Abstract and Introduction

Abstract

Despite being the mainstay of pain management, opioids are limited in their clinical utility by adverse effects, such as tolerance and paradoxical hyperalgesia. Research of the past 15 years has extended beyond neurons, to implicate central nervous system immune signaling in these adverse effects. This article will provide an overview of these central immune mechanisms in opioid tolerance and paradoxical hyperalgesia, including those mediated by Toll-like receptor 4, purinergic, ceramide, and chemokine signaling. Challenges for the future, as well as new lines of investigation will be highlighted.

Introduction

Opioids have been used to treat pain for millennia and are presently used to treat acute pain (eg, trauma or surgery) and chronic pain, including headache.[1,2] Opioids are widely used for acute management of migraine, with a recent survey finding current opioid use among 16% of respondents, and prior use by 14% of respondents.[3] Notably, opioids have been increasingly prescribed for acute headache in emergency departments (35% of cases) over the last decade.[4] Despite inducing potent analgesia, clinical utility is limited by several adverse effects, including tolerance and paradoxical hyperalgesia. There is a possibility that opioid-induced hyperalgesia may manifest as opioid medication overuse headache. While these adverse effects can be partly explained by neuronal mechanisms,[5,6] relatively recent advances also point to central immune signaling, predominantly mediated by glia – immunocompetent cells in the central nervous system (CNS), including microglia and astrocytes.[7,8]

That the effects of opioids might extend beyond neurons was first recognized when a significant increase in the expression of the astrocyte activation marker glial fibrillary acidic protein (GFAP) was found in the ventral tegmental area after long-term systemic morphine administration.[9] The astrocyte response to morphine was shown not to be an epiphenomenon, but causally linked to adverse opioid effects. Song and Zhao[10] demonstrated that morphine tolerance was abolished if the astrocyte inhibitor fluorocitrate was delivered intrathecally, corresponding with attenuation of spinal morphine-elevated GFAP expression. Subsequent studies have also identified opioid-induced upregulation of both astrocyte and microglial activation markers in multiple brain and spinal cord regions, with consequences for opioid pharmacodynamics.[10–18]

The molecular, cellular, and behavioral consequences of opioid-induced central immune signaling have been widely studied since these seminal investigations. Particular emphasis has been placed on the cell types involved, the signaling mechanisms and pathways, and the behavioral correlates. This review is not intended to provide an exhaustive account of the interactions between opioids and central immune signaling (for review, see Hutchinson et al[8] and Thomas and Hutchinson[19]). While such signaling results in other consequences with great relevance to opioid management of pain, such as addiction, the central immune mechanisms have recently been reviewed elsewhere[20,21] and are beyond the present scope. Instead, we will focus on those mechanisms relevant to opioid analgesia, namely tolerance and hyperalgesia, which typically follow repeated opioid administration.

Immunocompetent Cells of the CNS

Studies toward the end of the last century have led to an appreciation of the bidirectional signaling that occurs between neurons and a host of immunocompetent cells present in the CNS, including glia (microglia, astrocytes, and oligodendrocytes), endothelial cells, perivascular macrophages, and infiltrating peripheral immune cells. These cells are now known to be far more than passive bystanders or components of the extracellular matrix, and have been shown to modulate neurotransmission within the CNS.

The majority of research has focused on the role of astrocytes and microglia. Astrocytes are the most abundant cell type in the CNS. In addition to providing structural support, promoting formation of the blood–CNS barrier, and regulating cerebral blood flow, astrocytes contribute to synaptic transmission, provide trophic support, and promote repair of neuronal systems. They also maintain homeostasis in the extracellular environment by regulating neurotransmitter and ion concentrations in the synaptic cleft.[22] Microglia are the tissue-specific phagocytes of the CNS. They exhibit constitutive and regional heterogeneity throughout the parenchyma, presumably to coordinate diverse responses to
insult. The cytoarchitecture of microglia under a basal surveillance state allows them to continuously sample the extracellular space for perturbations. A transition to reactive gliosis involves changes in cell number, morphology, phenotype, motility, expression of membrane-bound and intracellular signaling proteins (e.g., mitogen-activated protein kinases [MAPKs]), and release of immunoregulatory products, such as cytokines and chemokines. A common marker of astrogliosis, as noted above, is increased expression of GFAP, which is indicative of altered morphology. Microgliosis is often correlated with increased expression of CD11b (complement receptor CR3) and Iba1. It should be noted that the functional relationship between the expression of such markers and changes in cell responses is currently unclear, and in certain circumstances, there seems to be no obvious connection between the two. Nonetheless, numerous studies have now documented glial responses to opioids in sites relevant for pain and analgesia, such as the dorsal spinal cord and periaqueductal gray (PAG).

Endothelial and peripheral immune cells are also critical contributors to central immune signaling. As they contribute to formation of the blood–brain barrier, endothelial cells have the potential to respond to both peripherally and centrally circulating opioids. Indeed, recent studies from our group have demonstrated that CNS endothelial cells respond in a proinflammatory manner to opioids and their metabolites in vitro and in vivo. While a recent study demonstrated that splenocytes traffic into the CNS following repeated systemic morphine, the functional consequences are not understood. Potential contributions to CNS signaling are further unclear, given the widespread view that opioids suppress peripheral immune function.

Neurons are traditionally regarded as responding solely to neuronally derived neurotransmitters, and more recently as submissive participants in central immune signaling. However, neurons express a wide range of immune receptors and ligands that can be upregulated under certain conditions, and are hence capable of autocrine and paracrine immune signaling. Such neuronal mechanisms may have relevance for opioid-induced central immune signaling.

Mechanisms of Immune Activation by Opioids

Immunocompetent cells of the CNS, such as glia, express a wide array of receptors that allow them to directly respond a variety of stimuli, including opioids. It should be noted that both microglia and astrocytes can additionally respond indirectly to opioids (i.e., as a downstream consequence of opioid effects on neurons). For example, though the extracellular mediator is unknown, knockout of neuronal protein kinase C gamma (PKCy) was shown to attenuate morphine-elevated expression of GFAP. Furthermore, the neuronal peptides (e.g., calcitonin gene related peptide [CGRP]) and neuronal chemokines (e.g., CCL2 and CX3CL1) increase glial reactivity following repeated morphine administration. However, immune cells in the CNS also express opioid receptors and Toll-like receptors that facilitate direct interaction with opioids.

Classical Opioid Receptors

Microglia and astrocytes are widely accepted to express μ, δ, and κ opioid receptors, based on mRNA and protein expression, although regional heterogeneity may exist. In vitro, activation of μ-opioid receptors by morphine enhances Toll-like receptor 4 (TLR4)-mediated pro-inflammatory signaling via the PKCε-Akt-ERK pathway in microglia. Conversely, morphine signaling at μ-opioid receptors expressed by CNS endothelial cells attenuates TLR4-mediated pro-inflammation. Direct investigation of the functional role of opioid receptors expressed by immune cells is clearly complex and requires further investigation. However, this task is complicated by the fact that some of the common pharmacological tools used to infer opioid receptor activity also have activity at TLR4 receptors. To date, naloxone is suggested to be the only opioid receptor antagonist to be identified as devoid of TLR4 effects, based on studies of HEK293 cells.

Toll-like Receptors

Several lines of evidence, including some retained opioid actions in triple opioid receptor knockout mice and non-stereoselective actions, suggested a non-classical site of action for opioids at immune cells. Such a site was identified as TLR4 by Hutchinson et al. TLR4 is one of a family of 13 single transmembrane receptors that recognize a diverse range of moieties or "patterns." For TLR4, these include exogenous stimuli (e.g., lipopolysaccharide [LPS] of Gram-negative bacteria) and endogenous danger signals (danger-associated molecular patterns; alarmins) to trigger an innate immune response. Upon recognition of such patterns, TLR4 dimerizes with the co-receptors CD14 and MD2, and signals through adaptor proteins, including MyD88 and TRIF which phosphorylate MAPKs and NFκB, resulting in pro-inflammatory mediator production. Expressed basally by both microglia and sensory neurons of trigeminal ganglia and dorsal root ganglia (DRG), TLR4 can be upregulated by astrocytes following certain inflammatory stimuli. Morphine, the prototypical opioid, has been demonstrated to non-stereoselectively bind the LPS "binding pocket" on MD2 and to signal through TLR4 in a manner parallel to that of...
To date, every clinically relevant opioid screened (including morphine, oxycodone, methadone, remifentanil, etc.) binds to the TLR4/MD2 heterodimer. Notably, the major morphine non-opioid metabolite morphine-3-glucuronide (M3G) also binds to the TLR4/MD2 heterodimer, which likely accounts for its pro-nociceptive properties. Since opioids signal non-stereoselectively at TLR4 (in contrast to classical opioid receptors), the receptor can be selectively blocked by the (+)-opioid antagonists, (+)-naloxone, and (+)-naltrexone, without affecting opioid analgesia. Though opioid signaling at other TLRs has not been yet characterized, TLR2 (which also signals through MyD88) has been recognized to contribute to microglial reactivity by morphine.

Consequences of Opioid-induced Central Immune Signaling for Analgesia

The activation of opioid receptors and TLRs on immune cells results in functional consequences for opioid analgesia. Analgesic consequences of opioid-induced central immune signaling are well established following repeated opioid administration, but less so following acute administration. For example, chronic but not acute morphine increases spinal cord GFAP expression. Yet central immune signaling does play a role in acute opioid actions, as attenuation of glial function or cytokine production potentiates acute analgesia. While these results may at first glance appear at odds with each other, it bears recognition that GFAP is a cytoplasmic structural protein, not a signaling molecule or transcription factor that regulates glial proinflammatory mediator production. Hence, disconnects between GFAP expression and induction/release of astrocyte proinflammatory substances are to be expected.

Opioid Tolerance

Opioid tolerance is characterized by reduced sensitivity to an opioid agonist, and is usually manifested by the need to escalate doses to achieve the desired effect. The majority of mechanistic investigations have focused solely on neurons, but the role of microglia and astrocytes in opioid tolerance has been demonstrated by several groups (summarized in the Figure). Pan-blockade of microglia and astrocyte reactivity (using agents such as minocycline, propentofylline, or fluorocitrate, which are now recognized to lack cell selectivity), either systemically, intracerebroventricularly, or intrathecally, attenuates tolerance. More recent advances have examined the mechanisms by which central immune signaling supplements our understanding of systems and receptor adaptations underlying opioid tolerance.
Central immune mechanisms of opioid tolerance and opioid-induced hyperalgesia. Activation of Toll-like receptor 4 (TLR4) signaling, production of sphingosine-1 phosphate (S1P), and peroxynitrite (ONOO$^-$; formed by the interaction of superoxide [O$_2^-$] and nitric oxide [NO]) downstream of ceramide, as well as the purinergic receptors P2X4R and P2X7R have been shown to induce production of pro-inflammatory mediators such as tumor necrosis factor (TNF), interleukin-1$\beta$ (IL-1$\beta$), IL-6, IL-18, complement factor c5a, CX3CL1 (also known as fractalkine), and tissue plasminogen activator (tpa) in microglia, astrocytes, and neurons. These immune mediators are also pro-nociceptive, and hence act as an opponent process of neuronally mediated opioid analgesia. Production is largely mediated by NF$\kappa$B activation, and phosphorylation of the mitogen-activated protein kinases p38 and extracellular regulated kinase (ERK). Anti-inflammatory signaling via the cannabinoid 2 (CB2) and IL-10 (IL-10R) receptors oppose opioid tolerance by inhibiting pro-inflammatory mediator production. In addition, S1P and ONOO$^-$ dysregulate glutamate homeostasis by downregulating expression of both the glutamate transporter GLT-1 and the processing enzyme glutamine synthase (GS) in astrocytes. Microglia-derived IL-18 signals at astrocyte IL-18 receptor (IL-18R), leading to D-serine release by astrocytes, which can facilitate N-methyl-D-aspartate (NMDA) receptor activation. The chemokine receptor CXCR4 can induce heterologous desensitization of $\mu$-opiod receptors (MOR) in neurons, mediated at least in part via activation of Src family kinases (SFK). Neuronal CXCL12 signaling at neuronal CXCR4 also contributes to opioid induced hyperalgesia. $\mu$-opioid receptor (MOR)-signaling increases P2X4R expression, triggering brain derived neurotrophic factor (BDNF) release from microglia. Neuronal TrkB receptor activation by BDNF downregulates the principal neuronal potassium-chloride cotransporter, KCC2, which increases the intracellular Cl$^-\$ concentration in lamina I neurons. This leads to disinhibition, where Cl$^-$ is effluxed rather than influxed through GABA$A$ and glycine channels, resulting in opioid-induced hyperalgesia. *Indicates mechanisms specific to opioid induced hyperalgesia.

**Systems Adaptations.** Several central immune mechanisms representing systems adaptation have been described for opioid tolerance. However, the central immune mechanism having received the most study is opioid induction of pro-nociceptive immune mediators in the CNS (see Grace et al$^{[7]}$ for review of pro-nociceptive mechanisms) as an opponent process of neuronally mediated opioid analgesia. Following
repeated opioid administration, such pro-nociceptive mediators accumulate.\[38,61,63\] That they oppose opioid analgesia, resulting in tolerance, has been demonstrated by many groups, where blockade of immune mediator signaling (such as by tumor necrosis factor [TNF], interleukin [IL]-1β, IL-6, CX3CL1, complement factor C5a, tissue plasminogen activator) prevents and/or reverses tolerance.\[17,38,61,64-67\] Intracellular regulators of pro-inflammatory mediator production, such as NFκB, and the MAPKs p38 and ERK are activated by morphine and contribute to opioid tolerance.\[30,36,68,69\] Furthermore, anti-inflammatory treatments, such as CB2 agonists or elevation of IL-10, also oppose opioid tolerance.\[58,61,63,70,71\] Microglia and astrocytes are considered to be the principal source of cytokines contributing to opioid tolerance in the CNS,\[16,72\] although contributions of other cell types such as CNS endothelial cells that release pro-inflammatory factors in response to morphine cannot be discounted.\[29\]

TLR4 signaling has been identified as a key regulator of such mechanisms of tolerance, as blockade in the lumbar spinal cord and PAG not only attenuates tolerance,\[11,47,73\] but also concomitant pro-inflammatory mediator production.\[30,73\] In further support of a role for TLR4, receptor activation by non-opioid ligands induces a state of “naïve tolerance.”\[11,54,74\] The role of TLR4 in opioid tolerance has recently been disputed, but these discrepancies may be explained by several key experimental variables. For example, in vitro work in the HEK293-hTLR4 reporter cell line found only minor activation following opioid agonist stimulation.\[75\] However, the shortcomings of the sole use of NFκB activation as a surrogate for TLR4 signaling have been recently critiqued.\[76\] Acute blockade of TLR4 by (+)-naloxone failed to attenuate tolerance in vivo,\[77\] but this acute dosing paradigm may not account for longer-acting neuroinflammatory metabolites (e.g., M3G\[29\]) that were likely blocked by our repeated (+)-naloxone-dosing regimen.\[47\] Other studies have explored tolerance and other adverse opioid effects in mouse strains with mutations in the Tlr4 gene.\[77-79\] Not only are some TLR4 agonists documented to signal around point mutations,\[80\] but also developmental, compensatory signaling pathways may also be activated in the absence of TLR4,\[7\] leaving conclusions based solely on mutant mouse models and even knockout mouse models in need of further investigation.

The ceramide pathway has been implicated in opioid tolerance. Ceramide is generated by enzymatic hydrolysis as well as from de novo synthesis. While repeated morphine increases microglia and astrocyte ceramide expression,\[81,82\] the mechanisms are not well understood, though links between opioid-induced TLR4 signaling have been posited.\[58,82\] Nonetheless, inhibition of ceramide biosynthesis attenuates morphine tolerance.\[81\] Ceramide signaling is responsible for the production of two key mediators: sphingosine-1 phosphate (S1P) and peroxynitrite (ONOO−); formed by the interaction of superoxide (O2−) and nitric oxide (NO). Inhibitor studies have implicated both mediators in morphine tolerance, which is due to dysregulation of glutamate homeostasis, activation of NFκB, and phosphorylation of p38 and ERK, leading to pro-inflammatory cytokine release.\[82-84\] This mechanism converges with other studies demonstrating that increased production of reactive oxygen and nitrogen species contributes to opioid tolerance.\[85-87\]

Purinergic signaling also contributes to opioid tolerance. In addition to morphine-induced upregulation of P2X4R and P2X7R on microglia, genetic and pharmacological blockade of these receptors attenuates tolerance.\[42,88,89\] Receptor activation results in production of pro-inflammatory/pro-nociceptive mediators as an opponent process of neuronally mediated opioid analgesia. Attenuation of receptor signaling results in decreased expression of CD11b and Iba1,\[42,88\] though as noted above, such expression changes are not necessarily predictive of function. Morphine also phosphorylates p38, upregulates the pro-inflammatory cytokine IL-18 in microglia, and increases the excitability of postsynaptic terminals in the spinal dorsal horn in a P2X7R-dependent fashion.\[88,89\] Whereas brain derived neurotrophic factor (BDNF) release by P2X4R-stimulated microglia induces disinhibition of second-order nociceptive projection neurons in the spinal dorsal horn, this mechanism was shown to be absent from morphine tolerance.\[77\] Astrocye reactivity appears to be downstream of microglial purinergic signaling, as astrocytes do not express such receptors, yet attenuation of P2X4R signaling decreased morphine-induced GFAP expression.\[42\] IL-18 is one example of a microglia-to-astrocyte signal induced by morphine. IL-18 is exclusively expressed by microglia (as noted above), whereas IL-18R is upregulated exclusively by astrocytes after repeated morphine administration.\[89\] Despite the indirect activation of astrocytes secondary to microglial IL-18 release, astrocyte signaling remains crucial to tolerance, as morphine tolerance is attenuated by intrathelial inhibition of IL-18 binding.\[89\] In response to IL-18 signaling, astrocytes release D-serine, facilitating N-methyl-D-aspartate (NMDA) receptor activation.\[89\]

Several questions regarding the role of purinergic signaling in opioid tolerance remain unanswered. The first is how opioids regulate purinergic receptor expression. Horvath and DeLeo\[43\] argued for regulation by µ-opioid receptors, based on attenuated P2X4R expression when morphine was co-administered with CTAP. However, we have since demonstrated that CTAP is not selective for µ-opioid receptors, but is also an antagonist at the TLR4/MD2 heterodimer.\[29\] Hence, it remains unclear whether TLR4 or µ-opioid receptors regulate purinergic receptor expression. The second question is how purinergic receptors regulate morphine-induced microglial reactivity, since blockade of such receptors decreases Iba1 and CD11b expression,\[42,88\] suggesting a regulatory role that is independent of both µ-opioid receptors and TLR4 in the presence of morphine. Finally, the mechanism by which ATP is released during morphine tolerance is unknown. Several authors have speculated that morphine may induce ATP release from neurons and astrocytes, but this is yet to be demonstrated.\[42,88,89\]
Opioid potency is decreased in females, compared with males.

Future Directions and Conclusions

The last decade has seen major advances that have extended our mechanistic understanding of the role for central immune signaling in the counter regulation of opioid analgesia. In addition to addressing those questions remaining for known mechanisms, we further suggest several new lines of inquiry that should be investigated in the future.

Opioid potency is decreased in females, compared with males. Yet, as with most preclinical research, sexual dimorphism in opioid-
induced central immune signaling has received little attention. Several studies suggest a potential contribution. Chronic administration of the female sex hormone estradiol in male rats and ovariectomized female rats increased the LPS-stimulated inflammatory response in hippocampal microglia in vivo and increased proinflammatory cytokine transcription in vivo. Furthermore, the estradiol metabolites, estradiol-3-glucuronide, and estradiol-17-glucuronide not only dock to MD2 in silico, but also activate TLR4 signaling in vitro and induce allodynia in vivo. These data predict elevated glial reactivity in females at basal levels and upon stimulation, potentially resulting in exaggerated opioid-induced central immune signaling, though this has yet to be tested. However, intrathecal administration of the TLR4 ligand LPS failed to induce allodynia in female mice, and shown to be mediated by the male sex hormone testosterone. How these factors interact to influence opioid analgesia remains to be elucidated. It is also worth noting that the sexually dimorphic effects of opioids may have particular implications for headache treatment, as migraine/severe headache (~3:1; female : male) and medication overuse headache (~13:1; female : male) are more prevalent among females.

As opioids are prescribed during chronic pain, which has a significant central immune basis per se, a potential for mechanistic interactions resulting in exacerbated pain exists. We and others have demonstrated that enduring pain resulting from spinal cord injury, peripheral inflammation, peripheral nerve damage, and surgery is enhanced by a short course of opioids around the time of initial trauma. Owing to the fact that the long-term effects (≥1 year) of opioid therapy related to pain, function, or quality of life have been not been evaluated in the clinical literature, this may be an underrecognized factor contributing to chronic pain. However, such an interaction has been identified in the surgery literature where intraoperative remifentanil (a TLR4 agonist) was associated with chronic pain at the conclusion of these studies, approximately 1 year after cardiac surgery. Such pain has been correlated with exaggerated central immune signaling in preclinical models, but the mechanisms have not been investigated. As discussed above, these processes may also intersect with opioid medication overuse headache.

Following a trend within the pain field more generally, there has been scrutiny over the translational potential of glial pathophysiology identified in preclinical studies. Critiques have highlighted the purported physiological, pathological, and pharmacological response differences between primary microglia derived from rodents and humans. However, there is a paucity of human data on such glial responses, as highlighted and discussed by several other groups. It has also been suggested that species differences are not the only critical variable under study, and the call for validation of rodent studies with studies in primary human microglia is restricted by commercially available human microglia that largely derive from first trimester fetuses, for which information on sex, age, patient history, delays between death and cell isolation, and post-mortem procedures are not available. Further, it should be recognized that first trimester human fetuses do not have microglia representative of infants or adults, further clouding interpretation of results from human compared with rodent studies.

Endeavors to thoroughly characterize functional differences between rodent and human glia are nonetheless of paramount importance to inform translational pharmacology. While scarce, the clinical data regarding opioid-induced central immune signaling is promising. Markers microglial and astrocyte reactivity were elevated in post-mortem brain samples of opioid addicts, compared with controls. Indirect evidence has also been documented where peripheral blood mononuclear cells were isolated from human samples, stimulated ex vivo with TLR agonists and the subsequent release of IL-1β measured. IL-1β release was elevated in chronic pain patients, as compared with that of pain-free individuals, and was elevated further in chronic pain patients receiving opioids. There is obviously a clear need for further clinical investigation.

One potential approach to clinical validation is to undertake clinical trials. Since there is some overlap between the central immune mechanisms of opioid tolerance/hyperalgesia and neuropathic pain, many of the pharmacological therapies that have been proposed to treat pain may be useful adjuvants to improve the clinical efficacy of opioids. Indeed, there are early indications that this may be a fruitful approach, as the phosphodiesterase inhibitor pentoxifylline decreased postoperative opioid requirements.

Despite being the mainstay of pain management, the clinical utility of opioids is severely limited by adverse effects. There is convincing pre-clinical evidence that opioids induce central immune signaling that profoundly contributes to the adverse effects of tolerance and hyperalgesia. While clinical evidence is emerging, translation remains a high priority for the future. Nonetheless, addressing opioid-induced central immune signaling may be of vital importance for this area of high unmet medical need.

Sidebar

Statement of Authorship

Category 1

a. Conception and Design
   Peter M. Grace, Steven F. Maier
b. Acquisition of Data
Peter M. Grace, Linda R. Watkins

c. Analysis and Interpretation of Data
Peter M. Grace, Linda R. Watkins

Category 2

a. Drafting the Manuscript
Peter M. Grace

b. Revising It for Intellectual Content
Peter M. Grace, Steven F. Maier, Linda R. Watkins

Category 3

a. Final Approval of the Completed Manuscript
Peter M. Grace, Steven F. Maier, Linda R. Watkins

References


3. Buse DC, Pearlman SH, Reed ML, Serrano D, Ng-Mak DS, Lipton RB. Opioid use and dependence among persons with migraine:


2008;24:479–496.


9. Beitner-Johnson D, Guitart X, Nestler EJ. Glial fibrillary acidic protein and the mesolimbic dopamine system: Regulation by chronic


11. Eidson LN, Murphy AZ. Blockade of Toll-like receptor 4 attenuates morphine tolerance and facilitates the pain relieving properties of

2013;93:573–578.

13. Holdridge SV, Armstrong SA, Taylor AM, Cahill CM. Behavioural and morphological evidence for the involvement of glial cell activation


42. Horvath RJ, Romero-Sandoval EA, De Leo JA. Inhibition of microglial P2X4 receptors attenuates morphine tolerance, Iba1, GFAP and mu opioid receptor protein expression while enhancing perivascular microglial ED2. Pain. 2010;150:401–413.


This website uses cookies to deliver its services as described in our *Cookie Policy.* By using this website, you agree to the use of cookies.

close