Neuroinflammation is induced by tongue-instilled ZnO nanoparticles via the Ca\textsuperscript{2+}-dependent NF-κB and MAPK pathways

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Abstract

Background: The extensive biological applications of zinc oxide nanoparticles (ZnO NPs) in stomatology have created serious concerns about their biotoxicity. In our previous study, ZnO NPs were confirmed to transfer to the central nervous system (CNS) via the taste nerve pathway and cause neurodegeneration after 30 days of tongue instillation. However, the potential adverse effects on the brain caused by tongue-instilled ZnO NPs are not fully known.

Methods: In this study, the biodistribution of Zn, cerebral histopathology and inflammatory responses were analysed after 30 days of ZnO NPs tongue instillation. Moreover, the molecular mechanisms underlying neuroinflammation in vivo were further elucidated by treating BV2 and PC12 cells with ZnO NPs in vitro.

Results: This analysis indicated that ZnO NPs can transfer into the CNS, activate glial cells and cause neuroinflammation after tongue instillation. Furthermore, exposure to ZnO NPs led to a reduction in cell viability and induction of inflammatory response and calcium influx in BV2 and PC12 cells. The mechanism underlying how ZnO NPs induce neuroinflammation via the Ca\textsuperscript{2+}-dependent NF-kB, ERK and p38 activation pathways was verified at the cytological level.

Conclusion: This study provided a new way how NPs, such as ZnO NPs, induce neuroinflammation via the taste nerve translocation pathway, a new mechanism for ZnO NPs-induced neuroinflammation and a new direction for nanomaterial toxicity analysis.

Keywords: Zinc oxide nanoparticles, Taste nerve, Neuroinflammation, Calcium ion

Background

Over the past few decades, nanotechnology has developed rapidly and emerged as a promising technique for various materials science, biomedical and daily life applications. Zinc oxide nanoparticles (ZnO NPs) are frequently used in dental materials, such as toothpaste \[1\], mouthwash, dental resin, root canal flings \[2\] and implant surface coatings \[3\]. The increased biological applications of ZnO NPs in stomatology provides a potential route for ZnO NPs to translocate into the human body. Despite great progress in nanotechnology, the effects of ZnO NPs exposure on the human body, in general and specifically on the brain, are not well understood. The central nervous system (CNS), which plays a commander-like role in our body, integrates received information to coordinate and influence all other bodily activities.

The routes of ZnO NPs transfer to the CNS include blood brain barrier (BBB) pathway \[4\], placental barrier pathway \[5, 6\], gastrointestinal absorption pathway \[7, 8\] and sensory nerve translocation pathway \[9, 10\]. At present, large numbers of studies have shown that intranasal instillation of nanoparticles can be transported along the olfactory nerve and trigeminal nerve into CNS, resulting in neurotoxicity \[11–14\]. The taste nerve pathway is similar to the olfactory nerve pathway,
which can identify the characteristics of different foods and transmit sensory signals to CNS. Therefore, nanoparticles tongue instillation is likely to be uptaken by taste bud cells and transported into CNS via the taste nerve pathway. In a previous study, we demonstrated that ZnO NPs and titanium dioxide NPs (TiO\textsubscript{2} NPs) can be taken up by taste buds and translocated into the brain via taste nerves (chorda tympani (CT) and glossopharyngeal nerves), providing a new pathway for NPs to translocate into the brain [15]. Furthermore, NPs deposited onto the brain induce oxidative damage and degenerate learning and memory, resulting in neurodegeneration. By definition, neurodegeneration disturbs the properties of the CNS and therefore affects neuronal function, which is associated with many neurodegenerative diseases, such as Alzheimer’s disease (AD), frontotemporal lobar dementia (FTLD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS). Increasing evidence shows that neurodegenerative diseases are partially caused and promoted by neuroinflammation. Furthermore, neuropathologists have found that glial cells, including astrocytes and microglia, are activated in each of these disorders, accompanied by low-to-moderate levels of inflammatory mediators in the parenchyma [16]. NPs may accelerate the process of neurodevelopmental disorders and neurodegenerative diseases by promoting inflammation, reactive oxygen species (ROS), microglial activation and neuronal loss [17]. Many studies have indicated that exposure to ZnO NPs can induce significant inflammatory responses in various organs, such as lung [18–20], heart [21] and brain. Tian [22] reported that ZnO NPs induced inflammatory responses in the brains of mice and further impaired their learning and memory abilities. Thus, the neurodegeneration caused by tongue-instilled ZnO NPs and TiO\textsubscript{2} NPs may be associated with neuroinflammation. Therefore, this study aimed to investigate whether tongue-instilled ZnO NPs induce inflammatory responses in the CNS and to evaluate the potential molecular mechanisms underlying this process.

**Results**

**Characterization, identification, and stability of ZnO NPs**

The physical and primary particle sizes and morphology of ZnO NPs were measured by TEM. The TEM micrographs demonstrated that ZnO NPs were prismatic-shaped, with a diameter of 42.31 ± 17.94 nm (Fig. 1a). The hydrodynamic size of ZnO NPs in a DW suspension was 232.8 ± 53.55 nm, and the size distribution was centralized (Fig. 1b). The Raman spectra of ZnO NPs samples showed peaks at 437 and 584 cm\textsuperscript{-1}, which confirmed the molecular structure of ZnO NPs (Fig. 1c). Furthermore, the zeta potential of ZnO NPs was 14.6 mV at pH 7.0, and the specific surface area was 27.342 (m\textsuperscript{2}/g). The physicochemical properties of ZnO NPs are summarized in Table 1.

**Biodistribution of ZnO NPs**

To assess ZnO NPs penetration in rat tissues, the Zn concentrations in nerve and brain tissue as well as those in blood were quantitatively analysed by ICP-MS. The Zn levels in the tongue instillation group were significantly higher than those in the gavage and control groups, and the Zn levels in the sub-brain regions were ranked as follows: cerebral cortex > hippocampus > cerebellum > brain stem pattern (F(2, 51) in nerve, cerebellum, brain stem, cerebral cortex, hippocampus were 3.482, 1.475, 3.672, 3.893, 3.315, respectively) (Fig. 1d). However, in the tongue instillation and gavage groups, there were no significant increases in the blood Zn concentrations compared with that in the control group (F(2, 50) = 1.351) (Fig. 1e). These results confirmed that most of ZnO NPs enter the rat brain via the taste nerve pathway but not via blood and the digestive tract when administered via the tongue.

**Neuroinflammation is induced by ZnO NPs in the rat brain**

To evaluate the inflammatory responses induced by ZnO NPs, the relative gene expression levels of key cytokines, including TNF-\textalpha, IL-1\beta, IL-6, IL-10, IFNG and NOS2, in the cerebral cortex were measured after the exposure. TNF-\textalpha, IL-1\beta and NOS2 were significantly upregulated in the tongue instillation group compared to that in the other groups (F(2, 51) = 5.476, 3.855, 5.767) (Fig. 2a). Furthermore, the TNF-\textalpha and IL-1\beta levels were measured by ELISA, revealing significantly high TNF-\textalpha (F(2, 48) = 5.372) and IL-1\beta (F(2, 50) = 3.985) levels induced by ZnO NPs tongue instillation for 30 days (Fig. 2b). In addition, to exclude the effect of influence factors (ie, anesthesia procedure) on the experimental results, a complementary study was performed. The animals were divided into five groups of six animals each, including the DW instillation group, the ZnO NPs tongue instillation group, the ZnO NPs gavage group, the DW gavage group and the background group (the animals don’t receive any treatment). The analyzed results in the DW gavage group and the background group were no different with DW instilled group, which indicated the neuroinflammation was induced by ZnO NPs tongue instillation and the other treatment in our study (ie, anesthesia procedure) would not affect our results (Additional file 1: Figure S5).

**ZnO NPs-induced histopathological and immunohistochemical changes**

Histopathological analyses of the cerebral cortex revealed that tongue instillation exposure to ZnO NPs induced no apparent changes in brain histology when
compared with the gavage and control groups. However, sparser hippocampal tissues were observed in both ZnO-treated groups compared to that in the control (Fig. 3a). Thus, ZnO NPs in the brain did not affect the cellular integrity or tissue morphology but still caused some minor damage. What's more, fungiform papilla hyperemia was observed in ZnO tongue instillation group (Fig. 4), which may hint the sensory and taste function of tongue was affected.

Pathological changes in brain neurons were also examined using the Nissl staining method. Decreased numbers of Nissl bodies and shrunken nuclei were found in

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**Table 1** Summary of physicochemical properties of ZnO nanoparticles

| Particles | Morphology | Primary size (nm)<sup>a</sup> | Hydrodynamic size (nm)<sup>b</sup> | Zeta potential (mV)<sup>c</sup> | Surface area (m²/g)  |
|-----------|------------|-----------------------------|-----------------------------|-------------------|-------------------|
| ZnO       | Hexagonal  | 42.31 ± 17.94               | 232.8                       | 14.6              | 27.342            |

<sup>a</sup>The primary nanoparticle size was measured by transmission electron microscope

<sup>b</sup>Nanoparticles were dispersed in DW and measured by dynamic light scattering instrument

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the cortex ($F(2, 42) = 3.751$) and hippocampus ($F(2, 42) = 4.133$) after ZnO NPs tongue instillation, which showed that neurons were damaged in this group (Fig. 3b).

Immunostaining of the cortex and hippocampus with anti-GFAP and anti-CD11b indicated that GFAP ($F(2, 42) = 3.698$) and CD11b ($F(2, 42) = 4.572$) expression was increased in the tongue instillation group compared to that in the other groups, suggesting that ZnO NPs stimulate astrocyte and microglial activation or proliferation after translocation to the brain (Fig. 5a and b). P2X7 receptor expression was also increased in the ZnO NPs tongue instillation group ($F(2, 42) = 6.824$), which might be associated with inflammatory responses in the brain (Fig. 5c).

**Dose-dependent and time-dependent cytotoxicity induced by ZnO NPs**

To elucidate the mechanism of brain inflammation, BV2 and PC12 cells were selected as models for analysis at the cellular and molecular levels. To investigate the cytotoxic effects of ZnO NPs, the viabilities of BV2 and PC12 cells were determined by the MTT and LDH assays. Within 3 h of treatment, ZnO NPs exerted no strong toxic effects on BV2 cells or PC12 cells. Moreover, no significant viability changes were observed in either cell line at concentrations below 10 μg/mL at any time point. However, when the concentrations reached 20 μg/mL, an obvious decrease in cell viability was observed after 6 h of treatment. Henceforth, cell survival rates decrease as time and ZnO NPs concentrations increase. Furthermore, BV2 cells showed more sensitivity to ZnO NPs treatment, as their viability decreased earlier and the survival rate was lower than those in PC12 cells (Fig. 6a and b).

The LDH assay reflects membrane integrity, which can indirectly assess cell viability. As shown in Fig. 6c and d, the LDH activity was increased by ZnO NPs in a concentration- and time-dependent manner. The largest increase was observed at concentrations of 30 μg/mL at every time point ($P < 0.05$) except for 1 h. Dose- and time-dependent reductions in BV2 and PC12 cell viability were observed after exposure to ZnO NPs.

**ZnO NPs were taken up by BV2 and PC12 cells**

To evaluate ZnO NPs distribution and the induction of cellular ultrastructural changes, BV2 and PC12 cells...
were incubated with 30 μg/mL ZnO NPs for 1 h. TEM analysis revealed that ZnO NPs were taken up by PC12 cells and located in the vesicles. What's more, a sunken with ZnO NPs in the cytomembrane was observed in BV2 cells, which showed the cell was phagocytosing NPs. However, numerous vacuoles but not granular NPs were observed in the cytoplasm of BV2 cells (Fig. 6e).

**ZnO NPs-induced proinflammatory gene alterations and cytokine release**

To clarify the inflammatory responses to ZnO NPs in BV2 and PC12 cells, the levels of the proinflammatory genes TNF-α, IL-1β, IL-6, IL-10, IFNG and NOS2 were measured by qRT-PCR. After ZnO NPs exposure, significantly increased gene expression levels of TNF-α, IL-1β, IL-6 and NOS2 were observed at 1, 3, and 6 h (P < 0.05), and a significant up-regulation of IL-10 gene expression was observed at 3 and 6 h (P < 0.05) after 30 μg/mL ZnO NPs treatment in BV2 cells (Fig. 7a). PC12 cells treated with 30 μg/mL ZnO NPs also exhibited increased TNF-α, IL-6 and NOS2 gene expression at 1, 3 and 6 h (P < 0.05), but no proinflammatory genes were significantly altered at 12 h (Additional file 2: Figure S1A).

Furthermore, TNF-α and IL-1β in the cellular supernatants were measured. The secretion of both cytokines was significantly increased (P < 0.05) after ZnO NPs stimulation in BV2 cells at a dose ≥ 20 μg/mL, peaking at 3 and 6 h (Fig. 7b). In addition, ZnO NPs also induced the secretion of TNF-α in PC12 cells (P < 0.05) (Additional file 2: Figure S1B), while the cytokine release dropped after 12 h of exposure in both cell lines. These data demonstrated that ZnO NPs dose-dependently induce expression cytokine and proinflammatory gene expression, especially after 6 h of exposure.

**ZnO NPs increase intracellular Ca^{2+} concentrations**

We detected intracellular Ca^{2+} levels after exposure to 30 μg/mL ZnO NPs, revealing that calcium ions were detected rapidly (after 1 h) in BV2 (F(4, 55) = 3.571) and
PC12 cells (F(4, 55) = 3.079) (Fig. 8a and Additional file 3: Figure S2A).

P₂X₇, an ionotropic receptor, was detected by western blot to elucidate the factors underlying the intracellular Ca²⁺ increase. P₂X₇ expression was increased in a dose-dependent manner in both cell lines at 1, 3, and 6 h after ZnO NPs treatment. However, P₂X₇ expression was drastically reduced at 12 h, which might be caused by the low cell viability observed at 12 h (Fig. 8c and Additional file 3: Figure S2C).

Furthermore, cell ultrastructural changes indicated that the calcium stores (mitochondria and endoplasmic reticulum) were injured after treatment with 30 μg/mL ZnO NPs. The dilatational endoplasmic reticulum and the empty and swollen mitochondria were observed in the cytoplasm (Fig. 8d and Additional file 3: Figure S2D).

Mechanism underlying ZnO NPs-induced inflammation
To elucidate the possible molecular mechanisms involved in ZnO NPs-induced toxicity, western blot analysis was used to evaluate the protein expression of NF-κB, ERK and p38. As shown in Fig. 9a, the phosphorylation of ERK in BV2 cells was initially increased after incubation with 30 μg/mL ZnO NPs for 1 h (F(5, 48) = 3.079). Meanwhile, significantly increased phosphorylation levels of NF-κB, ERK and p38 were observed in both cell lines after 30 μg/mL ZnO treatment beginning at 3 h and continuing through 12 h (P < 0.05) (Fig. 9 and Additional file 4: Figure S3). Furthermore, total NF-κB in the cytoplasm was decreased, accompanied by increased NF-κB phosphorylation in the cytoplasm, suggesting that NF-κB p65 translocates into the nucleus and leads to NF-κB activation (Fig. 9 and Additional file 4: Figure S3). These analyses suggest that the NF-κB, ERK and p38 pathways participate in ZnO NPs-induced inflammation in a dose-dependent manner.

Intracellular Ca²⁺ plays an important role in ZnO NPs-induced inflammation
To test the possibility that ZnO NPs-induced inflammation is mediated by calcium-dependent pathways, the cells were treated with A839977 (a P₂X₇ receptor antagonist) and BAPTA-AM (an intracellular Ca²⁺ chelator). According to previous experiments, the cytotoxicity, inflammation response and increase in the expression of inflammation-related pathways peaked at 6 h after treatment with 30 μg/mL ZnO NPs; thus, 6 h of 30 μg/mL ZnO NPs treatment was used in the inhibition experiments. As shown in Fig. 10e and Additional file 5: Figure S4E, the 30 μg/mL ZnO NPs-induced increase in NF-κB, ERK and p38...
phosphorylation was significantly inhibited by BAPTA-AM in both cell lines ($P < 0.05$). The cytokine release and proinflammatory gene expression levels nearly returned to baseline (Fig. 10b, c and f and Additional file 5: Figure S4B, C and F) following the down-regulation of NF-κB, ERK and p38 phosphorylation, and the survival rates also increased.

Fig. 5 Immunohistochemical analysis of brain tissues in rats. Sections of brain tissues were stained with GFAP (a), CD11b (c) and P₂RX₇ (e). Cell counting analysis of astrocytes and microglia in the cerebral cortex and hippocampus (b & d, number/cm²). The relative values of P₂RX₇ expression in the cerebral cortex and hippocampus (f). GFAP, CD11b and P₂X7 receptor expression was increased in the tongue instillation group compared to that in the other groups. The red arrows show the positive cells. Results shown as means ± SD from three independent experiments, compare with control group, *$P < 0.05$, **$P < 0.01$. ($n = 5$). Abbreviations: ZnO: zinc oxide nanoparticles; GFAP: glial fibrillary acidic protein; P₂RX₇: P₂X purinoceptor 7; SD: Standard deviation.
Fig. 6 (See legend on next page.)
after BAPTA-AM pre-treatment, suggesting that excess intracellular Ca\textsuperscript{2+} activates the NF-κB, ERK and p38 pathways, ultimately leading to inflammatory responses and apoptosis. However, the levels of ZnO NPs-induced cytokine release and proinflammatory gene expression were not significantly decreased after treatment with A839977. Furthermore, in A839977 treatment group, the phosphorylation levels of NF-κB, ERK and p38 were not much difference compare with control group, perhaps due to the failure of intracellular Ca\textsuperscript{2+} levels to return to baseline by blocking P\textsubscript{2}X\textsubscript{7} receptor (Fig. 10d and Additional file 5: Figure S4D). Together, these results indicate that intracellular Ca\textsuperscript{2+} plays an important role in ZnO NPs-induced neuroinflammation.

**Discussion**

The wide applications of NPs have raised concerns regarding their safety when released into the environment and the human body. Previous studies indicated that the toxicological effects of NPs were associated with their size [23–25], shape [26–28], surface area [29], ζ-potential [8, 25] and surface modifications [30–32]. Characterization analysis indicated that ZnO NPs used in our study are small in size and have large surface areas, positive charges and good dispersion, allowing easier cellular uptake than NPs of larger sizes, smaller surface areas and agglomerative clustering. Therefore, ZnO NPs have more surface activity and enhanced adsorption properties, which might have unusual biological effects when compared to other substances that we contact in daily life.

In our previous study, we demonstrated that ZnO NPs and TiO\textsubscript{2} NPs can transfer into the CNS via taste nerves after tongue instillation for 30 days. Although tongue-instilled ZnO NPs and TiO\textsubscript{2} NPs didn’t cause detectable influence on rat growth and development, they induced oxidative damage, learning and memory degeneration and neurodegeneration in a Wistar rat model [15]. Based on the complicated relationships between neuroinflammation and neurodegenerative disease [16], we speculated that the neurodegeneration caused by the tongue instillation of ZnO and TiO\textsubscript{2} NPs may be associated with neuroinflammation. Therefore, this study aimed to investigate whether tongue-instilled ZnO NPs induce inflammatory responses in the CNS and the potential molecular mechanisms underlying this process.

The first step to assessing the neuroinflammation induced by ZnO NPs was to determine their fate in the brain. The tongue instillation of ZnO NPs for 30 days significantly increased the Zn content in the brain, and the Zn levels in the sub-brain regions were ranked as follows: cerebral cortex > hippocampus > cerebellum > brain stem. The concentrations of Zn in liver, spleen, kidney, and brain tissues were reportedly increased substantially after 13 weeks of repeated oral ZnO NPs administration (536.8 mg/kg BW), while no significant differences in Zn concentrations were observed between the vehicle control and ZnO NPs (134.2 mg/kg BW) administered orally [33]. The exposure concentrations of ZnO NPs were more than the 500 mg/kg generally administered via gavage in toxicology studies [6, 34, 35]. Thus, increasing brain Zn concentrations requires a much higher dose of ZnO NPs and iterative administration through gavage treatment than other exposure methods. After receiving a single dose of 2000 mg/kg ZnO NPs by oral gavage, the highest plasma Zn levels were observed at 24 h, but the Zn levels were barely altered in the 50 mg/kg treatment group at this time point [36]. However, in our study, the Zn levels in the tongue instillation group were significantly higher than those in the gavage and control groups in the taste nerve and sub-brain regions, while there was no significant increase in blood Zn levels, indicating that most of ZnO NPs are transferred to the brain via taste nerves but do not enter the digestive tract or blood.

The deposited NPs in the brain would damage neuronal function and induce neuronal injury. Histopathological analyses of the brain revealed that exposure to ZnO NPs via tongue instillation did not affect cellular integrity or tissue morphology. However, the Nissl method, used to highlight important structural features of neurons, indicated that neurons were damaged after ZnO NPs tongue instillation. Wang et al. [37] also found that intranasally instilled TiO\textsubscript{2} NPs induced pathological changes and abnormal neuronal arrangement in the hippocampus and CNS. Healthy and integrated brain neurons are essential for learning and memory. Dose- and time-dependent reductions in the viabilities of BV2
Fig. 7 The inflammatory responses to ZnO NPs in BV2 cells. The mRNA relative values of cytokines gene expressions in BV2 cells (a). The gene expression levels of TNF-α, IL-1β, IL-6 and NOS2 were significant up-regulation after ZnO NPs exposure at 1, 3, and 6 h, and IL-10 gene expression was significantly increased at 3 and 6 h. The concentration of TNF-α and IL-1β in BV2 cells culture supernatants were performed by Elisa analysis (b). The secretion of both cytokines was significantly increased after ZnO NPs stimulation in BV2 cells at a dose ≥ 20 μg/mL, peaking at 3 and 6 h. Results shown as means ± SD from three independent experiments, compare with control group, *P < 0.05, **P < 0.01. Abbreviations: ZnO: zinc oxide nanoparticles; SD: Standard deviation.
and PC12 cells were observed after exposure to ZnO NPs. The results from this study demonstrated that ZnO NPs had a severe neural toxicological effect. In addition, fungiform papilla hyperemia was observed in ZnO tongue instillation group. The damage of lingual papilla and neurons may affect the taste sensations, which need to be further study.

The activation of glial cells, including astrocytes and microglia, which are the first line of defence against the entry of foreign particles or infectious agents, can be

**Fig. 8** Effect of ZnO NPs on the intracellular Ca\(^{2+}\) expression of P\(_{2X}\) purinoceptor 7 and ultrastructure changes in BV2 cells. (a) BV2 cells were treated with ZnO NPs (30 \(\mu\)g/mL) for 1, 3, 6 or 12 h, cells were stained with Ca\(^{2+}\) indicator Fluo4-AM (green) and DAPI (blue). Scale bar represents 200 \(\mu\)m. Compare the mean fluorescence intensity of Ca\(^{2+}\) measured in BV2 cells (b), and an obvious increase in the Ca\(^{2+}\) fluorescence (green) was observed in a time-dependent manner. The expression of P\(_{2X}\) receptor was increased in a dose-dependent manner after treating with ZnO NPs in BV2 cells at doses of 0, 5, 10, 15, 20 or 30 \(\mu\)g/mL for 1, 3, 6 or 12 h (c). The dilatational endoplasmic reticulum and the empty and swollen mitochondria were observed after ZnO NPs (30 \(\mu\)g/mL) for 6 h treatment in BV2 cells (d). The yellow arrows show the dilatational endoplasmic reticulum and the red arrows show the swollen mitochondria. Results shown as means ± SD from three independent experiments, compare with control group, *\(P<0.05\), **\(P<0.01\). Abbreviations: cont: control; ZnO: zinc oxide nanoparticles; P\(_{2X}\): P\(_{2X}\) purinoceptor 7; DAPI: 4,6-diamino-2-phenyl indole; SD: Standard deviation
identified by GFAP and CD11b, respectively. In present study, ZnO NPs stimulate astrocyte and microglial activation or proliferation after translocation to the brain. Overactivated glial cells may induce ROS, release nitric oxide (NO) and inflammatory cytokines, and induce neurotoxicity in response to extraneous nanoparticles [38]. Furthermore, activated astrocytes and microglia are regarded as characteristics of neuroinflammatory diseases [39]. TNF-α and IL-1β release and proinflammatory gene expression alterations in the brain after 30 days of tongue instillation. TNF-α release is always accompanied by glial cell activation, leukocyte infiltration and neuronal apoptosis [40, 41]. IL-1β is central to the inflammatory process as well, attributed to the increased permeability of the BBB to circulating immune cells, and is directly responsible for the decline in tissue function [42]. What’s more, cytotoxicity was examined in accordance with the intracellular levels of inflammation in our vitro study. These findings indicated that inflammatory response might be a key route in the neurotoxicity of ZnO NPs.

BV2 cells showed more sensitive to the treatment of ZnO NPs compared with PC12 cells, as their viability decreased earlier and the survival rate was lower than those in PC12 cells. Meanwhile, ZnO NPs induced severer inflammatory responses in BV2 cells. TEM images showed that ZnO NPs were taken up by PC12 cells and located in the vesicles, while numerous vacuoles but not granular NPs were observed in the cytoplasm of BV2 cells. In our previous study, we determined that the intracellular ZnO NPs initially remained in lysosomes and then released zinc ions as rapidly as possible in BV2 cells [43]. Microglial cells function like macrophages by acting as a first line of defence against foreign particle or
ZnO NPs-induced inflammation is mediated by calcium-dependent pathways in BV2 cells. BV2 cells were preincubated for 1 h with A839977 (200 nM) and BAPTA-AM (20 μM) before ZnO NPs treatment. After the cells were treated with ZnO NPs at a dose of 30 μg/ml for 6 h.

Summary of cell viability (a), TNF-α (b), and IL-1β (c) release and the expression of proinflammatory genes (f). The concentration of Ca²⁺ in BV2 cells, cells were stained with Ca²⁺ indicator Fluo4-AM (green) and DAPI (blue) (d). Scale bar represents 200 μm. Compare the mean fluorescence intensity of Ca²⁺ measured in BV2 cells (g). Total proteins of BV2 cells were extracted, and the levels of P2X7, NF-κB, ERK, and p38 signaling pathway molecules were analyzed via Western Blot (e). The gray value was semiquantitative as shown in histogram (h). The 30 μg/ml ZnO NPs-induced cytotoxicity, increase in proinflammatory cytokine release and NF-κB, ERK and p38 phosphorylation was significantly inhibited by BAPTA-AM. Results shown as means ± SD from three independent experiments, compare with control group, *P < 0.05, **P < 0.01. Abbreviations: cont: control; ZnO: zinc oxide nanoparticles; P2X7; P2X purinoceptor 7; DAPI: 4,6-diamino-2-phenylindole SD: Standard deviation.

**Fig. 10** ZnO NPs-induced inflammation is mediated by calcium-dependent pathways in BV2 cells. BV2 cells were preincubated for 1 h with A839977 (200 nM) and BAPTA-AM (20 μM) before ZnO NPs treatment. After the cells were treated with ZnO NPs at a dose of 30 μg/ml for 6 h.
infectious agent entry, which have powerful phagocytic ability and lysosomal system after being activated. ZnO NPs release zinc ions as soon as possible once they locate in lysosomes, so that it is hardly to observe the grainy ZnO NPs in BV2 cells. Fukui et al. [44] indicated that cellular uptake of the ZnO NPs could release numerous Zn\(^{2+}\), which caused stronger oxidative stress, and the increase of intracellular Zn\(^{2+}\) was the main reason to induce toxicity. Compare with PC12 cells, ZnO NPs can be taken up faster and more by BV2 cells, and then release zinc ions to play a role in inducing cytotoxicity, which may be the reason why BV2 cells showed more sensitivity to the treatment of ZnO NPs.

In all eukaryotic cells, finely tuned changes in Ca\(^{2+}\) modulate a variety of intracellular functions, and disruption of Ca\(^{2+}\) handling leads to cell death [45]. Changes in intracellular calcium ion concentrations are closely association with the progression of inflammation. Previous studies found that NPs increased intracellular Ca\(^{2+}\) concentrations and further contributed to the inflammatory response [46, 47]. Herein, calcium ion levels were significantly increased after exposure to 30 \(\mu\)g/mL ZnO NPs in both BV2 and PC12 cells, prompting the consideration that Ca\(^{2+}\) influx might be a key factor in ZnO NPs-induced neuroinflammation. Ca\(^{2+}\) influx is essential for TNF-\(\alpha\) and IL-1\(\alpha\) release in murine microglial cells (MG5) and murine peritoneal macrophages, respectively [48, 49]. Previous studies have shown that the executive functions of activated microglia, such as phagocytosis [50], as well as the release of NO, TNF-\(\alpha\), IL-6 and IL-12 are Ca\(^{2+}\)-dependent [51–53].

ZnO NPs induce membrane lipid peroxidation and lead to calcium influx through membrane channels, inducing further cytotoxicity [54]. Ca\(^{2+}\) increases can be elicited by both Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx through plasma membrane channels, which can be divided into four classes: (i) voltage-operated Ca\(^{2+}\) channels, (ii) ligand-gated Ca\(^{2+}\) channels, (iii) second messenger-operated channels, (iv) store-operated Ca\(^{2+}\) entry [45]. Among these classes, purinergic receptors are the most noticeably expressed ligand-gated Ca\(^{2+}\)-permeable channels in glial cells and control several glial functions, such as cytokine release, in Ca\(^{2+}\)-dependent pathways [51]. Furthermore, purinergic receptors are also involved in neuronal physiology and pathology, causing neurodegenerative diseases [55, 56].

The P\(_2\)X purinoceptor 7 receptor (P\(_2\)RX7) stands out as the single member of the P\(_2\)XR family that plays an important role in multiple inflammatory and immune responses via the inward flux of Na\(^+\) and Ca\(^{2+}\) and the outward flux of K\(^+\), activating inflammasomes and ultimately contributing to inflammation and host defence [57]. Previous studies indicated that P\(_2\)RX7 activates glial cells and promotes the release of proinflammatory factors and chemokines, such as IL-6, TNF, and CXCL2 from mouse microglia [58–60]; MCP-1, CCL2, and IL-1\(\beta\) from rat astrocytes [61, 62]; and IL-8 from rat C6 glioma cells [63]. From the findings above, we hypothesized that P\(_2\)RX7-mediated Ca\(^{2+}\) influx participate in ZnO NPs-induced neuroinflammation. In the ZnO NPs tongue instillation group, a significant increase in P\(_2\)RX7 expression in the cerebral cortex and hippocampus. What’s more, we observed increased P\(_2\)X7 protein expression accompanied by high Ca\(^{2+}\) concentrations in BV2 and PC12 cells. These results confirmed our hypothesis. In addition, the overloading of intracellular Ca\(^{2+}\) may destroy Ca\(^{2+}\) stores and further induces apoptosis [64]. Mitochondria and endoplasmic reticulum are the Ca\(^{2+}\) stores in the cytoplasm, which stockpile more than 90% of Ca\(^{2+}\) inside the cells. TEM analysis indicated significantly damaged mitochondria and endoplasmic reticulum after treatment with 30 \(\mu\)g/mL ZnO NPs. In these cells, numerous vacuoles were observed in the cytoplasm, the endoplasmic reticulum was dilatational and the mitochondria were empty and swollen. Such ultrastructural changes were also previously reported upon treatment with anatase TiO\(_2\) NPs and PEGylated multi-walled carbon nanotubes [65, 66].

To explore the possible molecular mechanisms underlying ZnO-NPs-mediated neuroinflammation, we measured the expression levels of regulators involved in the inflammation pathway, including NF-\(\kappa\)B, ERK and p38. NF-\(\kappa\)B is a protein complex involved in cellular responses to stimuli, such as stress, cytokines and NPs [67], and results in inflammation, apoptosis, tumorigenesis, and various autoimmune diseases. In addition, ERK and p38 are members of MAPKs, are important for transducing signals to the nucleus and also play key roles in the regulation of many cellular responses, such as inflammation, proliferation, differentiation and apoptosis [68]. Some studies have indicated that NPs, such as ZnO, Al\(_2\)O\(_3\), TiO\(_2\), CeO\(_2\), Ag, CuO and quantum dots, induce inflammation due to NF-\(\kappa\)B [69–71], ERK [72, 73] and/or p38 [74–76] activation. In our study, we also observed similar results, as the NF-\(\kappa\)B, ERK and p38 pathways were activated in a dose-dependent manner after ZnO treatment in both cell lines. Furthermore, the activation strength and duration of the ERK pathway differed between the two cell lines, with ERK activation occurring very early (at 1 h) in BV2 cells. As indicated previously, BV2 cells act as the first line of defence against the entry of foreign particles or infectious agents, which potentially contributed to the differential activation of inflammation signalling pathways.

Ca\(^{2+}\) is known to play a key role in activating several signalling pathways, such as MAPKs and redox-sensitive transcription factors, including NF-\(\kappa\)B, which can lead to the production of proinflammatory molecules and
mediators. Previous studies indicated that ultrafine particles and TiO2 NPs may exert proinflammatory effects by modulating intracellular calcium concentrations, NF-κB activation, and cytokine production via an ROS-mediated mechanism [77, 78]. In another study, Ca2+ influx via ROS-activated TRPM2 mediated the amplification of ERK activation and NF-κB nuclear translocation, which led to CXCL8 production [79]. Ratner also found that P. aeruginosa PAO1 stimulated increases in Ca2+, and the response stimulated p38 and ERK signalling cascades, resulting in NF-κB activation and IL-8 expression [80]. ROS might be a key mediator of inflammatory conditions, potentially stimulated by increased intracellular Ca2+, and further activate the NF-κB, ERK and p38 pathways, resulting in inflammation. Our previous studies also found that ZnO NPs induced oxidative damage in the brain after tongue instillation for 30 days, and ROS levels were enhanced in BV2 cells after 10 μg/mL NPs treatment [15, 81]. Furthermore, enhanced Ca2+ levels can lead to the activation of protein kinase C (PKC), which is involved in the activation of NF-κB and ERK [82–84]. Thus, we speculated that ZnO NPs induce neuroinflammation via the Ca2+-dependent NF-κB, ERK and p38 activation pathways.

To further elucidate how ZnO NPs-induced inflammation is mediated by calcium-dependent pathways, A839977 and BAPTA-AM were used to block P2RX7 expression and Ca2+ increase. BAPTA-AM but not A839977 prevented the NF-κB, ERK and p38 activation, proinflammatory gene upregulation, TNF-α and IL-1β release and cell viability decrease induced by ZnO NPs. Intracellular Ca2+ analysis indicated that BAPTA-AM inhibited Ca2+ increases in the cytoplasm, while A839977 did not. As previously mentioned, the P2X7 receptor is not the only channel to mediate Ca2+ influx, as multiple channels participate in this process. ATP-induced biphasic Ca2+ mobilization is mediated by calcium-dependent pathways, A839977 and BAPTA-AM were used to block P2RX7 expression and Ca2+ increase. BAPTA-AM but not A839977 prevented the NF-κB, ERK and p38 activation, proinflammatory gene upregulation, TNF-α and IL-1β release and cell viability decrease induced by ZnO NPs. Intracellular Ca2+ analysis indicated that BAPTA-AM inhibited Ca2+ increases in the cytoplasm, while A839977 did not. As previously mentioned, the P2X7 receptor is not the only channel to mediate Ca2+ influx, as multiple channels participate in this process. ATP-induced biphasic Ca2+ mobilization is mediated by P2Y receptors (0–5 min), P2X receptors (5–30 min) and internal Ca2+ stores (30 min-3 h) [48]. Daniel F. Gilbert et al. [85] found that ATP appears to more selectively induce P2X than P2Y receptor-operated Ca2+ entry and then activates downstream proinflammatory signalling in BV2 cells. During thioglycolate-elicited macrophage activation, PGE2 selectively impairs P2Y but not P2X Ca2+ mobilization, while this effect is absent in lipopolysaccharide (LPS)-activated cells [86]. Therefore, the relative importance of Ca2+ influx versus Ca2+ mobilization depends on the stimulus and the cell type. These results indicated that Ca2+ increase is essential for ZnO NPs-induced neuroinflammation via the NF-kB, ERK and p38 activation pathways. A model of ZnO NPs-induced neuroinflammation in the CNS via the taste nerve pathway and the related mechanisms underlying ZnO NPs-induced neuroinflammation described herein are shown in Fig. 11.

Conclusion
In summary, this study demonstrated that ZnO NPs can be transported to the brain via the taste nerve after 30 days of tongue instillation and induce glial cell activation and inflammatory responses in the CNS. Moreover, ZnO NPs can induce inflammatory responses via the Ca2+-dependent NF-κB, ERK and p38 activation pathways in BV2 and PC12 cells. In general, this study provided a new way for how NPs, such as ZnO NPs, induce neuroinflammation via the taste nerve translocation pathway (sensory nerves pathway), a new mechanism for ZnO NPs-induced neuroinflammation and a new direction for nanomaterial toxicity analysis. In addition, the findings of this study could provide more useful toxicological information and references for security application of nanomaterials, and some information to prevent and cure neurodegenerative diseases.

Methods
Characterization of ZnO NPs
ZnO NP powder was purchased from SigmaAldrich (CAS number: 1314-13-2, USA). The physical and primary particle sizes and morphology were determined using transmission electron microscopy (TEM; JEOL, Tokyo, Japan). Raman spectra were acquired at room temperature using a Raman spectrometry system (Jobin-Yvon T64000, France). The hydrodynamic size agglomerates and charge measurements of ZnO NPs in distilled water (DW) were determined by dynamic light scattering (DLS) using the Zetasizer Nano ZS instrument (Malvern, Malvern, UK). Additionally, the specific surface areas of the NPs were measured by Brunauer-Emmett-Teller adsorption analysis on a Micromeritics ASAP 2010 M+C instrument (Micromeritics Co, GA, USA).

Animals and treatment
Male Wistar rats (4 weeks old) weighing 130–150 g were purchased from the Animal Center of Southern Medical University (Guangzhou, China). One week prior to beginning the experiment, the rats were housed under controlled environmental conditions (23 ± 1 °C room temperature, 60 ± 10% relative humidity and a 12 h/12 h light/dark cycle). Rodent diet and water were provided ad libitum.

The rats were randomly allocated into three groups with comparable weights: the control group, the ZnO NPs tongue instillation group and the ZnO NPs gavage group. The exposure procedure referred to the intranasal instillation procedure in other intranasal instillation studies [37, 87]. The concentration of ZnO NPs exposure was 50 mg/kg body weight (BW), which was based on the doses of oral administration in other animal models [15, 33]. ZnO NPs were dispersed in DW (50 mg/mL) and sonicated for at least 30 min before the administration. Rats of the control and ZnO NP
instillation groups were weighed and anaesthetized under 1% pentobarbital sodium via intraperitoneal injection (48 mg/kg) before instillation. The rats were held in the lateral position, and their tongues were pulled from the corner of their mouth. Then, a 50 mg/mL suspension of ZnO NPs (50 mg/kg BW) was instilled onto the surface of the tongue with a microsyringe, and the control group was instilled with an equal amount of DW. The instillation procedure lasted approximately one hour, and the tongue was then rinsed with DW to clean the remainder of the NPs. For the ZnO gavage group, the rats were treated with a 50 mg/mL suspension of ZnO NPs (50 mg/kg BW) by oral gavage and anaesthetized in the same manner 1 h later to eliminate the effects of anaesthesia. These protocols were performed every other day for 30 days.

After 30 days of treatment, animals from each group (n = 5) were anaesthetized and exsanguinated. After blood collection, the animals were perfused with 100 mL of ice-cold saline (0.9% NaCl in deionized water with 10 U/mL heparin) and fixed in ice-cold 4% paraformaldehyde. Fixed tissue samples were analysed by histopathology examination.
ICP-MS elemental analysis of tissues and blood
The levels of Zn in the blood or sub-brain regions were quantified by inductively coupled plasma mass spectrometry (ICP-MS). The collected tissues and blood were pre-digested in concentrated nitric acid overnight, completely digested after the addition of H2O2 and heated to 160 °C. The solutions were heated again to 120 °C until the remaining nitric acid evaporated to nearly dryness. The resulting solutions were finally diluted to 2 mL with 1% nitric acid and 0.1% Triton-100 and analysed using ICP-MS. The detection limit of ICP-MS was 0.05–0.8 mg/L Zn for method validation.

Inflammatory responses in the rat brain
The gene expression levels of TNF-α, IL-1β, IL-6, IL-10, IFNG and NOS2 were determined by qRT-PCR. The expression level of each target gene was normalized to the GAPDH mRNA content and analysed using the 2-ΔΔCT method. The primers for qRT-PCR are shown in Table 2.

Table 2 Primer sequences specific for rats used in the qRT-PCR analysis

| Gene  | Forward primer | Reverse primer |
|-------|----------------|----------------|
| TNF-α | ACTGAAACTTCCGAGGTATTG | GCTTGGTGTGGTCTAGCAG |
| IL-1β | TGCAAGTGCTGAGAGCCTA | ATCTGGACAGCCCAAGTC |
| IL-6  | ATTGTATGAACAGCGATGATGC | CCAGAGCAGATTTTCAAT |
| IL-10 | CCAGTCAGCCAGACCCACAT | GGGGCATCACTTCTACCA |
| IFNG  | AGCAACAGTAAAGCAAAAAAG | AAGCCAGACGTTTGGC |
| NOS2  | AGCGGCCACACCCTCCTTGG | CACACAAGTTTGGTCTGGC |
| GAPDH | AGTGCCAGCCGCTGTCATA | GATGTTGATGGTCTTCCGT |

on a LightCycler480 Sequence Detector System (Roche, Switzerland).

Cerebral cortex levels of TNF-α and IL-1β in the three groups were measured by an enzyme linked immunosorbent assay (ELISA) kit specific for rats (Biolegend, San Diego, CA). The assays were performed strictly according to the manufacturer’s instructions. Absorbance was read using a microplate reader (M5, Molecular Devices, USA) at a wavelength of 450 nm.

Histopathological and immunohistochemical examination of the tongue and brain
The fixed tongue and brain tissues were dehydrated in a graded series of ethanol and xylene solutions, embedded in paraffin blocks and sectioned at a 4 μm thickness using a microtome. Haematoxylin & eosin (H&E) and Nissl staining were performed using standard laboratory procedures. Finally, the sections were observed, and photos were acquired using a light microscope (Bx51, Olympus, Japan).

Furthermore, IHC analysis was also carried out to identify specific neurotoxic effects. Astrocyte and microglial cell activation in the brain sections was detected by glial fibrillary acidic protein (GFAP; Cell Signaling Technology (CST), USA) and CD11b (Abcam, USA), respectively. Anti-P2X purinoceptor 7 (P2RX7; Abcam) was used to label the ionotropic P2X7 receptor. Photographs of positive staining (brown) were captured in randomly selected fields of both the prefrontal cortex and the hippocampus and counted by ImageJ software.

Cell culture
The BV2 and PC12 cell lines were purchased from CBCAS (Cell Bank of the Chinese Academy of Sciences, Shanghai, People’s Republic of China) and maintained in DMEM containing 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO2 humidified incubator. The culture medium was replaced every other day, and cells were passaged upon reaching approximately 80% confluence.

ZnO NPs were diluted to 100 μg/mL from a 1 mg/mL stock solution in the culture medium and sonicated for at least 30 min to prevent aggregation before the cells were treated.

For inhibition experiments, cells were preincubated for 1 h with the following substances: A839977 (200 nM; MCE, USA), a P2RX7 receptor antagonist, and BAPTA-AM (20 μM; MCE, USA), an intracellular Ca2+ chelator, before ZnO NPs treatment.

MTT assay and LDH measurement
Cell survival rates following treatment with different concentrations of ZnO NPs (5, 10, 15, 20, 30, 40, 50 and 60 μg/mL) were evaluated using the MTT assay. Briefly,
BV2 and PC12 cells were seeded onto 96-well culture plates at a density of 5 × 10^4 cells/well and allowed to attach overnight. Then, the cells were exposed to various concentrations of ZnO NPs for 1, 3, 6, 12, 24 and 48 h. Cell viability was evaluated using the MTT assay (n = 6), and the absorbance was measured using a microplate reader at a wavelength of 570 nm.

Lactate dehydrogenase (LDH) leakage is based on the measurement of LDH activity in the extracellular medium, which releases from damaged cells. After exposure to ZnO NPs, the culture medium was removed and centrifuged at 3000 rpm for 5 min to obtain the cell-free supernatant. The LDH activity in the medium was determined using a commercial LDH kit (Nanjing Jiancheng, Nanjing, China) according to the manufacturer’s protocol, and the absorption was measured using a microplate reader at a wavelength of 450 nm.

Inflammatory responses at the cellular level

BV2 and PC12 cells were seeded on 6-well plates, treated with ZnO NPs for a specific amount of time, and rinsed twice with PBS before TRIzol reagent was added to extract the RNA. The subsequent steps were performed in accordance with the qRT PCR detection method described for the in vivo study. The primers used for qRT PCR in PC12 and BV2 cells are shown in Tables 2 and 3, respectively.

TNF-α and IL-1β in the culture medium after ZnO NPs treatment were measured by ELISA using the same protocol as that used to measure cytokine release in vivo.

Ultrastructural changes in the cells

For the TEM examination, BV2 and PC12 cells were seeded on 6-well plates and incubated with 30 μg/mL ZnO NPs for amount of time, and rinsed twice with PBS before collection and then removed from plate by scraping. After fixing with glutaraldehyde, cells were post-fixed in 1% osmium tetroxide, dehydrated, and embedded in epon. Thin sections were collected on uncoated copper grids, stained with uranyl acetate and lead citrate and then examined with TEM instrument.

Calcium imaging

After incubating with 30 μg/mL ZnO NPs for 1, 3, 6 and 12 h in BV2 and PC12 cells, intracellular calcium levels were analyzed using calcium kit (Fluo4-AM, Dojindo, Japan) according to the manufacturer’s protocol. The fluorescence of calcium were observed under an automatic fluorescence microscope (BX63, Olympus, Japan).

Western bolt

After treatmenting with ZnO NPs, cells were washed twice with PBS, and then RIPA lysis buffer (Beyotime, Haimen, China) with protease inhibitor and phosphatase inhibitors was added to extract the protein. The bicinchoninic acid (BCA) protein assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, USA) was used to determine protein concentration. An equal amount of protein was separated using 8% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany) and blocked with TBST containing 5% skim milk for 1 h at room temperature and then incubated overnight at 4 °C with specific antibodies including: anti-P 2X7 (1:1000, abcam), NF-κB p65, phospho-NF-κB p65, p44/p42 MAPK, phospho- p44/p42 MAPK, p38 MAPK, phospho-p38 MAPK (1:1000, CST) and GAPDH (1:1000, Proteintech, USA). The blots were then washed by TBST, exposed to HRP-conjugated secondary antibodies (1:1000, CST, USA) for 1 h, and the antigen-antibody complex was detected with chemiluminescent HRP substrate (Millipore) by a chemiluminescence measuring instrument (Tanon 5200, Shanghai, China).

Statistical analysis

All analyzes were performed in SPSS 19.0 software and the results are expressed as the means ± SD. All data were statistically analyzed by analysis of variance (ANOVA). A homogeneity-of-variance test was performed, and Least—Significant Difference (LSD) and Dunnett’s T3 tests were used when equal variance was assumed or not. P-values less than 0.05 were considered significant.

Table 3 Primer sequences specific for mice used in the qRT-PCR analysis

| Gene   | Forward primer   | Reverse primer   |
|--------|------------------|------------------|
| TNF-α  | GCCACTCCCCAAAAAGATTG | GCTCTCACCTTGTTT |
| IL-1β  | TGCCAACCTTGGACAGTATG | TGATAGCTGCTGAGGAC |
| IL-6   | GTGGCCCTTGGAGCTGATG | CGAATTTGCATGCAACACT |
| IL-10  | TGCCCGCAAGAAATCAAGGACAT | CACCTCCACCTGCTCCACTG |
| IFNG   | AGCAAGGGCCAAAAAGGATGC | TCATTGAAATGCTTGGCGCTG |
| NOS2   | CGCTCTAGTGGAAAGGCGGCC | TGATAGCCACCCACACAGAC |
| GAPDH  | CTTGCCGGCAGCGTATCCTC | ATGAAGGGTGCTGATGAGC |

Additional files

Additional file 1: Figure S5. Zn level and the release of cytokines in tissue and blood. The concentrations of Zn in the nerves (CT and glossopharyngeal nerve), sub-brain regions (A) and blood (B). The Zn levels of nerve and sub-brain regions in the tongue instillation group were significantly higher than other four groups, and there were no significant increases in the blood Zn concentrations compared with that in other groups. The concentration of TNF-α(C) and IL-1β(D) were analyzed in the rat brain of five groups. The concentrations of TNF-α and IL-1β were significantly increased in ZnO NPs tongue instillation group compared with other four groups. Results shown as means ± SD, compared with background group, *P < 0.05, **P < 0.01. (n = 6). (TIF 30505 kb)

Additional file 2: Figure S1. The inflammatory responses to ZnO NPs in PC12 cells. The mRNA relative values of cytokines gene expressions in
Additional file 3: Figure S2. The expression of involved mechanism of inflammation after treatment with ZnO NPs. PC12 cells were treated with ZnO NPs at a dose of 0, 5, 10, 15, 20 or 30 μg/mL for 1, 3, 6 or 12 h. Total proteins were extracted, and the levels of NF-κB, ERK and p38 signaling pathway molecules were analyzed via Western Blot (A). The gray value was semiquantitative as shown in histogram (B). The phosphorylation levels of NF-κB, ERK and p38 were significantly increase after ZnO NPs treatment. Results shown as means ± SD from three independent experiments, compare with control group, *P < 0.05, **P < 0.01. Abbreviations: ZnO: zinc oxide nanoparticles; SD: Standard deviation. (TIF 46023 kb)

Additional file 4: Figure S3. Effect of ZnO NPs on the intracellular Ca²⁺, expression of P₂X₇ receptor and ultrastructure changes in PC12 cells. PC12 cells were treated with ZnO NPs (30 μg/mL) for 1, 3, 6 or 12 h. Cells were stained with Ca²⁺ indicator Fluo4-AM (green) and DAPI (blue). Scale bar represents 200 μm. Compare the mean fluorescence intensity of Ca²⁺ in PC12 cells (A). The gene expression levels of TNF-α and IL-1β in PC12 cells culture supernatants were also measured via ELISA (B). The phosphorylation levels of P₂X₇ receptor and ultrastructure changes in PC12 cells. Re- sults shown as means ± SD from three independent experiments, compare with control group, *P < 0.05, **P < 0.01. Abbreviations: cont: control; ZnO: zinc oxide nanoparticles; P₂X₇: P₂X purinoceptor 7; DAPI: 4,6-diamino-2-phenyl indole SD: Standard deviation. (TIF 32619 kb)

Additional file 5: Figure S4. ZnO NPs-induced inflammation is mediated by calcium-dependent pathways in PC12 cells. PC12 cells were pre-incubated for 1 h with AB39977 (200 nM) and BAPTA-AM (20 μM) before ZnO NPs treatment. After the cells were treated with ZnO NPs at a dose of 30 μg/mL for 6 h. Summary of cell viability (A), TNF-α (B) and IL-1β (C) release and the expression of proinflammatory genes (D). The concentration of Ca²⁺ in PC12 cells, cells were stained with Ca²⁺ indicator Fluo4-AM (green) and DAPI (blue). Scale bar represents 200 μm. Compare the mean fluorescence intensity of Ca²⁺ measured in PC12 cells (B). The dilatational endoplasmic reticulum and the empty and swollen mitochondria were observed after ZnO NPs (30 μg/mL) for 6 h treatment in PC12 cells (D). The yellow arrows show the dilatational endoplasmic reticulum and the red arrows show the swollen mitochondria. Results shown as means ± SD from three independent experiments, compare with control group, *P < 0.05, **P < 0.01. Abbreviations: cont: control; ZnO: zinc oxide nanoparticles; P₂X₇: P₂X purinoceptor 7; DAPI: 4,6-diamino-2-phenyl indole; SD: Standard deviation. (TIF 41705 kb)

Abbreviations
AD: Alzheimer’s disease; ALS: Amyotrophic lateral sclerosis; BBB: Blood brain barrier; BET: Brunner emmett teller; Ca²⁺: Calcium ions; CNS: Central nervous system; CT: Chorda tympani; DLS: Dynamic light scattering; FLD: Frontotemporal lobar dementia; GFP: G lactin fibrillary acid protein; H&E: Haematoxylin & eosin; HRP: Horseradish peroxidase; IC: Inductively coupled plasma; LDH: Lactate dehydrogenase; NO: Nitric oxide; P₂X₇: P₂X purinoceptor 7; PD: Parkinson’s disease; PM: Particulate material; ROS: Reactive oxygen species; TEM: Transmission electron microscopy; TiO₂: NPs: Titanium dioxide nanoparticles; ZnO NPs: Zinc oxide nanoparticles

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Availability of data and materials
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request. Online supporting information are available.

Authors’ contributions
All authors contributed to the idea and design of this article. HL, AC and XL performed the experiments, generated the data and discussed the findings. JL, XW and YK provided their technical assistance for cell culture methods and involved in cytological experiment. HL wrote the draft manuscript, which was revised by XW and LS. LS established the manuscript in final form. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments were performed in compliance with the regulations and guidelines of the National Ethics Committee on Animal Welfare of China. The approval number provided by the Southern Medical University ethical committee was L2017002.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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