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Intranasal Delivery of Cell-Penetrating anti-NF- κ B Peptides (Tat-NBD) Alleviates Infection-Sensitized Hypoxic-Ischemic Brain Injury

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Abstract

Perinatal infection aggravates neonatal hypoxic-ischemic (HI) brain injury and may interfere with therapeutic hypothermia. While the NF- κ B signaling pathway has been implicated in microglia activation in infection-sensitized HI, the current therapeutic strategies rely on systemic intervention, which could impair neonatal immunity and increase the risk of severe infection. To devise a brain-targeted anti-NF- κ B strategy, we examined the effects of intranasal delivery of tat-NBD peptides in two animal models of neonatal infection-sensitized HI. Kinetics experiments showed that tat-NBD peptides entered the olfactory bulbs rapidly (10–30 min) and peaked in the cerebral cortex around 60 min after intranasal application in P7 rats. Further, intranasal delivery of 1.4 mg/kg tat-NBD, which is only 7% of the intravenous dose in past studies, markedly attenuated NF- κ B signaling, microglia activation, and brain damage triggered by HI with 4 or 72 h pre-exposure to the bacterial endotoxin lipopolysaccharide (LPS). In contrast, intranasal delivery of mutant tat-NBD peptides or systemic application of minocycline failed to block LPS-sensitized HI injury. Yet, intranasal delivery of up to 5.6 mg/kg tat-NBD peptides immediately after pure-HI insult showed litter protection, likely due to its rapid clearance from the brain and inability to inhibit parenchymal plasminogen activators. Together, these results suggest a novel therapy of infection-sensitized HI brain injury in newborns.

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Keywords

Hypoxia-Ischemia; Neuroinflammation; NF- κ B; Microglia

INTRODUCTION

Hypoxic-ischemic encephalopathy, affecting 6 per 1000 live births in developed countries, is an important cause of neonatal mortality and neurological disabilities (Volpe, 2008). A large body of evidences indicated that perinatal infection aggravates HI brain injury and increases the risk of cerebral palsy (Stoll et al., 2004; Khwaja and Volpe, 2008; Leviton et al., 2010). Moreover, a recent study showed that asphyxiated infants with intrauterine infection (chorioamnionitis) were less responsive to hypothermia treatment, raising the possibility that infection-sensitized HI may have more complex pathological mechanisms and require specialized therapies (Wintermark et al., 2010). Consistent with this idea, it is known that the microglia activation in pure-HI is secondary to tissue damage in a delayed “sterile inflammation” manner, whereas its onset in infection-sensitized HI involves microbes and may occur faster (Fig. 1A) (Dirnagl et al., 1999; Chen and Nunez, 2010). For example, although pure-HI and low-dose LPS-exposure alone are weak stimuli of NF- κ B signaling, their combination triggers NF- κ B activity rapidly (Fig. 1B). Furthermore, given its fast and intense activation, the NF- κ B pathway may be a useful therapeutic target in infection-sensitized neonatal HI brain injury (Lehnardt et al., 2003; Wang et al., 2009).

Tat-NBD, a 22 amino-acid cell-penetrating peptide containing the NF- κ B Essential Modulator (NEMO)/IKK γ -Binding Domain coupled to the transduction sequence of the HIV-TAT protein, is a potent NF- κ B inhibitor that attenuates inflammatory responses in multiple paradigms (May et al., 2000; Pizzi et al., 2009). Yet, intravenous delivery of tat-NBD peptides still requires a high dose to cross the blood-brain-barrier (BBB) to reach the central nervous system, which may weaken general immunity and increase the risk of severe infection that already accounts for 26% of neonatal death. Hence, there is a need to develop brain-targeted anti-NF- κ B therapy of infection-sensitized neonatal HI injury for safety concerns.

To this end, we examined the effects of intranasal delivery of tat-NBD peptides in two animal models of infection-sensitized HI injury: namely, the Rice-Vannucci procedure of HI after 4 or 72 h pre-exposure to low-dose LPS stimulation (Eklind et al., 2005). Intranasal delivery is a powerful method of transporting protein and peptides into the central nervous system (Dhuria et al., 2010; Alcala-Barraza et al., 2010; Akpan et al., 2011). The efficient nose-to-brain delivery is mediated in part through the continuous open channels formed by olfactory ensheathing cells that are situated between olfactory nerves and the cerebrospinal fluid space (Li et al., 2005). In the present study, we showed that intranasal delivery of tat-NBD peptides at <10% of the intravenous dose in past studies provided strong protection of LPS-sensitized HI brain injury, but is less effective against pure-HI insults. Together, these results suggest a novel preferential therapy of infection-sensitized HI brain injury in neonates.

MATERIAL AND METHODS

Animal surgery, brain damage quantification, and intranasal delivery

LPS-sensitized neonatal HI was performed as previously described (Eklin et al., 2005; Yang et al., 2012). Briefly, 0.3 mg/kg LPS was injected intraperitoneally to Wistar rat pups of either sex at 4 or 72 h before the induction of hypoxia following unilateral carotid ligation (The Rice-Vannucci procedure). During the hypoxic challenge (10% oxygen for 90 min), pups were placed in glass jars in a 37° C water bath. HI was performed in seven-day-old pups, and randomized for receiving tat-NBD peptide or control treatments at 10 min after the conclusion of hypoxic stress. The extents of tissue loss in the hippocampus, striatum, and cerebral cortex at 7 d recovery were measured against their counterparts in the opposite hemisphere by a researcher unaware of the treatment. Intranasal delivery of regular or biotin-labeled tat-NBD peptides (YGRKKRRQRR-TALDWSWLQTE) and mutant tat-NBD (YGRKKRRQRR-TALDASALQTE) was performed as previously described (Dhuria et al., 2010). Briefly, rat pups were anesthetized and put on their backs over a heating pad (38°C). Using a 10µL Hamilton syringe connected with PE10 polyethylene tubing, we injected 2 µl peptide solution (2.5 µg /µl) to the left and right nares of each animal, alternating at 2 min-interval, for a total volume of 12 µl. The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and in compliance with the ARRIVE guidelines (Animals in Research: Reporting *In-Vivo* Experiments; Kilkenny et al., 2010).

Immuno-detection of biotin TAT-NBD

A commercial streptavidin Agarose resin kit (Thermo Scientific, Waltham, MA) was used to pull-down biotin-label tat-NBD peptides for HRP-conjugated chemiluminescence detection.

Immunohistochemistry and 2,3,5-triphenyltetrazolium chloride (TTC) stain

In-vivo TTC staining was performed as described (Sun et al., 2012). For immunohistochemistry, the following antibodies were used: mouse anti-CD11b/c (OX42; Serotec, Raleigh, NC); rabbit anti-Iba1 (Wako, Osaka, Japan).

Electrophoresis mobility shift assay (EMSA) and zymography

The NF-κB EMSA was performed using a commercial kit (Lightshift Chemiluminescence kit; Thermo Scientific, Waltham, MA) as previously performed (Yang et al., 2012). The plasminogen activator and matrix metalloproteinase zymograms were performed as previously described (Yang et al., 2009).

Magnetic resonance spectroscopy (MRS)

31P magnetic resonance spectra of the whole brain were acquired on a Bruker 7T Biospec system (Bruker, Billerica, MA) using a transmit/receive surface coil and ISIS localization with a 4 sec repetition time, 4 repetitions of 60 averages each. The four individual spectra were averaged and imported into jMRUI for analysis using the AMARES algorithm (Vanhamme et al., 1997). The ratios of metabolites to either total phosphorus or to ATP were calculated for both control (n=4) and 72 h LPS-exposure group (n=3).

Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA extraction, cDNA preparation, and quantitative PCR were performed as previously described (Sun et al., 2012). The qPCR (SYBR green) of rat *Tspo*, *IL-1 β* , and *IL-6* cDNAs were detected using the following primers and normalized to the level of β -*actin*.

Tspo: 5'-CTATGGTTCCTTGGGTCTCTAC-3', 5'-AGGCCAGGTAAGGATACAGCAAG-3'; *IL-1 β* : 5'-CTTTCGACAGTGAGGAGAATGAC-3', 5'-CAAGACATAGGTAGCTGCCACAG-3'; *IL-6*: 5'-GGAGAGGAGACTTCACAGAGGAT-3', 5'-AGTGCATCATCGCTGTTTCATAC-3'; β -*actin*: 5'-GGCACCACACTTTCTACAATGA-3', 5'-AGTGGTACGACCAGAGGCATAC-3'.

Statistical analysis

Values are represented as mean $\pm$ SD or SEM (when $n = 10$). Quantitative data were compared between different groups using two-sample (unpaired) *t*-test assuming equal variance or one-way ANOVA followed by Newman-Keuls multiple comparisons for post-hoc test.

RESULTS

The addition of LPS pre-exposure to HI triggers acute NF- κ B activation

Previous studies have shown that HI after 4 or 72 h pre-exposure to low-dose LPS (0.3 mg/kg, IP) causes greater brain damage in both rat and mouse pups (Eklind et al., 2005; Sun et al., 2012). We also showed that HI with 4 h LPS pre-exposure (LPS4h/HI) induced rapid NF- κ B activation (Yang et al., 2012), but whether HI with 72 h LPS pre-exposure (LPS72h/HI) has the same effect is yet to be tested. Here, using electrophoresis mobility shift assay (EMSA) with NF- κ B probes we show that the LPS72h/HI insult—but not pure-HI and 4 or 72 h LPS-exposure *per se*—induced the NF- κ B activity specifically in the carotid artery-ligated hemisphere (R*) at 4 h recovery (Fig. 1B). These results indicated that LPS4h/HI (a model of acute infection-HI) and LPS72h/HI (a model of HI with sub-acute infection) both induced rapid NF- κ B activation, which may contribute to brain injury in these models.

However, LPS4h/HI and LPS72h/HI insults have distinct effects on HI-triggered plasminogen activator induction (Adhami et al., 2008; Yang et al., 2009). We confirmed that the LPS4h/HI insult greatly reduced both tissue-type (tPA) and urinary-type (uPA) plasminogen activator activities at 4 h recovery (Yang et al., 2012) (Fig. 1C). In contrast, the inhibitory effect on plasminogen activators disappeared in LPS72h/HI insult (Fig. 3E). These results suggested that LPS4h/HI and LPS72h/HI insults have overlapped, as well as, unique pathogenic mechanisms.

Intranasal delivery of low-dose tat-NBD mitigates 4h LPS-sensitized HI injury

To devise brain-targeted anti-NF- κ B intervention, we tested the protective effect of intranasal delivery of 1.4 mg/kg tat-NBD in P7 rat pups, which is only 7% of the

intravenous dose (20 mg/kg) used in previous studies of neonatal HI brain injury (Nijboer et al., 2008a). The pups that received tat-NBD peptides intranasally showed no discernible abnormalities or growth delay. To assess brain distribution after intranasal delivery, we used immunoblotting to detect the appearance biotin-labeled tat-NBD peptide in the olfactory bulbs and the cerebral cortex. This analysis showed that tat-NBD peptides entered the olfactory bulbs as early as 10 min after intranasal application and disappeared after 30 min. The peak of tat-NBD peptide distribution in the cerebral cortex was close to 60 min after intranasal application (Fig. 1D). These results indicated fast transport and rapid clearance of tat-NBD peptides in the brain after intranasal delivery, which may influence its therapeutic effect.

Intranasal delivery of tat-NBD blocks LPS4h/HI-induced brain injury and NF- κ B signaling

Next, we compared LPS4h/HI-induced brain damage in rat pups receiving intranasal delivery of saline (PBS) or tat-NBD peptides. To mimic neuroprotective therapy that can be initiated in the neonatal intensive care unit, we applied treatments at 10 min after the hypoxia stress and examined the brains at 24 h or 7 d recovery. Triphenyltetrazolium chloride (TTC) staining showed discernible cortical infarction in saline-treated, but not tat-NBD-treated pups, at as early as 24 h recovery ($n=4$ for each; Fig. 1F, G). By 7 d after LPS4h/HI, saline-treated rats showed much larger brain atrophy than tat-NBD-treated animals (Fig. 1H, I). Quantification showed that 40% of the cerebral cortex, 51% of the hippocampus, and 36% of the striatum was degenerated in saline-treated rats, while the extent of tissue loss was 6% in the cerebral cortex, 7% in the hippocampus, and 6% in the striatum in tat-NBD-treated animals (Fig. 1E, $n=10$ for each). These results amounted to 85% reduction of LPS4h/HI-induced brain atrophy by acute intranasal delivery of tat-NBD peptides.

The most likely mechanism of tat-NBD-mediated brain protection is the inhibition of NF- κ B signaling and microglia activation (Lehnardt et al., 2003; Wang et al., 2009). Consistent with this notion, EMSA showed that intranasal delivery of tat-NBD peptides abolished LPS4h/HI-induced acute NF- κ B activity (Fig. 2A, $n > 6$ for each). In contrast, systemic administration of minocycline failed to do so at a dose (45 mg/kg) that was reported to prevent white-matter injury following brain injection of LPS (Fan et al., 2005) (Fig. 2B).

We then used quantitative RT-PCR to ensure the inhibition by tat-NBD peptides (1.4 mg/kg) on LPS4h/HI-induced pro-inflammatory cytokine synthesis. This analysis showed that, while low-dose LPS-exposure *per se* had no effect, dual LPS4h/HI insult greatly increased the mRNA levels of *IL-1 β* , *IL-6*, and *Tspo* (Translocator protein of 18 kDa, a marker for microglia activation; Martin et al., 2010; Choi et al., 2011) in the brain at 24 h recovery, which were markedly attenuated by intranasal delivery of tat-NBD peptides ($p < 0.05$, $n=4$ for each; Fig. 2C). Similarly, while saline-treated pups showed many activated, Iba1/OX42 double-positive microglia/macrophages in the cerebral cortex at 24 h recovery, contralateral hemispheres and the ipsilateral cortex in tat-NBD-treated pups only showed ramified OX42(–) microglia while the OX42(+) amoeboid microglia were confined in the corpus callosum (Fig. 2D–I). To confirm that the observed protection is due to NF- κ B inhibition, we also examined the effects of mutant tat-NBD peptides lacking the NEMO-binding property

(May et al., 2000). As expected, intranasal application of mutant tat-NBD peptides was unable to prevent LPS4h/HI-induced brain injury and the induction of MMP-9 at 24 h recovery (Svedin et al., 2007; Adhami et al., 2008) (Fig. 2J, K). Together, these results suggested that tat-NBD peptides prevent LPS4h/HI-induced brain injury and microglia activation through anti-NF- κ B intervention.

Intranasal delivery of tat-NBD attenuates 72 h LPS-sensitized HI brain injury

Past studies have shown that 72 h LPS pre-exposure increases HI brain injury in neonates, in contrast to its protective pre-conditioning effect in adults (Eklind et al., 2005; Sun et al., 2012). Some scientists suggested that the longer incubation period of bacterial endotoxin in LPS72h/HI is closer to intrauterine infection in human infants. Hence, we also examined the effect of intranasal delivery of tat-NBD peptides under LPS72h/HI insults.

Our analysis suggested that LPS72h/HI has more complex pathogenic mechanisms than acute LPS4h/HI insult. First, we found that LPS-exposure in P4 rat pups retarded their growth (Fig. 3A). By P7, the body-weights of 72 h LPS-treated pups were significantly lighter than those of control littermates (30%-increase versus 54%-increase from P4, $n=20$; $p < 0.001$). Yet, control and 72 h LPS-treated rats have a similar resting-state phosphorus spectrum (Fig. 3B). Magnetic resonance spectroscopy (MRS) showed that saline-treated pups had the mean pH=7.2, [PCr]/[Pi]=31.6%, and [ATP]/[Pi]=21.7% ($n=4$), and 72 h LPS-treated pups had the mean pH=7.3, [PCr]/[Pi]=30%, and [ATP]/[Pi]=20.5% ($n=3$; all $p > 0.05$). These results suggest that while 72 h LPS-exposure impairs the growth of rat pups, it does not deplete the high-energy phosphate pool in neonatal brains.

EMSA showed that intranasal application of 1.4 mg/kg tat-NBD peptides at 10 min after the LPS72h/HI insult still blocked the acute NF- κ B activity (Fig. 3C), suggesting that the anti-NF- κ B effect of tat-NBD peptides endures a longer period of exposure to bacterial endotoxin. However, the overall protective effect of this therapy was reduced in LPS72h/HI insults. Compared to the saline treatment, intranasal tat-NBD peptides decreased the extent of tissue loss from 38% to 25% in the cerebral cortex, from 43% to 30% in the hippocampus, and from 35% to 24% in the striatum (Fig. 3D, $n=10$ for each). These data amount to 32% reduction of LPS72h/HI-induced brain atrophy ($p = 0.02$). While significant, this level of protection is more modest compared with its effect following acute LPS4h/HI insults (85% reduction of brain atrophy). The different extents of protection despite similar inhibition on acute NF- κ B signaling suggest that LPS72h/HI insult involves more complex pathological mechanisms.

Consistent with this notion, while LPS4h/HI blocks plasminogen activator induction (Fig. 1C), LPS72h/HI lacked this effect especially in the induction of uPA activity, which was preserved after intranasal delivery of tat-NBD peptides ($n=6$ for each; Fig. 3E, F). Similarly, the inhibitory effect of tat-NBD on LPS72h/HI-induced MMP-9 activation became inconsistent (Fig. 3G, H; $p > 0.05$ with $n=4$). Together, these results suggest that HI insult with 72 h pre-exposure to LPS induces multiple pathological mechanisms, and thus inhibition of the NF- κ B signaling pathway only provides partial protection.

Immediate intranasal delivery of tat-NBD has little benefit in pure-HI brain injury

Because pure-HI is a weaker stimulus of acute NF- κ B activity when compared to LPS4h/HI or LPS72h/HI insults (Fig. 1B), it raises the question whether early intranasal application of tat-NBD peptides after pure-HI insult provides protection. We examined this issue and found that intranasal application of 1.4 mg/kg tat-NBD at 10 min after pure-HI only reduced < 20% brain atrophy ($p > 0.05$), and a higher dose at 5.6 mg/kg did not show greater protection ($n=10$ for each, Fig. 4A-D).

We previously reported that pure-HI insults trigger rapid induction of plasminogen activators as a key mechanism of brain damage, inferred by the strong protection with therapeutic application of the Plasminogen Activator Inhibitor-1 protein (Yang et al., 2009). Thus, we examined the effect of intranasal delivery of tat-NBD peptides on HI-induced plasminogen activators. Plasminogen zymography showed that acute intranasal delivery of 1.4 or 5.6 mg/kg tat-NBD peptides failed to suppress the HI-induced tPA and uPA activity at 4 h recovery (Fig. 4E, F). Neither did it prevent MMP-9 activation at 24 h recovery (Fig. 4G, H). These results suggest that early intranasal delivery of tat-NBD peptides has a greater efficacy against infection-sensitized HI brain injury.

DISCUSSION

Several evidences suggest that the NF- κ B signaling pathway may be an important therapeutic target in infection-sensitized neonatal HI brain injury (Wang et al., 2009; Yang et al., 2012). While systemic application of tat-NBD peptides—a potent anti-NF- κ B reagent—yielded mixed results in previous studies of pure-HI injury (van den Tweel et al., 2006; Nijboer et al., 2008b; van den Kooij et al., 2010), the present study is the first that employs intranasal drug-delivery and compares their efficacy in pure- and LPS-sensitized HI models. Our results suggest that the nose-to-brain therapy is particularly effective against infection-sensitized HI injury. These results shed new insights into the mechanism of neonatal HI brain injury and have clinical implications.

Infection-sensitized neonatal HI: an unmet medical need and a scientific enigma

It is well established that perinatal infection predicts worse neurological outcomes in infants with HI brain injury (Dammann et al., 2002; Stoll et al., 2004). Further, the treatment of infection-sensitized HI injury remains a great challenge due to unique properties of neonates. First, although preterm neonates have a high incidence of intrauterine infection, they are excluded from therapeutic hypothermia due to side effects. Even term infants whose placentas showed signs of infection were less responsive to the hypothermia treatment (Wintermark et al., 2010). Second, using general immune-suppression to control infection-sensitized HI brain injury may increase the risk of severe infection, which already accounts for 26% of neonatal mortality (Lawn et al., 2005). Similarly, past studies have shown that while early application of tat-NBD peptides at 0 and 3 h after HI offers protection, extended treatment at 0, 6 and 12 h aggravates the brain damage (van den Tweel et al., 2006; Nijboer et al., 2008b). Thus, to develop brain-targeted anti-inflammation therapy may benefit neonatal care.

There are also indications that pure-HI and infection-sensitized HI induce diverse pathological mechanisms. For example, while mouse pups lacking the myeloid differentiation primary response gene 88 (MyD88)—a critical mediator of innate immunity and the NF- κ B signaling pathway—have greater resistance to LPS-sensitized HI than wild-type mice, they are equally susceptible to pure-HI insults (Wang et al., 2009). Further, the combination of LPS pre-exposure and HI triggers acute NF- κ B activity in the brain, but pure-HI or LPS-stimulation alone lacks this effect (Fig. 1B and Yang et al., 2012). Together, these observations suggest divergent pathogenic processes between pure- and infection-sensitized HI brain injury with the NF- κ B pathway playing a particularly important role in the latter (Fig. 5).

Intranasal delivery of tat-NBD is a potent therapy of infection-sensitized HI injury

To test this hypothesis and devise a brain-targeted anti-NF- κ B therapy, here we examined the effects of intranasal delivery of tat-NBD peptides in experimental models of pure-HI and infection-sensitized HI injury. Intranasal delivery is a powerful method of transporting proteins and chemicals to the brain (Dhuria et al., 2010; Alcalá-Barraza et al., 2010; Akpan et al., 2011). Although details of the nose-to-brain transport are yet to be fully characterized, an important contributor may be the continuous open channels formed by olfactory ensheathing cells that enclose olfactory axons from the olfactory mucosa to the olfactory bulbs (Li et al., 2005). These open channels allow freer protein passage across BBB and into the cerebrospinal fluid space. We hypothesized that intranasal delivery of tat-NBD peptides requires a smaller dose and is particularly effective against LPS-sensitized HI injury. Our references are the previous reports showing that intraperitoneal application of 20 mg/kg tat-NBD peptides at 0 and 3 h after HI reduces brain damage (Nijboer et al., 2008b; van den Kooij et al., 2010). To simplify the therapeutic protocol and compare its efficacy in diverse backgrounds, we applied only one dose of tat-NBD peptides (1.4 mg/kg; 7% of the systemic dose) at 10 min after hypoxia with and without 4 or 72 h LPS pre-exposure, according to an established protocol (Eklind et al., 2005). The inclusion of both 4 and 72 h LPS exposure is aimed to mimic HI injury following acute or sub-acute perinatal infection, respectively.

Our results showed that intranasal delivery of tat-NBD peptides provides strong inhibition of acute NF- κ B activation with either a short (LPS4h/HI) or long (LPS72h/HI) interval between LPS-stimulation and the HI insult, while the extents of brain tissue preservation differ. In LPS4h/HI, this therapy provided 85% reduction of brain atrophy and almost completely blocked MMP-9 activation at 24 h recovery. In LPS72h/HI, it provided 32% reduction of brain atrophy and the inhibitory effect on MMP-9 was inconsistent. Interestingly, early intranasal delivery of tat-NBD peptides yielded very little protection of pure-HI insult and was unable to prevent MMP-9 activation. These results suggest that acute NF- κ B signaling induction is a specific response to combined infection/HI injury, while MMP-9 activation is a common downstream event in neonatal HI brain injury (Fig. 5). This scenario is consistent with the report showing that an elevated plasma level of MMP-9, presumably released from the injured brain, is a biomarker of neonatal encephalopathy (Bednarek et al., 2012).

Although our study using intranasal delivery of tat-NBD peptides did not show protection in pure-HI injury, several factors may account for the difference outcomes from previous studies using intraperitoneal injections (Nijboer et al., 2008a; van den Kooij et al., 2010). First, we only applied tat-NBD once at 10 min after hypoxia, while the previous studies used two injections (20 mg/kg) at 0 and 3 h recovery. Accordingly, intranasally delivered tat-NBD peptides entered the brain rapidly and peaked at around 1 h recovery, while more tat-NBD peptides were found in the brain at 3 h than 1 h recovery in previous studies (Nijboer et al., 2008a). In view of the rapid onset of NF- κ B activity in LPS-sensitized HI, the fast and short-lived brain distribution of tat-NBD peptides by intranasal delivery may lead to strong protection in this condition, but weaken its effect under pure-HI insults. Second, pure-HI brain injury triggers fast induction of plasminogen activators, which are not direct inhibitory targets of tat-NBD peptides. The combination of rapid clearance and an inability to block plasminogen activators may explain why early intranasal application of tat-NBD peptides has little protection in pure-HI insults. Future studies are needed to determine whether different intervention timings by intranasal delivery of tat-NBD peptides can provide protection against pure-HI insults.

Intranasal delivery of tat-NBD as an adjunct therapy of complex neonatal HI injury

If pure-HI and HI with 4 h LPS pre-exposure (LPS4h/HI) represent two prototypes of neonatal brain injury, HI with 72 h LPS pre-treatment (LPS72h/HI) is more complex and contains pathogenic mechanisms of both, such as rapid induction of plasminogen activators and the NF- κ B pathway. Further, the smaller pups following 72 h LPS-exposure may have less reserve to replenish the brain energy after HI insults (Cady et al., 2008). These complex pathogenic mechanisms can explain why intranasal delivery of tat-NBD peptides potently blocks acute NF- κ B signaling, but is less effective in preserving brain tissue after LPS72h/HI insults. Because human diseases are also complex and often involve multiple pathological mechanisms, the LPS72h/HI model may be a particularly useful paradigm to test potential synergy between intranasal tat-NBD delivery and other neuroprotection therapies, such as hypothermia, for clinical application. To this end, future studies are warranted to determine the efficacy, pharmacokinetics, and toxicology profiles for intranasal delivery of tat-NBD peptides in neonatal HI brain injuries.

In summary, results of the present study highlight the complexity of pathogenesis in neonatal HI brain injury and suggest that immediate intranasal delivery of tat-NBD peptides is a preferential therapy of infection-sensitized HI injury.

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Highlights

1. Infection drastically alters pathogenic mechanism of hypoxic-ischemic brain injury.
2. Intranasal delivery of anti-NFkB peptides blocks infection-sensitized HI injury.
3. Results suggest a safe, brain-targeted anti-neuroinflammatory therapy.

Figure 1.

Intranasal tat-NBD delivery blocks infection-sensitized HI injury (LPS4h/HI). **A**, Scheme for different properties of neuroinflammation between pure- and infection-sensitized HI brain injury (modified with permission from Dirnagl et al., 1999). **B**, EMSA showed acute NF-κB activity on the carotid artery-ligated hemisphere (right, R; asterisks) at 4 h after HI with 4 or 72 h LPS pre-exposure (0.3 mg/kg, IP). Note the absence of NF-κB activity following pure-HI and LPS-exposure alone or using mutant NF-κB probes (Mut). **C**, Plasminogen zymogram showed induction of tPA and uPA activities by pure-HI insults at 4

h recovery, which was greatly attenuated by the LPS4h/HI insult. **D**, Immunoblotting showed that biotin-conjugated tat-NBD peptides entered the olfactory bulbs at 10-30 min and the cerebral cortex at 60 min after intranasal administration. **E**, Quantification of tissue loss (as the percentage of counterparts in the opposite hemisphere) in the cerebral cortex, hippocampus, and striatum at 7 day recovery following intranasal saline (PBS) or tat-NBD peptide (1.4 mg/kg) treatment at 10 min after LPS4h/HI injury (n=10 for each group). **F, G**, Representative TTC-stained brain slices at 24 h after LPS4h/HI insult and the PBS or tat-NBD treatment. Note the pale, infarcted areas in the cerebral cortex of saline-treated rat pups. **H, I**, Brain photographs of rats treated with saline or tat-NBD at 7 d after LPS4h/HI insult. Red arrows point to unilateral tissue loss in the carotid artery-ligated hemisphere.

Figure 2. Tat-NBD blocks NF-κB signaling and microglia activation following LPS4h/HI injury. **A**, **B**, EMSA showed that intranasal administration of tat-NBD peptides, but not intraperitoneal injection of minocycline (45 mg/kg), blocks the acute NF-κB activity at 4 h after LPS4h/HI injury. **C**, RT-PCR showed that intranasally applied tat-NBD peptides significantly decreased LPS4h/HI-induced *IL-1β*, *IL-6*, and *Tspo* transcripts at 24 h recovery (n=4 each). Note that the low-dose LPS exposure was insufficient to induce pro-inflammatory cytokines in the brain. **D-I**, Immunostaining detected numerous OX42/Iba1 double-positive microglia/

macrophages in the cerebral cortex (Ctx) of PBS-treated pups at 24 h recovery (E, H). In contrast, Iba1(+) microglia retained ramified processes and OX42(+) cells were confined in the corpus callosum (CC) in the opposite hemisphere (D, G) or in pups receiving tat-NBD treatment (F, I). **J, K**, Only wild-type tat-NBD peptides, but not mutant tat-NBD peptides, blocked LPS4h/HI-induced MMP-9 activation at 24 h recovery (n=4 each). **: $p < 0.01$ compared to untouched (UN) subjects.

Figure 3. Tat-NBD attenuates neonatal HI injury sensitized by 72 h LPS-exposure (LPS72h/HI). **A, B**, P7 rat pups after 72 h LPS-exposure (0.3 mg/kg, IP) showed a similar phosphorus magnetic resonance spectrum (MRS) in the brain, despite significantly smaller body-weight gain ($n=20$ for each in A, and 3–4 in B). **C**, Intranasal delivery of tat-NBD (1.4 mg/kg) blocks the NF- κ B activity at 4 h after LPS72h/HI injury. **D**, The tat-NBD treatment significantly reduced brain atrophy at 7 d after LPS72h/HI injury. The p -value was determined by t -test ($n=10$ each). **E, F**, Intranasal delivery of tat-NBD peptides was unable to prevent tPA and

uPA activities at 4 h after LPS72h/HI insults (n=6 each). Shown in E are the results of two separate samples, and in F, the quantification of tPA and uPA activities. **G, H**, Intranasal delivery of tat-NBD had inconsistent effects on LPS72h/HI-induced MMP-9 activity at 24 h recovery, ranging from near-complete inhibition (#1) to no effect (#2 in G). Consequently, quantification showed only a trend of reduction of MMP-9 activation by tat-NBD treatment ($p = 0.06$; $n=4$ for each).

Figure 4.

Acute intranasal delivery of tat-NBD peptides failed to protect against pure-HI injury. **A, B, C**, Photographs of the rat pup brains 7 days after pure-HI injury and immediate intranasal administration of saline (**A**) or tat-NBD peptides at the dose of 1.4 (**B**, 1X) or 5.6 mg/kg (**C**, 4X). **D**, Quantification of brain atrophy showed that intranasal delivery of up to 5.6 mg/kg tat-NBD peptides was ineffective against pure-HI insults. **E-H**, Intranasal delivery of tat-NBD failed to attenuate HI-induced tPA and uPA activities at 4 h (n=5 each) or MMP-9 activity at 24 h recovery (n=4 each).

Figure 5.

Intranasal delivery of tat-NBD peptides is a potent therapy of infection-sensitized HI. Note that the induction of NF-κB signaling is a specific early (4 h) event after combined LPS/HI insults, while the MMP-9 activation is a later event (around 24 h recovery; see Svedin et al., 2007; Adhami et al. 2008; Yang et al., 2009) in either pure- or LPS-sensitized HI injury. Hence, early intranasal administration of tat-NBD peptides has greater protection of infection-sensitized HI.