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In vivo fluorescent sensing of salicylate-induced change of zinc ion in auditory cortex of rat brain

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Abstract: This study demonstrates a fluorescent method for in vivo sensing of the dynamic change of Zn\(^{2+}\) concentration in auditory cortex microdialysate induced by salicylate with N’-(7-nitro-2,1,3-benzoazadiazole-4-yl)-N,N,N’-tris(pyridine-2-ylmethyl) ethane-1,2-diamine (NBD-TPEA) as a probe. The excellent property of NBD-TPEA probe makes it possible to achieve a high selectivity for Zn\(^{2+}\) sensing with the co-existence of amino acids and other metal ions as well as the species commonly existing in the cerebral system. To validate the method for in vivo fluorescence sensing of Zn\(^{2+}\) in rat brain, we pre-mix the microdialysates in vivo sampled from auditory cortex with NBD-TPEA probe and then perfuse the mixtures into a fluorescent cuvette for continuous-flow fluorescence detection. The method demonstrated here shows a linear relationship between the signal output and Zn\(^{2+}\) concentration within the concentration range from 0.5 \(\mu\)M to 4 \(\mu\)M, with a detection limit of 156 nM (\(S/N = 3\)). The basal level of extracellular Zn\(^{2+}\) in auditory cortex microdialysate is determined to be 0.52 \(\pm\) 0.082 \(\mu\)M (\(n = 4\)). This value is increased by the injection of 100 mg/mL of salicylate (1 \(\mu\)L/min, 5 min, i.p.), reaches a peak at the time point of 90 min, and levels off with time. Such an increase is attenuated by the injection of MK-801, a potent and specific NMDA receptor antagonist, after the pre-injection of 100 mg/mL salicylate for 5 min. This study offers a fluorescent method for in vivo sensing of Zn\(^{2+}\) in rat brain that could be useful for investigation on chemical processes involved in brain functions.
Understanding of the chemical processes involved in the salicylate-induced physiological and pathological events is of great importance since, as one kind of anti-inflammatory component of aspirin, salicylate has been widely used to treat various ailments. However, excessive salicylate would induce a series of diseases, such as allergic reactions, hepatic and renal dysfunction, tinnitus and hearing loss. For example, early attempts have demonstrated that systemic injection of 250 mg/kg of salicylate can significantly reduce the sound evoked field output of the rat cochlea, in which salicylate competes with the contribution of cytoplasmic chloride to nonlinear capacitance of sensory outer hair cells. On the other hand, in the salicylate-induced physiological and pathological events, a comprehensive chemical process has been proposed to be involved. For instance, the injection of high doses of salicylate evokes excessive glutamate efflux from inner hair cells into cochlear, which not only results in cochlea excitotoxicity but also leads to temporary hearing threshold shift. Moreover, the injection of salicylate could decrease cochlear perilymph ascorbate of guinea pigs, as demonstrated in our early study, suggesting that ascorbate may be involved in the chemical processes of the salicylate-induced tinnitus. In spite of these early studies, the chemical processes involved in the salicylate-induced physiological and pathological events remain to be further explored and understood.

As one kind of the most abundant transition metal elements that presents in all organs and bodies, Zn\(^{2+}\) has been demonstrated to play critical roles in many physical and pathological processes, especially in brain function activity. For instance, in central nervous system, Zn\(^{2+}\) acts as an important endogenous neuromodulator for glutamate transmission. During forebrain ischemia, considerable Zn\(^{2+}\) was reported to release from synaptic vesicles of glutamatergic neuronal terminal to extracellular fluid, which would cause neuronal death. In spite of these attempts, a close survey of literature indicates that there is no report on the role of Zn\(^{2+}\) that plays in the salicylate-induced physiological and pathological events. In this
context, a method that is capable of in vivo sensing of Zn$^{2+}$ level remains very essential. However, the high chemical complexity inherent in the cerebral system, the requirement of spatial resolution of the investigation on chemical issues in the physiological and pathological events, as well as the less theoretical and instrumental demanding of neurotechnologies unfortunately render difficulties in applying the methods reported so far for Zn$^{2+}$ measurement.\textsuperscript{10}

Very recently, one of our authors has demonstrated that the NBD-TPEA probe displays a high selectivity and sensitivity towards Zn$^{2+}$ due to its strong affinity of all nitrogen atoms of pyridine and amine towards Zn$^{2+}$.\textsuperscript{11} Such a property of NBD-TPEA probe potentially enables its promising application for effective sensing of Zn$^{2+}$ in the cerebral system. To explore such potentiality, we sample the brain dialysates with in vivo microdialysis technique and pre-mix the microdialysates with the fluorescent probe and finally perfuse the mixtures into a cuvette for continuous-flow fluorescence detection, as shown in Scheme 1. The combination of sample pre-mixing with continuous-flow fluorescence detection facilitates signal readout recording in a continuous-flow system, eliminates background contamination, and minimizes fluorescent bleaching, while to some extent warrants the time resolution. The method demonstrated here is highly selective and sensitive and could thus be used for monitoring the dynamic change in Zn$^{2+}$ concentration involved in various brain function activity such as salicylate-induced physiological and pathological events.

\textbf{Experimental}

\textbf{Chemicals and solutions}

Dopamine (DA), lactate, glucose, sodium ascorbate (AA), uric acid (UA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT), dizocipline [(+)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5, 10-imine] maleate (MK-801),
and amino acids (99%) were all purchased from Sigma-Aldrich. Sodium salicylate was obtained from Sinopharm Chemical Reagent Co., Ltd. Other chemicals were of at least analytical grade and used as received. Artificial cerebrospinal fluid (aCSF) used as the perfusion solution for in vivo microdialysis and in vitro experiments was prepared by mixing NaCl (126 mM), KCl (2.4 mM), KH$_2$PO$_4$ (0.5 mM), MgCl$_2$ (0.85 mM), NaHCO$_3$ (27.5 mM), Na$_2$SO$_4$ (0.5 mM), and CaCl$_2$ (1.1 mM) in Milli-Q water and the solution pH was adjusted to 7.4. All aqueous solutions were prepared with Milli-Q water (18.2 MΩ-cm).

**Synthesis of NBD-TPEA**

Synthesis of NBD-TPEA probe was conducted with the a procedure reported previously.$^{11}$ Briefly, N,N,N’-Tris(pyridine-2-ylmethyl) ethane-1,2-diamine (TPEA, 2754 mg, 8.26 mmol), K$_2$CO$_3$ (1142 mg, 8.26 mmol), and 4-chloro-7-nitro-2,1,3-benzoxadiazole (4-CINBD, 1814 mg, 9.18 mmol) were mixed in 200 mL of tetrahydrofuran (THF) and the mixture was stirred overnight at room temperature. After that, the solvent was collected and evaporated under vacuum. The crude product obtained was chromatographed on silica gel by eluting with acetate/methanol (v/v, 8:1) to give an orange oil product with 63% yield.

![Synthesis of NBD-TPEA](attachment:synthesis.png)

**In vivo microdialysis and fluorescent detection**

Surgeries for in vivo microdialysis were performed as reported previously.$^{12}$ Adult male Sprague-Dawley rats (350-400 g) were purchased from Center of Health Science, Peking University and housed on a 12:12 h light-dark scheduler with food and water *ad libitum*. The animals were anaesthetized with chloral hydrate (345 mg/kg, i.p.) and positioned onto a stereotaxic frame. The microdialysis guide cannulas were carefully implanted in the auditory cortex (AP = -4.0 mm, L = 6.5 mm from bregma, V = 2.0 mm from the surface of the skull).
using standard stereotaxic procedures. The guide cannula was in place with three skull screws and dental acrylic. Stainless steel dummy blockers were inserted into the guide cannulas and fixed until the insertion of the microdialysis probe (CMA, dialysis length, 2 mm; diameter, 0.24 mm). Throughout the surgery, the body temperature of the animals was maintained at 37 °C with a heating pad. Immediately after the surgery, the rats were placed into a warm incubator individually until they recovered from the anesthesia. The rats were allowed to recover for at least 24 h before in vivo microdialysis sampling. Prior to in vivo fluorescent measurements, the microdialysis probe was implanted in the auditory cortex and was allowed to equilibrate for at least 90 min by continuously perfusing aCSF at 1 µL min⁻¹.

In vitro fluorescent spectrum was recorded on a Hitachi F-4600 spectrometer (Hitachi Co. Ltd. Japan) with a Xe lamp as the excitation source at room temperature. In vivo continuous-flow fluorescence detection was performed in a RF-20A xs fluorescence detector cell (Shimadzu Co. Ltd., Japan) with Xe lamp as the excitation source at room temperature. The excitation wavelength was set as 469 nm, and the emission wavelength was set as 550 nm. To detect Zn²⁺ in the microdialysates in vivo sampled from the auditory cortex, we first mixed 30 µL of auditory cortex microdialysates with 30 µL of NBD-TPEA (10 µM) dissolved in DMSO/aCSF (v/v, 1:99, pH 7.4). After 5 min, the mixtures were perfused into a fluorescence cell with microinjection pump (CMA 100, CMA Microdialysis AB, Stockholm, Sweden) at a perfusion of rate of 3 µL min⁻¹ for continuous-flow fluorescent detection. The basal level of Zn²⁺ in the microdialysates was obtained from the fluorescence response of the mixtures containing microdialysates and NBD-TPEA probe (normal group). To study the change of Zn²⁺ level in the microdialysates induced by salicylate injection, 100 mg/mL of sodium salicylate dissolved in phosphate buffer (0.1 M, pH 7.4) was injected into the rats (350 mg/kg, i.p.). Such a dose was reported to produce an animal model of tinnitus. The microdialysates were collected, mixed and detected with the same procedures employed for
the microdialysates from the normal group (salicylate group). To elucidate the salicylate-induced change of Zn$^{2+}$ level, in the separate experiments, we injected 1 mg/mL MK-801 dissolved in phosphate buffer (0.1 M, pH 7.4) into the animals (5 mg/kg, i.p.) after the animals were injected with 100 mg/mL salicylate for 5 min, and the microdialysates were thus collected, mixed, and detected with the same method employed for the normal and salicylate groups (Sal/MK-801 group). During the surgery and in vivo microdialysis sampling, the body temperature of the animals was maintained at 37°C with a heating pad and anesthetic was supplemented if necessary.

ICP-MS analysis

Inductively coupled plasma mass spectrometry (ICP-MS) was carried out on an Agilent 7700x series ICP-MS instrument. For ICP-MS analysis, 20 µL of the microdialysate was diluted to 2 mL with Milli-Q water. Agilent ICP-MS calibration standards (multi-element) was used for preparing the calibration curves, of which Zn$^{2+}$ concentrations were 0, 0.02, 0.1, 0.5, 1, 5, 10, 50 and 100 ppb (µg/mL), and Cd$^{2+}$ concentration were 0, 0.001, 0.01, 0.1, 1 and 10 ppb (µg/mL). The isotope detected was $^{66}$Zn and $^{111}$Cd, and both readings were made in He gas mode.

Results and discussion

Fluorescent response and selectivity

In DMSO/aCSF (v/v, 1:99, pH 7.4) mixture, NBD-TPEA itself shows a weak fluorescence with $\lambda_{ex}$ and $\lambda_{em}$ at 469 nm and 550 nm, respectively, as shown in Fig. 1 A. The weak emission could be attributed to the presence of two amine groups at TPEA (Fig. 1 B, inset, red dash circle) as reported previously. TPEA connects to the 4-amino-7-nitro-2,1,3-benzoxadiazole (ANBD) fluorophore via an ethylene group and thus
quenches the emission of ANBD fluorophore via a space photoinduced electron transfer (PET) process. However, when Zn$^{2+}$ was added into the mixture of DMSO/aCSF containing NBD-TPEA, the fluorescent emission intensity of NBD-TPEA was largely enhanced, as displayed in Fig. 1 B (red curve). Such an enhancement was mainly caused by the PET block effect induced by the coordination of Zn$^{2+}$ to all N atoms of TPEA, which leads to the recovery of the fluorescence of ANBD fluorophore. In addition to its application for Zn$^{2+}$ imaging in living cells with excellent biocompatibility and cell permeability, this mechanism is envisaged to be particularly useful for in vivo sensing of Zn$^{2+}$ in the cerebral system, as described below.

To explore such possibility, we systematically studied the selectivity of the method for Zn$^{2+}$ sensing over other kinds of metal ions, amino acids, and physiologically important species in the cerebral system. To do this, each kind of the species was separately added into 10 µM of NBD-TPEA in the DMSO/aCSF mixture. As displayed in Fig. 2, no obvious fluorescence enhancement was observed upon the addition of each kind of metal ions, (with an exception of the addition of Zn$^{2+}$ and Cd$^{2+}$) (A), amino acids (B), and physiologically important species (C). We also studied the effect of metal ions, amino acids and physiologically important species on the Zn$^{2+}$-enhanced fluorescent intensity of NBD-TPEA and found that negligible signal changes were observed when each kind of metal ions (Fig. S1), amino acids (Fig. S2), and physiologically important species (Fig. S3) was added into the mixture of 10 µM of NBD-TPEA and 10 µM of Zn$^{2+}$ in DMSO/aCSF. However, the addition of Cd$^{2+}$ into the same mixture clearly decreases the fluorescent intensity (data not shown), which may be caused by the competitive coordination of Cd$^{2+}$ and Zn$^{2+}$ with NBD-TPEA. Note that, the interference of Cd$^{2+}$ was not considered here because of its low level in the brain microdialysate (i.e., at a nanomolar level), as determined with the traditional ICP-MS in our study (data not shown). Furthermore, no significant change of the
fluorescence response of 10 µM NBD-TPEA in DMSO/aCSF (v/v, 1:99, pH 7.4) at 550 nm was observed upon consecutive excitation with 469 nm laser light for 60 min with an interval of 1 min (data not shown), suggesting the good photostability of NBD-TPEA probe employed in this study. The high selectivity and excellent photostability of the NBD-TPEA probe form a strong basis for in vivo sensing of Zn\textsuperscript{2+} in the cerebral system.

**Fluorescent sensing of Zn\textsuperscript{2+}**

In order to validate the selective fluorescent mechanism demonstrated above for in vivo sensing of Zn\textsuperscript{2+} in rat brain, we premixed the brain microdialysates with the probe and perfused the resulting mixtures into a fluorescent cuvette with a volume as small as 30 µL for continuous-flow fluorescent sensing (Scheme 1). With the method, we obtained a time resolution of 30 min (30 µL, with 1 µL/min as a perfusion rate for in vivo microdialysis). Such a time resolution, although remained to be further improved in our future study, could meet the requirement for understanding the chemical process involved in the salicylate-induced physiological and pathological events such as tinnitus since previous attempts have demonstrated that the development of salicylate-induced tinnitus was a slow process.\textsuperscript{4a,b} Moreover, the method demonstrated here also facilitates the fluorescent signal readout recording with less fluorescence bleaching because of the easily switchable property and continuous-flow feature of the samples in the micro-volume fluorescent cuvette. As displayed in Fig. 3, the method shows a well-defined response toward Zn\textsuperscript{2+} and the relative fluorescence intensity \((F-F_0)/F_0\) was linear with the concentration of Zn\textsuperscript{2+} with a dynamic linear range within the concentration range from 0.5 µM to 4 µM \(((F-F_0)/F_0= 0.8471 \ C/µM +0.1397, R^2 = 0.9844)\). Where, \(F_0\) represents the fluorescence intensity of blank NBD-TPEA solution, and \(F\) represents the fluorescent intensity of the mixtures of NBD-TPEA probe and
Zn standards (or the microdialysates for in vivo analysis). The detection limit was calculated to be 156 nM ($S/N = 3$). Remarkably, the use of the relative fluorescence intensity (i.e., $(F-F_0)/F_0$) as the signal readout for the calibration eliminates background contamination that may be inherent in in vivo sensing of Zn$^{2+}$ (possibly all kinds of trace amount of metal ions) in rat brain since the contamination may come from labwares, reagents, and environmental contributions within the whole operation procedure.$^{14a}$ To achieve the $(F-F_0)/F_0$ value, in conventional fluorescent method, both $F$ and $F_0$ were read out at the maximum fluorescent emission wavelength, which need to be accurately defined under a provided maximum excitation wavelength. However, in the method demonstrated here, the easily switchable and continuous-flow character of the solutions in the cuvette further facilitates the fluorescent signal readout since both the $F$ and $F_0$ values reach their steady-state values in the continuous-flow cell and could be easily defined and read out. Moreover, the continuous-flow character of the solutions containing the fluorescence probe in the cuvette could minimize the fluorescent bleaching as compared with in the quiescent solution.

**Application for selective sensing of salicylate-induced Zn$^{2+}$ in rat brain**

To demonstrate the application of the our method for selectively sensing Zn$^{2+}$ in rat brain, we collected the brain microdialysates, pre-mixed the microdialysates with NBD-TPEA in DMSO/aCSF (v/v, 1:99, pH 7.4) and perfused the resulting mixture into a fluorescent cuvette for continuous-flow fluorescent detection (Scheme 1). As shown in Fig. S4 A and Fig. 4 A (black curve), the pre-mixing of 30 µL of the microdialysate from auditory cortex of normal group leads to an obvious increase in the fluorescent intensity, suggesting the presence of Zn$^{2+}$ in the auditory cortex microdialysate. The fluorescent intensity remains almost unchanged as function of the time from 0 min to 150 min for in vivo microdialysis in the auditory cortex, as displayed in Fig. 4 A (black curve), suggesting that the basal level of
auditory cortex Zn$^{2+}$ does not change in the normal group in the time scale employed in this study. The basal level of Zn$^{2+}$ in the auditory cortex microdialysate was determined to be $0.52 \pm 0.082 \ \mu$M ($n = 4$). To ensure the result obtained with the fluorescent method, we further determined the basal level of Zn$^{2+}$ with the traditional ICP-MS, in which the signal obtained for aCSF (i.e., without addition of microdialysates) was subtracted to minimize the background contamination. We found that the result obtained with our method was almost consistent with the value determined with traditional ICP-MS method (i.e., $0.64 \pm 0.10 \ \mu$M, $n = 4$). We shall note that, the basal level of Zn$^{2+}$ in the brain microdialysates reported so far remain different, ranging from 19 nM to several hundred nanomolar or even higher.$^{14}$ The difference might be due to the difference in animal models, brain regions, and experimental conditions (for example, the probes and perfusion rates used for in vivo microdialysis) employed for in vivo sensing of Zn$^{2+}$, as reported previously.$^{14}$

To further study the change of Zn$^{2+}$ level induced by salicylate, 100 mg/mL of sodium salicylate in phosphate buffer (0.1 M, pH 7.4) was intraperitoneally injected into the animals (350 mg/kg, i.p.). This dose of sodium salicylate has been reported to be able to induce tinnitus.$^{2a,3a}$ The microdialysates were analyzed with the method with the same procedures employed for those sampled from the normal animals. As illustrated in Fig. S4 B and Fig. 4 A (blue curve), the injection of salicylate into the animals leads to an increase in the level of extracellular Zn$^{2+}$ in auditory cortex microdialysate from the point of 60 min. The level of Zn$^{2+}$ reaches its maximum (i.e., $1.20 \pm 0.24 \ \mu$M, $n = 4$) at the time point of 90 min and then levels off with time after salicylate injection. Previous attempts have demonstrated that the development of salicylate-induced tinnitus was a slow process,$^{4a,b}$ and the results obtained here suggest that Zn$^{2+}$ in auditory cortex might be involved in the development of salicylate-induced tinnitus.

To further confirm the involvement of auditory cortex Zn$^{2+}$ in the development of
salicylate-induced tinnitus, we injected 1 mg/mL of MK-801, a potent and specific NMDA receptor antagonist that blocks both the excitatory and toxic actions of NMDA,\textsuperscript{15} into the animals (5 mg/kg, i.p., in 0.1 M phosphate buffer, pH 7.4) after the injection of 100 mg/mL of salicylate in phosphate buffer (0.1 M pH 7.4) for 5 min (Sal/MK-801 group) and the concentration of Zn\textsuperscript{2+} in the microdialysate was detected with the SPM-CFFD method with the same procedures for the salicylate group. As illustrated in Fig. S4 C and Fig. 4 A (red curve), the concentration of Zn\textsuperscript{2+} in the microdialysates of the MK-801 group first increases from 0 min to 90 min, and then decreases after 90 min. This change was rather different from the results obtained for the salicylate group, suggesting that the salicylate-induced increase in the extracellular Zn\textsuperscript{2+} level was suppressed after the injection of MK-801.

For better comparison, we summarized the auditory cortex Zn\textsuperscript{2+} levels of normal, salicylate, and Sal/MK-801 groups recorded with our SPM-CFFD method. As illustrated in Fig. 4 B, the auditory cortex Zn\textsuperscript{2+} level does not significantly increase both in salicylate (blue bars) and Sal/MK-801 (black bars) group at the time point of 30 min, as compared with basal levels of normal group (white bars). However, the auditory cortex Zn\textsuperscript{2+} levels in both salicylate and Sal/MK-801 groups increase obviously from 60 min to 150 min, as compared with basal levels of normal group. Two-way ANOVA followed by a post hoc Tukey test indicated significant differences at each time points (from 60 min to 150 min) between salicylate group and normal group, and between Sal/MK-801 group and normal group ($F = 30.46, P < 0.01; P = 0.01, F_{(5, 18)} = 2.77$). Note that, at the time point of 90 min, although the auditory cortex Zn\textsuperscript{2+} level of Sal/MK-801 group was higher than that of salicylate group, there is no significant difference between salicylate group and Sal/MK-801 group. At the time point of 120 min, there were significant differences in the auditory cortex Zn\textsuperscript{2+} levels among normal group, salicylate group and Sal/MK-801 group. These results demonstrated that the attenuation of the salicylate-induced increase of auditory cortex Zn\textsuperscript{2+} level is mainly
caused by the intraperitoneal injection of MK-801. It has been reported that, MK-801, as an NMDA receptor antagonist, is competitive to combine with NMDA rather than common neurotransmitters such as glutamate.\textsuperscript{15} We speculated that the injection of MK-801 may effectively reduce the NMDA receptor excitotoxicity and eventually inhibit the oxidative damage of auditory cortex and, as a result, attenuate the salicylate-induced increase of the extracellular Zn\textsuperscript{2+} level in the auditory cortex. These results demonstrate that the SPM-CFFD method developed here could be used for in vivo sensing of Zn\textsuperscript{2+} in the cerebral system and will find interesting applications in the investigations on brain functions.

**Conclusion**

By using NBD-TPEA as the selective fluorescence probe and in vivo microdialysis as the sampling technique, we have successfully developed a fluorescent method for in vivo sensing of extracellular Zn\textsuperscript{2+} level in auditory cortex and its change induced by salicylate. To accomplish in vivo fluorescence sensing with simple signal readout recording, minimal fluorescent bleaching, less background contamination and acceptable time resolution, the microdialysates continuously sampled from auditory cortex were pre-mixed with the NBD-TPEA probe, and the mixtures were perfused into a fluorescent cuvette for continuous-flow fluorescence detection to form a new method. The method is highly selective and sensitive for sensing of dynamic change in the Zn\textsuperscript{2+} level in the brain microdialysates and will find interesting applications in the investigations on brain functions.

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References


**Scheme 1.** Schematic illustration of sample pre-mixing and continuous-flow fluorescent detection for in vivo sensing of Zn$^{2+}$.
Fig. 1 (A) Excitation (black curve) and emission (red curve) spectra of 10 µM of NBD-TPEA in DMSO/aCSF (v/v, 1:99, pH 7.4). (B) Fluorescent emission spectra of 10 µM of NBD-TPEA in the absence (black curve) and presence (red curve) of 10 µM of Zn²⁺ in DMSO/aCSF (v/v, 1:99, pH 7.4). Inset, the proposed binding mode of Zn²⁺ with NBD-TPEA.
Fig. 2 (A) Fluorescence emission spectra of 10 μM of NBD-TPEA in the absence (black curves) and presence of each kind of metal ions (A), amino acids (B), and physiologically important species (C) in DMSO/aCSF (v/v, 1:99, pH 7.4). $\lambda_{ex} = 469$ nm. The concentrations for metal ions were 100 μM for $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, and 10 μM for other metal ions. The concentrations of each kind of amino acids were 100 μM. The concentrations for physiologically important species were 5-HT (10 μM), AA (200 μM), DA (10 μM), DOPAC (10 μM), glucose (10 mM), lactate (1 mM) or UA (80 μM). Black curve overlaps with the curves with the colors rather than blue and red. $\lambda_{ex} = 469$ nm.
Fig. 3

Fig. 3 Typical fluorescence-time response obtained with the method for the Zn$^{2+}$ standards. A 30 µL of different concentrations of Zn$^{2+}$ in aCSF (pH 7.4) was pre-mixed into 30 µL of NBD-TPEA (10 µM) in DMSO/aCSF (v/v, 1:99, pH 7.4) for 5 min, and the resulting mixtures were perfused into a fluorescent cell for continuous-flow fluorescent detection. Flow rate, 3 µL min$^{-1}$. The final concentrations of Zn$^{2+}$ were indicated in the figure.
Fig. 4 (A) Dynamic changes of auditory cortex Zn$^{2+}$ in the normal (black curve), salicylate (blue curve) and Sal/MK-801 (red curve) groups ($n = 4$). All brain microdialysates (with a perfusion rate of 1 µL min$^{-1}$) were pre-mixed with 30 µL of NBD-TPEA (10 µM) in DMSO/aCSF (v/v, 1:99, pH 7.4) for 5 min, and the resulting mixtures were perfused into a fluorescent cell for continuous-flow fluorescent detection. Perfusion rate for the mixtures was 3 µL min$^{-1}$. Other conditions were the same as those in Fig. 3. (B) Comparison of the dynamic changes of auditory cortex Zn$^{2+}$ levels of normal group (white bars), salicylate group (blue bars), and Sal/MK-801 group (black bars). Date presented as mean ± SD. The asterisks (*$P < 0.01$,) indicate significant differences among auditory cortex Zn$^{2+}$ levels of normal group, salicylate group, and Sal/MK-801 group.
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