuphine represses LID in the non-human primate model of PD with moderate to severe stable parkinsonism and reproducible LID. This is achieved without compromising the anti-parkinson action of L-dopa or altering plasma L-dopa levels. Nalbuphine given with L-dopa is also safe and well tolerated with chronic administration. The sedation that can arise at high doses of nalbuphine monotherapy does not occur with L-dopa co-administration. Importantly, the motor behavioral response of the animals to nalbuphine correlates with the reversal of specific molecular markers in the striatum associated with LID indicating target engagement at effective doses.

Alterations in opioid transmission have been recognized in the context of LID both experimentally in rodents and primates as well as in the brains of patients with PD and LID^{7–16}. Accordingly, a number of pharmacological agents with various degrees of specificity for different opioid receptors have been tested pre-clinically and in limited clinical studies with mixed results. This is likely due to the tendency of most agents to target different opioid receptors at higher doses thus losing specificity. For example, available data indicate that mu opioid receptor antagonism is needed for an anti-LID response but stimulating kappa opioid receptors would be beneficial as well^{24–28}. Therefore, a compound that antagonizes mu receptors but loses specificity at higher doses leading to blockade of kappa receptor mediated signaling as well can lose its anti-LID efficacy. This phenomenon is exemplified by a U-shaped dose-response relationship of the anti-LID effect of the mu receptor antagonist ADL5510 (Koprach *et al.*, 2011) likely due to loss of receptor specificity. The mixed pharmacology of nalbuphine, whereby it antagonizes mu receptors, but also stimulates kappa receptors, appears to be an important and unique property for its anti-LID effect.

The amelioration of dyskinesia severity in animals treated with nalbuphine and L-dopa is associated with improvements in molecular changes in the striatum that are known to occur with LID. Notably, the marked increase in FosB expression with LID models and postmortem PD brains^{36, 37, 43–45} is diminished in animals treated with nalbuphine and L-dopa compared to those that received L-dopa alone. The significance of this finding is the

observation that viral vector mediated over-expression of FosB in the striatum of 6-hydroxy-dopamine (6-OHDA) lesioned rats induces abnormal involuntary movements in the absence of repeated L-dopa administration suggesting that increased expression of this transcription factor per se can promote LID³⁷. Thus, it is conceivable that the reduction in

FosB expression by nalbuphine therapy in the present study plays a significant role in ameliorating LID. Additionally, the increased expression of prodynorphin and dynorphin A that occurs in the context of LID in animal models and in PD brains^{11–14, 36, 38}, the elevated expression of Cdk5 associated with LID^{40, 41}, as well as the phosphorylation of DARPP-32 at Thr34 that is activated in models of LID^{42, 46}, are also significantly curtailed with nalbuphine co-administration consistent with the reduced severity of LID in these animals.

Several lines of evidence point to the direct striatonigral pathway as the primary neural circuitry generating LID^{40, 47}. In LID models, sensitivity of D1 dopamine receptor mediated signaling through the direct striatonigral pathway correlates with the severity of dyskinetic movements, whereas D2 receptor levels that are upregulated by denervation are not impacted by L-dopa therapy⁴⁰. Repeated administration of a D1 agonist to 6-OHDA-lesioned rats increases prodynorphin mRNA expression in D1 dopamine receptor-expressing GABAergic medium spiny neurons (MSNs) of the direct pathway⁴⁸, and these are the same neurons that also exhibit FosB induction correlating with LID in rodents³⁶. Additionally, following denervation and repeated L-dopa therapy, D1 receptor stimulation linked to activation of adenylate cyclase and increased generation of cAMP leads to activation of protein kinase A (PKA) and phosphorylation of DARPP-32 at Thr34, ultimately regulating transcription of key molecular markers of LID including FosB and dynorphin. Accordingly, genetic inactivation of DARPP-32 in medium spiny neurons of the direct pathway but not in neurons of the indirect pathway of mice results in less L-dopa induced involuntary movements^{49, 50}. Our present findings support the direct pathway playing a significant role in LID mechanisms since the behavioral improvement in dyskinesia severity with nalbuphine was associated with normalization of the molecular indices of aberrant modulation of these striatonigral neurons including p-Thr34-DARPP-32, dynorphin A and FosB expression. Clinically, the dyskinesia producing potential of L-dopa and its beneficial anti-parkinson effects have long been suspected to be mediated by different pathways in the basal ganglia⁵¹. The ability of nalbuphine to repress LID but not impact the anti-parkinsonian action of L-dopa is consistent with this view and is essential for an effective anti-LID agent.

The profile of DARPP-32 phosphorylation regulated by nalbuphine co-therapy with L-dopa in the present study is also in agreement with D1-mediated signaling through the direct striatonigral pathway largely contributing to LID^{40, 47}. As a key regulator of signaling in striatal neurons, DARPP-32 plays a critical role in D1 receptor mediated transmission by modulating the state of phosphorylation and activity of a variety of downstream physiological effectors and is itself activated through phosphorylation in models of LID^{42, 46}. Experiments with striatal slice preparations and in vivo studies have shown that D1 receptor stimulation leads to a robust increase in p-Thr34-DARPP-32 in the striatum, specifically in D1 expressing striatonigral neurons but no change or decrease in p-Thr75-DARPP-32^{52, 53}. On the other hand, DARPP-32 phosphorylation at Thr75 occurs primarily through D2 signaling in D2 expressing striatal neurons⁵². Accordingly, striatal lysates of nalbuphine treated animals had less p-Thr34-DARPP-32 on Western blots compared to

animals that received only L-dopa prior to euthanasia, while p-Thr75-DARPP-32 levels were not different between the two groups. These findings suggest that nalbuphine modulates striatonigral pathway signaling in reducing LID.

The reduction of Cdk5 expression with nalbuphine therapy is consistent with previous observations that this kinase is up-regulated with LID in both primate and rat models^{40, 41} and is normalized with reduction of LID in rats⁴¹. Additionally, administration of a Cdk5 inhibitor reportedly reduces the severity of abnormal involuntary movements in hemiparkinsonian rats⁵⁴. Cdk5 appears to have complex regulatory effects in relation to striatal function. While dopaminergic signaling through cAMP - PKA activation induces increased phosphorylation of DARPP-32 at Thr34³⁹, Cdk5 phosphorylates DARPP-32 at Thr75 thereby converting DARPP-32 into an inhibitor of PKA⁵⁵. Accordingly, the increased expression of Cdk5 in dyskinetic animals has been proposed to represent a homeostatic response to hyperactivation of the D1-PKA pathway⁴⁰. But in addition to regulating postsynaptic signaling of dopamine in the striatum, Cdk5 may also regulate presynaptic dopamine release⁵⁶. Furthermore, Cdk5 is a direct target for the transcriptional activity of FosB⁵⁷ and, therefore, interventions that reduce FosB expression are expected to also decrease Cdk5 expression. Thus, the reduction in Cdk5 in animals treated with nalbuphine in the present study is the net effect of these mechanisms *in vivo* and correlates with decreased severity of LID.

Nalbuphine did not impact p-ERK, which is also activated by cAMP upstream of DARPP-2 in rodent models of LID^{49, 58}. Our finding is consistent with the observation that, unlike the sustained phosphorylation of DARPP-32 with chronic L-dopa administration, ERK activation is seen primarily following acute L-dopa administration and diminishes with chronic therapy with no differences detected between dyskinetic and non-dyskinetic parkinsonian primates⁴². Overall, the present findings indicate that the repression of LID with nalbuphine correlates with resetting of maladaptive changes within the striatum including modulating the activity of specific signaling molecules and regulating transcription.

The clinical implications of these findings for patients with PD are important. The anti-LID effect of nalbuphine is seen in the primate model at doses that are below its analgesic dose in humans, thus, providing a well established safety margin based on over three decades of prescribing this analgesic. This therapeutic profile of nalbuphine supports advancing it to clinical testing for LID. A rapid path to patients can be achieved under a repurposing paradigm using the current clinically available form of nalbuphine. The safety and efficacy will need to be tested in this patient population, with concomitant L-dopa treatment, guided by the dosing and treatment paradigms from these primate studies.

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References

1. Manson A, Stirpe P, Schrag A. Levodopa-induced-dyskinesias clinical features, incidence, risk factors, management and impact on quality of life. *J Parkinsons Dis.* 2012;2:189–198. [PubMed: 23938226]
2. Verhagen Metman L, Del Dotto P, van den Munckhof P, et al. Amantadine as treatment for dyskinesias and motor fluctuations in Parkinson's disease. *Neurology.* 1998;50:1323–1326. [PubMed: 9595981]
3. Snow BJ, Macdonald L, McAuley D, Wallis W. The effect of amantadine on levodopa-induced dyskinesias in Parkinson's disease: a double-blind, placebo-controlled study. *Clin Neuropharmacol.* 2000;23:82–85. [PubMed: 10803797]
4. Sawada H, Oeda T, Kuno S, et al. Amantadine for dyskinesias in Parkinson's disease: a randomized controlled trial. *PLoS One.* 2010;5:e15298. [PubMed: 21217832]
5. Brotchie JM. Nondopaminergic mechanisms in levodopa-induced dyskinesia. *Mov Disord.* 2005;20:919–931. [PubMed: 16007614]
6. Jenner P. Molecular mechanisms of L-DOPA-induced dyskinesia. *Nat Rev Neurosci.* 2008;9:665–677. [PubMed: 18714325]
7. Hanrieder J, Ljungdahl A, Falth M, et al. L-DOPA-induced dyskinesia is associated with regional increase of striatal dynorphin peptides as elucidated by imaging mass spectrometry. *Mol Cell Proteomics.* 2011;10:M111 009308.
8. Huot P, Johnston TH, Koprach JB, et al. The pharmacology of L-DOPA-induced dyskinesia in Parkinson's disease. *Pharmacol Rev.* 2013;65:171–222. [PubMed: 23319549]
9. Herrero MT, Augood SJ, Hirsch EC, et al. Effects of L-DOPA on preproenkephalin and preprotachykinin gene expression in the MPTP-treated monkey striatum. *Neuroscience.* 1995;68:1189–1198. [PubMed: 8544992]
10. Nisbet AP, Foster OJ, Kingsbury A, et al. Preproenkephalin and preprotachykinin messenger RNA expression in normal human basal ganglia and in Parkinson's disease. *Neuroscience.* 1995;66:361–376. [PubMed: 7477878]
11. Calon F, Birdi S, Rajput AH, et al. Increase of preproenkephalin mRNA levels in the putamen of Parkinson disease patients with levodopa-induced dyskinesias. *J Neuropathol Exp Neurol.* 2002;61:186–196. [PubMed: 11853020]
12. Henry B, Duty S, Fox SH, et al. Increased striatal pre-proenkephalin B expression is associated with dyskinesia in Parkinson's disease. *Exp Neurol.* 2003;183:458–468. [PubMed: 14552886]
13. Bishop C, Krolewski DM, Eskow KL, et al. Contribution of the striatum to the effects of 5-HT1A receptor stimulation in L-DOPA-treated hemiparkinsonian rats. *J Neurosci Res.* 2009;87:1645–1658. [PubMed: 19115412]
14. Tamim MK, Samadi P, Morissette M, et al. Effect of non-dopaminergic drug treatment on Levodopa induced dyskinesias in MPTP monkeys: common implication of striatal neuropeptides. *Neuropharmacology.* 2010;58:286–296. [PubMed: 19576910]
15. Bourdenx M, Nilsson A, Wadensten H, et al. Abnormal structure-specific peptide transmission and processing in a primate model of Parkinson's disease and L-DOPA-induced dyskinesia. *Neurobiol Dis.* 2014;62:307–312. [PubMed: 24148855]
16. Piccini P, Weeks RA, Brooks DJ. Alterations in opioid receptor binding in Parkinson's disease patients with levodopa-induced dyskinesias. *Ann Neurol.* 1997;42:720–726. [PubMed: 9392571]
17. Aubert I, Guigoni C, Li Q, et al. Enhanced preproenkephalin-B-derived opioid transmission in striatum and subthalamic nucleus converges upon globus pallidus internalis in L-3,4-dihydroxyphenylalanine-induced dyskinesia. *Biol Psychiatry.* 2007;61:836–844. [PubMed: 16950226]
18. Johansson PA, Andersson M, Andersson KE, Cenci MA. Alterations in cortical and basal ganglia levels of opioid receptor binding in a rat model of L-DOPA-induced dyskinesia. *Neurobiol Dis.* 2001;8:220–239. [PubMed: 11300719]
19. Mansour A, Fox CA, Burke S, et al. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. *J Comp Neurol.* 1994;350:412–438. [PubMed: 7884049]

20. Samadi P, Gregoire L, Bedard PJ. Opioid antagonists increase the dyskinetic response to dopaminergic agents in parkinsonian monkeys: interaction between dopamine and opioid systems. *Neuropharmacology*. 2003;45:954–963. [PubMed: 14573388]
21. Fox S, Silverdale M, Kellett M, et al. Non-subtype-selective opioid receptor antagonism in treatment of levodopa-induced motor complications in Parkinson's disease. *Mov Disord*. 2004;19:554–560. [PubMed: 15133820]
22. Klintonberg R, Svenningsson P, Gunne L, Andren PE. Naloxone reduces levodopa-induced dyskinesias and apomorphine-induced rotations in primate models of parkinsonism. *J Neural Transm*. 2002;109:1295–1307. [PubMed: 12373562]
23. Rascol O, Fabre N, Blin O, et al. Naltrexone, an opiate antagonist, fails to modify motor symptoms in patients with Parkinson's disease. *Mov Disord*. 1994;9:437–440. [PubMed: 7969211]
24. Henry B, Fox SH, Crossman AR, Brotchie JM. Mu- and delta-opioid receptor antagonists reduce levodopa-induced dyskinesia in the MPTP-lesioned primate model of Parkinson's disease. *Exp Neurol*. 2001;171:139–146. [PubMed: 11520128]
25. Koprach JB, Fox SH, Johnston TH, et al. The selective mu-opioid receptor antagonist adl5510 reduces levodopa-induced dyskinesia without affecting antiparkinsonian action in mptp-lesioned macaque model of Parkinson's disease. *Mov Disord*. 2011;26:1225–1233. [PubMed: 21465551]
26. Marin C, Bove J, Bonastre M, Tolosa E. Effect of acute and chronic administration of U50,488, a kappa opioid receptor agonist, in 6-OHDA-lesioned rats chronically treated with levodopa. *Exp Neurol*. 2003;183:66–73. [PubMed: 12957489]
27. Cox H, Togasaki DM, Chen L, et al. The selective kappa-opioid receptor agonist U50,488 reduces L-dopa-induced dyskinesias but worsens parkinsonism in MPTP-treated primates. *Exp Neurol*. 2007;205:101–107. [PubMed: 17335811]
28. Ikeda K, Yoshikawa S, Kurokawa T, et al. TRK-820, a selective kappa opioid receptor agonist, could effectively ameliorate L-DOPA-induced dyskinesia symptoms in a rat model of Parkinson's disease. *Eur J Pharmacol*. 2009;620:42–48. [PubMed: 19686730]
29. Berg D, Becker G, Reiners K. Reduction of dyskinesia and induction of akinesia induced by morphine in two parkinsonian patients with severe sciatica. *J Neural Transm*. 1999;106:725–728. [PubMed: 10907730]
30. Samadi P, Gregoire L, Bedard PJ. The opioid agonist morphine decreases the dyskinetic response to dopaminergic agents in parkinsonian monkeys. *Neurobiol Dis*. 2004;16:246–253. [PubMed: 15207281]
31. De Souza EB, Schmidt WK, Kuhar MJ. Nalbuphine: an autoradiographic opioid receptor binding profile in the central nervous system of an agonist/antagonist analgesic. *J Pharmacol Exp Ther*. 1988;244:391–402. [PubMed: 2826773]
32. Cao X, Liang L, Hadcock JR, et al. Blockade of cannabinoid type 1 receptors augments the antiparkinsonian action of levodopa without affecting dyskinesias in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated rhesus monkeys. *J Pharmacol Exp Ther*. 2007;323:318–326. [PubMed: 17630359]
33. Papa SM, Chase TN. Levodopa-induced dyskinesias improved by a glutamate antagonist in Parkinsonian monkeys. *Ann Neurol*. 1996;39:574–578. [PubMed: 8619541]
34. Wikberg T Simultaneous determination of levodopa, its main metabolites and carbidopa in plasma by liquid chromatography. *J Pharm Biomed Anal*. 1991;9:167–176. [PubMed: 1873308]
35. Saxer C, Niina M, Nakashima A, et al. Simultaneous determination of levodopa and 3-O-methyldopa in human plasma by liquid chromatography with electrochemical detection. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004;802:299–305.
36. Andersson M, Hilbertson A, Cenci MA. Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiol Dis*. 1999;6:461–474. [PubMed: 10600402]
37. Cao X, Yasuda T, Uthayathas S, et al. Striatal overexpression of DeltaFosB reproduces chronic levodopa-induced involuntary movements. *J Neurosci*. 2010;30:7335–7343. [PubMed: 20505100]

38. Engber TM, Susel Z, Kuo S, et al. Levodopa replacement therapy alters enzyme activities in striatum and neuropeptide content in striatal output regions of 6-hydroxydopamine lesioned rats. *Brain Res.* 1991;552:113–118. [PubMed: 1717109]
39. Greengard P, Allen PB, Nairn AC. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. *Neuron.* 1999;23:435–447. [PubMed: 10433257]
40. Aubert I, Guigoni C, Hakansson K, et al. Increased D1 dopamine receptor signaling in levodopa-induced dyskinesia. *Ann Neurol.* 2005;57:17–26. [PubMed: 15514976]
41. Kuan WL, Lin R, Tyers P, Barker RA. The importance of A9 dopaminergic neurons in mediating the functional benefits of fetal ventral mesencephalon transplants and levodopa-induced dyskinesias. *Neurobiol Dis.* 2007;25:594–608. [PubMed: 17188499]
42. Santini E, Sgambato-Faure V, Li Q, et al. Distinct changes in cAMP and extracellular signal-regulated protein kinase signalling in L-DOPA-induced dyskinesia. *PLoS One.* 2010;5:e12322. [PubMed: 20808799]
43. Tekumalla PK, Calon F, Rahman Z, et al. Elevated levels of DeltaFosB and RGS9 in striatum in Parkinson's disease. *Biol Psychiatry.* 2001;50:813–816. [PubMed: 11720701]
44. Fasano S, Bezard E, D'Antoni A, et al. Inhibition of Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) signaling in the striatum reverts motor symptoms associated with L-dopa-induced dyskinesia. *Proc Natl Acad Sci U S A.* 2010;107:21824–21829. [PubMed: 21115823]
45. Lindgren HS, Rylander D, Iderberg H, et al. Putaminal upregulation of FosB/DeltaFosB-like immunoreactivity in Parkinson's disease patients with dyskinesia. *J Parkinsons Dis.* 2011;1:347–357. [PubMed: 23933656]
46. Picconi B, Centonze D, Hakansson K, et al. Loss of bidirectional striatal synaptic plasticity in LDOPA-induced dyskinesia. *Nat Neurosci.* 2003;6:501–506. [PubMed: 12665799]
47. Feyder M, Bonito-Oliva A, Fisone G. L-DOPA-Induced Dyskinesia and Abnormal Signaling in Striatal Medium Spiny Neurons: Focus on Dopamine D1 Receptor-Mediated Transmission. *Front Behav Neurosci.* 2011;5:71. [PubMed: 22028687]
48. Gerfen CR, Engber TM, Mahan LC, et al. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science.* 1990;250:1429–1432. [PubMed: 2147780]
49. Santini E, Valjent E, Uziel A, et al. Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. *J Neurosci.* 2007;27:6995–7005. [PubMed: 17596448]
50. Bateup HS, Santini E, Shen W, et al. Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors. *Proc Natl Acad Sci U S A.* 2010;107:14845–14850. [PubMed: 20682746]
51. Mouradian MM, Heuser JJ, Baronti F, et al. Pathogenesis of dyskinesias in Parkinson's disease. *Ann Neurol.* 1989;25:523–526. [PubMed: 2774496]
52. Bateup HS, Svenningsson P, Kuroiwa M, et al. Cell type-specific regulation of DARPP-32 phosphorylation by psychostimulant and antipsychotic drugs. *Nat Neurosci.* 2008;11:932–939. [PubMed: 18622401]
53. Kuroiwa M, Bateup HS, Shuto T, et al. Regulation of DARPP-32 phosphorylation by three distinct dopamine D1-like receptor signaling pathways in the neostriatum. *J Neurochem.* 2008;107:1014–1026. [PubMed: 18823371]
54. Chagniel L, Robitaille C, Lebel M, Cyr M. Striatal inhibition of calpains prevents levodopa-induced neurochemical changes and abnormal involuntary movements in the hemiparkinsonian rat model. *Neurobiol Dis.* 2012;45:645–655. [PubMed: 22037042]
55. Bibb JA, Snyder GL, Nishi A, et al. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature.* 1999;402:669–671. [PubMed: 10604473]
56. Chergui K, Svenningsson P, Greengard P. Cyclin-dependent kinase 5 regulates dopaminergic and glutamatergic transmission in the striatum. *Proc Natl Acad Sci U S A.* 2004;101:2191–2196. [PubMed: 14769920]
57. Chen J, Zhang Y, Kelz MB, et al. Induction of cyclin-dependent kinase 5 in the hippocampus by chronic electroconvulsive seizures: role of [Delta]FosB. *J Neurosci.* 2000;20:8965–8971. [PubMed: 11124971]

58. Westin JE, Vercammen L, Strome EM, et al. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. *Biol Psychiatry*. 2007;62:800–810. [PubMed: 17662258]

FIGURE 1. Nalbuphine co-administration with L-dopa reduces LID.

(A) Time course of absolute LID scores with different doses of nalbuphine or vehicle (saline) injected along with L-dopa methyl ester. (B-D) Normalized LID scores. All but the lowest dose of nalbuphine significantly reduced LID in all analyses: time course of LID (B), peak LID scored at 50 min following L-dopa injection (C), and total LID scores calculated as the sum of scores from all post-injection time points (D). Data are the means $\pm$ SEM. One- or two-way ANOVAs followed by Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control.

FIGURE 2. Nalbuphine co-therapy does not affect the antiparkinson action of L-dopa or plasma L-dopa levels.

(A) Time course of absolute motor disability scores (MDS) with different doses of nalbuphine or vehicle (saline) injected along with L-dopa methyl ester. (B) Total MDS during the observation period (the sum of MDS from all post-injection time points) were unaffected by nalbuphine at all doses. Data are the means $\pm$ SEM. One-way ANOVAs followed by Dunnett's post-hoc test. None of the differences were significant. (C) Plasma L-dopa levels with and without nalbuphine co-administration. Blood samples were collected from two awake animals at 0, 60, 120, and 180 minutes following injection of either L-dopa methyl ester plus vehicle (saline) or L-dopa plus nalbuphine (0.13 mg/kg). Each treatment was repeated once for duplicate sampling. Data represent mean concentrations.

FIGURE 3. Chronic administration of nalbuphine has sustained anti-LID effect without impacting parkinsonian motor disability.

(A) Time course of absolute LID scores following L-dopa injection alone at baseline or co-administered with 0.13 mg/kg of nalbuphine after two weeks and one month of daily nalbuphine therapy. B-D. Nalbuphine significantly reduced dyskinesia at both time-points during the 30 day treatment period (midway and end) in all analyses: time course of LID scored at every 20 min interval (B), peak LID scored at 50 min following L-dopa injection (C), and total LID scores calculated as the sum of scores from all post-injection time points (D). This effect was no longer detectable after a one-week washout period (A-D). (E) Motor disability scores (MDS) were not affected by chronic nalbuphine therapy. Data are the

means $\pm$ SEM. One- or two-way ANOVAs followed by Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control.

FIGURE 4. Western blot analyses of striatal tissue lysates demonstrate reversal of LID-associated molecular changes by nalbuphine co-administration.

(A) Expression of FosB, dynorphin A and prodynorphin are significantly reduced by nalbuphine co-administration with L-dopa. (B) Quantification of FosB band intensity relative to β -actin in panel A. (C) Quantification of dynorphin A and prodynorphin band intensities relative to β -actin in panel A. (D) p-Thr34-DARPP-32 but not p-Thr75-DARPP-32 is significantly repressed by nalbuphine co-administration. Cdk5 expression is also reduced, while that of p-ERK is not different between the two groups of animals. (E) Quantification of p-Thr34-DARPP-32 band intensity relative to total DARPP-32 expression in panel D. (F) Quantification of p-Thr75-DARPP-32 band intensity relative to total DARPP-32 expression in panel D. (G) Quantification of Cdk5 band intensity relative to β -actin in panel D. (H) Quantification of p-ERK band intensity relative to β -actin in panel D. Each lane represents striatal tissue lysate from a separate animal. NB- = MPTP lesioned animals with LID given L-dopa but not nalbuphine prior to euthanasia; NB+ = MPTP lesioned animals with LID given both nalbuphine and L-dopa prior to euthanasia; p-T34-DARPP = p-DARPP-32 phosphorylated at threonine 34; p-T75-DARPP = p-DARPP-32 phosphorylated at threonine 75; Cdk5 = cyclin dependent kinase 5; p-ERK = phospho-Extracellular Signal-Regulated Kinase. Data represent the means $\pm$ SEM * $p < 0.05$; ** $p < 0.01$.

Table 1.

Subject Demographics and Clinical Data.

Monkey	Age (yrs)	Gender	MDS OFF	MDS ON	LID	Clinical Study	Molecular Study
1	5	F	21	4.7	11.5	Yes	No
2	5	F	20	7.2	15.7	Yes	No
3	12	M	17	7.4	3.9	Yes	No
4	5	M	26	8.6	13	Yes	Yes
5	7	M	20	6.1	5.9	Yes	Yes
6	7	M	24	11.6	8	Yes	Yes
7	7	M	18	6	2.5	No	Yes
8	12	F	21	4	7	No	Yes
9	9	F	17	3	5	No	Yes
10	8	M	20	5	6	No	Yes

Monkeys 1 to 6 were used for all the behavioral studies, and from this group, monkeys 4–6 were also euthanized for analysis of their brain tissue. Monkeys 7–10 were euthanized previously and their frozen (–80°C) brains were used to complete the molecular studies in a total of 7 animals. MDS OFF = motor disability score at baseline “off” state. MDS ON = motor disability score at the peak-dose effect of L-dopa in the “on” state. MDS ranges from 0–39, 0 = no impairment, 39 = extreme impairment. LID = dyskinesia score at peak-dose effect of L-dopa. LID score ranges from 0–27, 0 = no dyskinesia, 27 = severe and/or constant dyskinesia in all body segments. Data are means of three or more behavioral assessments to determine stability of the model.

Table 2:**Nalbuphine Monotherapy**

		Nalbuphine dose (mg/kg)				
		0	0.016	0.05	0.16	0.5
Time Course of MDS	0	19 ± 3	18 ± 3	20 ± 3	19 ± 3	19 ± 3
	30	19 ± 3	19 ± 3	20 ± 3	20 ± 4	20 ± 3
	50	19 ± 3	19 ± 3	21 ± 3	20 ± 4	21 ± 3
	70	19 ± 3	19 ± 3	21 ± 3	20 ± 4	21 ± 3
	90	19 ± 3	20 ± 3	21 ± 3	20 ± 4	21 ± 3
	110	20 ± 3	20 ± 3	21 ± 3	20 ± 3	21 ± 3
	130	20 ± 3	19 ± 3	21 ± 3	19 ± 4	21 ± 3
MDS	Total	116 ± 19	114 ± 19	122 ± 19	119 ± 21	123 ± 19
DENS	Total	1 ± 1	3 ± 1	5 ± 2	11 ± 5	21 ± 5*

Time course of motor disability scores (MDS) with different doses of nalbuphine or vehicle (saline), total MDS (the sum of MDS from all post-injection time points) and total DENS scores during the observation period are shown. Nalbuphine did not change MDS at any time point or total MDS. Data are the means ± SEM. One- or two-way ANOVAs with Dunnett's post-hoc test. None of the differences were significant. Total DENS scores were only significantly changed by the sedative effect of a high dose of Nalbuphine. One- or two-way ANOVAs followed by Dunnett's post-hoc test (*p < 0.05 versus control dose).