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Roles Played by the Na$^+$/Ca^{2+} Exchanger and Hypothermia in the Prevention of Ischemia-Induced Carrier-Mediated Efflux of Catecholamines into the Extracellular Space: Implications for Stroke Therapy

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Abstract

The release of [^{3}H]dopamine ([^{3}H]DA) and [^{3}H]noradrenaline ([^{3}H]NA) in acutely perfused rat striatal and cortical slice preparations was measured at 37 °C and 17 °C under ischemic conditions. The ischemia was simulated by the removal of oxygen and glucose from the Krebs solution. At 37 °C, resting release rates in response to ischemia were increased; in contrast, at 17 °C, resting release rates were significantly reduced, or resting release was completely prevented. The removal of extracellular Ca^{2+} further increased the release rates of [^{3}H]DA and [^{3}H]NA induced by ischemic conditions. This finding indicated that the Na$^+$/Ca^{2+} exchanger (NCX), working in reverse in the absence of extracellular Ca^{2+}, fails to trigger the influx of Ca^{2+} in exchange for Na$^+$ and fails to counteract ischemia by further increasing the intracellular Na$^+$ concentration ([Na$^+$]$_i$). KB-R7943, an inhibitor of NCX, significantly reduced the cytoplasmic resting release rate of catecholamines under ischemic conditions and under conditions where Ca^{2+} was removed. Hypothermia inhibited the excessive release of [^{3}H]DA in response to ischemia, even in the absence of Ca^{2+}. These findings further indicate that the NCX plays an important role in maintaining a high [Na$^+$]$_i$, a condition that may lead to the reversal of monoamine transporter functions; this effect consequently leads to the excessive cytoplasmic tonic release of monoamines and the reversal of the NCX. Using HPLC combined with scintillation spectrometry, hypothermia, which enhances the stimulation-evoked release of DA, was found to inhibit the efflux of toxic DA metabolites, such as 3,4-dihydroxyphenylacetaldehyde (DOPAL). In slices prepared from human cortical brain tissue removed during elective neurosurgery, the uptake and release values for [^{3}H]NA did not differ from those measured at 37 °C in slices that were previously maintained under hypoxic conditions at 8 °C for 20 h. This result indicates that hypothermia preserves the functions of the transport and release mechanisms, even under hypoxic conditions. Oxidative stress (H$_2$O$_2$), a mediator of ischemic brain injury enhanced the striatal resting release of [^{3}H]DA and its toxic metabolites (DOPAL, quinone). The study supports our earlier findings that during ischemia transmitters are released from the cytoplasm. In addition, the major findings of this study that hypothermia of brain slice preparations prevents the extracellular calcium concentration ([Ca^{2+}]$_o$)-independent non-vesicular transmitter release induced by ischemic insults, inhibiting Na$^+$/Cl$^-$-dependent membrane transport of monoamines and their toxic metabolites into the extracellular space, where they can exert toxic effects.

Keywords Ischemia · Dopamine release · Dopamine · Non-vesicular · DOPAL · Toxic · Na$^+$/Ca^{2+} exchanger · Monoamine transporter · Extracellular space · Hypothermia

Introduction

Cerebral ischemia is a pathological condition, during which blood flow to neurons is insufficient or completely blocked. Cerebral ischemia can be focal, such as when a brain artery is occluded, or global, such as after cardiac arrest. Ischemic stroke is the leading cause of brain dysfunction. However,

because the mechanism of stroke pathogenesis has not been clarified to date, no effective medical treatments for reversing sensory, motor and cognitive impairments due to stroke have been devised [1, 2]. Stroke is the second most prevalent cause of death and is the most common cause of permanent disability in adults [3].

The brain is a wired instrument, and its neurons process cabled information through synapses; however, neurons are also able to communicate with each other nonsynaptically, without synaptic contact [4–13]. Evidence has shown that nonsynaptic receptors and transporters are characterized by high affinity [5, 6] and have many implications for psychiatric issues, such as depression, changes in mood, changes in appetite, and psychiatric disorders. Therefore, it has been suggested that many drugs used to treat psychiatric diseases may exert their effects through diffusion into extracellular spaces, where they may mimic or influence the effects of endogenous ligands [5, 6]. Nonsynaptic chemical communication between neurons and between neurons and their target cells, via both pre- and postsynaptic sites, were also identified during the excellent studies by Agnati and Fuxe [14, 15], who used the term 'volume transmission' to denote non-synaptic transmission.

Extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$)-dependent release represents vesicular exocytotic release that is evoked by axonal action potentials, whereas the $[Ca^{2+}]_o$-independent release that can be measured at rest has been identified as being a non-exocytotic release from the cytoplasm, caused by the reversal of transporter activity [16–18]. Ischemic conditions (oxygen and glucose deficiencies) have previously been shown to result in the excessive production of reactive oxygen species (ROS) [19–22], oxidative stress (primarily during the reperfusion period) and the release of transmitters from the cytoplasm in a $[Ca^{2+}]_o$-independent manner [23–26]. Oxidative stress has been implicated as being the mechanism through which ischemia results in brain injury [21, 22]. This type of transmitter release from nonsynaptic varicosities [5, 12] into the extracellular space [21, 22] occurs even when the classical Ca^{2+}-dependent vesicular transmission is inhibited or impaired. Under this condition membrane transporters, when acting in reverse, are able to release signaling molecules from the cytoplasm, causing an increase in the extracellular concentrations of transmitters. This paper addresses this type of release and the possible methods for preventing it.

When a train of action potentials (APs) reaches a nerve terminal, any residual $[Ca^{2+}]$ above 500 nM begins to be extruded by the NCX, which has a low affinity ($K_d = 500$ nM) and a high capacity (5×10^3 Ca^{2+}/s) for Ca^{2+} [27]. However, when intracellular Na^+ levels increase excessively or strong membrane depolarization occurs, the NCX reverses and exports three Na^+ ions for each imported Ca^{2+} ion (the "reverse" mode of NCX activity [27, 28]). The expression patterns of 2 NCX isomers (NCX2 and NCX3) are primarily restricted to the brain, where they are presynaptically localized to synaptic terminals [29–31] and control the cytoplasmic Ca^{2+} levels in the nerve terminal, which increase from 0.1 (during resting conditions) to 20 μM [32, 33] (during an action potential).

Various studies have demonstrated that an NCX acting in reverse promotes the additional entry of Ca^{2+} [34] and apoptotic neuronal death during stroke and brain trauma. In response to neurotoxic glutamate release during ischemic insults [26, 35], when amino acid transporters (EAATs) are also inhibited, the excessive influx of Na^+ and Ca^{2+} [36] results in the reverse activation of the NCX and excitotoxicity. These findings indicate that the NCX may be involved in neurotoxicity.

In addition, there is convincing evidence that cytosolic dopamine (DA) is highly toxic following deamination by MAO, which converts DA to 3,4-dihydroxyphenylacetaldehyde (DOPAL) [37] and hydrogen peroxide (H_2O_2) [20], both of which have been shown to be neurotoxic [38, 39]. Cytosolic DA can also be auto-oxidized to form ROS (hydroxyl radicals, superoxide and H_2O_2) [20, 40]. Therefore, in this study, we measured the levels of DA and noradrenaline (NA) uptake and release from striatal and cerebral cortical slices, both at rest and in response to axonal activity, under normal and ischemic conditions and in the presence and absence of Ca^{2+} ions in the perfusion solution. Using high-performance liquid chromatography (HPLC) combined with scintillation spectrometry, we also studied whether the toxic metabolites of DA were released. The role played by the plasmalemmal exchanger, which is crucial for the clearance of cytoplasmic Ca^{2+}, during neurotoxicity and targeted treatments is controversial [28, 41]; therefore, we attempted to influence the excessive release of transmitters (DA and NA) by using NCX inhibitors under ischemic conditions, during which $[Na^+]_i$ is high, and the NCX is expected to operate in reverse. We also studied the effects of Ca^{2+} removal on the cytoplasmic release of transmitters in response to ischemia when extraneuronal Ca^{2+} would normally be exchanged for Na^+ and predicted to decrease $[Na^+]_i$.

Recently, 2-[2-[4(-nitrobenzyl-oxy)phenyl]ethyl]isothiourea (KB-R7943) was reported to potently inhibit both forms of NCX without affecting the Na^+-dependent transport systems [42]. Therefore, we studied the effects of KB-R7943 on the release of monoamines in response to ischemia.

Lowering the temperature depresses the metabolic rates [43] of neurons and prevents the resting release of transmitters induced by transporters operating in reverse [44–46]. Furthermore, in clinical practice, therapeutic hypothermia has been used with moderate levels of success, not only for stroke and myocardial infarction but also for neonatal encephalopathy [47, 48]. Mild to moderate hypothermia has

been shown [49–51] to have neuroprotective effects against ischemic and hypoxic insults [52, 53] in adults, in newborns [51, 54–56] and in animal experiments [57–59]. Therefore, we also studied the effects of hypothermia, induced by cooling slice preparations, on transmitter (DA and NA) release evoked by ischemia from rat striatal and cortical slices.

For the first time, this paper provides neurochemical evidence regarding the mechanism through which hypothermia inhibits the resting release of catecholamines from the cytoplasm, which may prevent the toxic effects of monoamine metabolites (aldehydes) in the extracellular space. The finding that hypothermia preserves the machinery necessary for transmitter function, even under hypoxic conditions, in human cortical slices provides further evidence of its neuroprotective effects.

Materials and Methods

Preparation of Rat Striatal and Cortical Slices

All experiments using Wistar rats (37–48 days old) were conducted with the permission of the local Animal Care Committee. The rats were lightly anaesthetized with diethyl ether and decapitated. The brain was removed and immediately placed in ice-cold Krebs solution that was continuously mixed with 95% O_2 and 5% CO_2. The Krebs solution used in this study contained the following in mmol/l: NaCl, 113; KCl, 4.7; $CaCl_2$ 2.5; $KHPO_4$, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5; ascorbic acid, 0.3; and Na_2EDTA, 0.03. The striatum or frontal cortex was dissected and cut transversely into 400-µm slices using a McIlwain tissue slicer. The slice preparations used in this study were previously described [60]. In our experiments, we did not compensate for the changes in osmolarity produced by the removal of glucose because we did not observe any differences in the release rates when glucose was not replaced [61].

In a series of experiments, olfactory bulb slice preparations were used, as described previously [62]. The olfactory bulb preparation is advantageous because the release of dopamine is dominant in this preparation.

Release of [3H]dopamine/[3H]noradrenaline

Briefly, after the striatal and frontal cortical slices were placed in Krebs solution, the tissue slices were incubated in 1 ml Krebs solution in the presence of either 5 µCi/ml [3H]DA or 5 µCi/ml [3H]NA at 37 °C for 45 min. After the loading period, the tissue was washed five times with Krebs solution, transferred to a thermoregulated superfusion apparatus [63], and superfused at 37 °C with a Krebs solution saturated with 95% O_2 + 5% CO_2. After a 20 min pre-perfusion treatment (flow rate: 0.5 ml/min), the effluent

samples were collected every three minutes. During the collection of the 3rd (9 min) and the 13th (39 min) samples, the tissue was stimulated (S_1 and S_2, respectively) with square-wave impulses, using a Grass S88 stimulator (Astro-Med, West Warwick, RI, USA). The striatal slices were stimulated at 30 V, 2 Hz, 2 ms for 2 min (240 shocks), and the cortical slices were stimulated at 20 V, 2 Hz, 2 ms for 3 min (360 shocks). These stimulation parameters proved to be supramaximal. Unless otherwise stated, the experimental conditions were not changed after the control collection periods, and the release of radioactivity was measured and expressed in disintegrations per gram of tissue (Bq/g). At the end of the experiment, the tritium contents of the tissues were determined, as previously described [64]. Using a computer programme, the fractional release (FR) values for [3H]DA (FRS_1) and [3H]NA (FRS_2) in response to stimulation were calculated as percentages of the total radioactivity present in the tissue at the beginning of stimulation; the basal resting release was determined during the collection periods before (FRR_1) and during ischemia (FRR_2). The fractional release (FR) values during the R_1 resting (FRR_1) or S_1 stimulation (FRS_1) periods served as internal controls for the calculations of the FRR_2/FRR_1 and FRS_2/FRS_1 ratios. Samples used to calculate R_1 and R_2 are indicated in legends. The extent of the cytoplasmic DA release during ischemia varied in experiments carried out at different time points, but always remained very high. Therefore, we tried to carry out experiments that addressed a problem within several weeks.

To prevent the metabolism of catecholamines by MAO during release experiments, the Krebs solution contained 10 µM pargyline. A thermoelectric device (Frigomix, B. Braun, Germany) was used to rapidly change the temperature of the bath solution. Ischemia was simulated by placing tissue slices in Krebs solution lacking glucose and mixing them with 95% N and 2.5% CO_2 [65]; ischemic conditions were simulated starting with the collection of the 7th fraction and maintained until the end of the experiments. When Ca^{2+}-free Krebs solution was used, $CaCl_2$ was omitted, and 1 mM EGTA was added to the Krebs solution.

The released [3H]NA and [3H]DA levels were measured using a Packard-Canberra TR 1900 liquid scintillation counter. The advantage of our acute slice preparations being maintained in a microperfusion system (100 µl) is that the slices are very thin (400 µm), and exchanging the perfusion fluid within the extracellular space is easily performed; furthermore, drugs may easily exert their effects, and oxygen and glucose removal is more efficient.

Chromatographic Analysis of Dopamine and Its Metabolites

The method described by Baranyi et al. [64] was used to study the metabolism of DA in slices loaded with 5 µC [3H]

DA. Under this condition, the Krebs solution did not contain pargyline. The slices were superfused, and the collected 3 min samples were analysed by high-performance liquid chromatography (HPLC) combined with liquid scintillation spectrometry.

Human Frontal Cortical Slice Preparation

Human frontal or temporalis cortical samples (n = 8) were obtained from bulk brain tissue that was removed during elective neurosurgery conducted on 8 patients. The tissues were randomly selected from brain surgeries that were performed at the National Institute of Clinical Neuroscience (Budapest, Hungary) and were used for histology. This study was not limited to any type of surgical procedure or day of the week, and no tissue was removed for the express purpose of this study. The ages of the patients varied from 31 to 68 years (mean 48.2 ± 10.8 years old). Daily medications were continued until surgical intervention. Standard premedication and induced anaesthesia procedures (patients were ventilated with a mixture of 40% oxygen plus 60% nitrous oxide) were used. Total intravenous anaesthesia, 1% propofol at a rate of 20 ml/h, was administered by perfusion. Fentanyl, at 0.05 mg for 20–30 min, and 2 mg cisatracurium every 1.5 h were also used. The patients underwent multimodal monitoring. The specimens were used for in vitro experiments within 2 h of collection. The operating centre was 45 min away from the laboratory. The slices (400 μm) were prepared from human brain samples (13.61 ± 1.26 mg, n = 16) after surgical interventions using a McIlwain tissue slicer. The slices were stimulated at a supramaximal voltage (2 Hz, 2 ms for 2 min (240 shocks)), as described for rat brain slices. In several cases, the slices were maintained at 8 °C for 20 h under hypoxic conditions to determine whether the catecholamine uptake and release machinery remained intact.

This study was conducted in strict accordance with institutional guidelines, taking the European Community Council Directive (86/609/EEC) into account. The experimental protocol was approved by the Semmelweis University Regional and the Institutional Committee of Science and Research Ethics (No. 116/2015). Informed consent was provided by those patients whose tissue samples were collected during operations and separated for histology.

Drugs

Levo-[7-^{3}H]-noradrenaline (specific activity = 44.5 Ci/mmol, Perkin Elmer, Boston, MA, USA) and 3,4-[7-^{3}H]-dopamine (specific activity = 60 Ci/mmol) were purchased from ART (St. Louis MO, USA). KB-R7943 (2-[2-[4(-nitrobenzyloxy)phenyl]ethyl]isothiourea) was obtained from Tocris Bioscience (Ellisville, MO). All other chemicals were obtained from Sigma (Budapest, Hungary).

Statistical Analysis

The statistical significance of the results was determined by Repeated Measures Analysis of Variance followed by multiple comparison method of Tukey-test. If the measured variables met the normality assumption, two-way factorial measures (FM ANOVA) analysis was performed (see Tables 1 and 3).

Student's t-test was used where appropriate (internal standards). A value of $p < 0.05$ was considered to be significant. Unless otherwise indicated, the data represent the mean $\pm$ S.E. (SEM).

Results

Nonvesicular Cytoplasmic Release of DA/NA in Response to Ischemia and Oxidative Stress

Plasmalemmal monoamine transporters exhibit a high degree of sequence homology [66]. Accordingly, noradrenergic/dopaminergic and serotonergic axon terminals are able to take up and release both monoamines (NA and DA) and serotonin [67–69], even in humans [70, 71]. Therefore, the release of DA and NA were measured separately in striatal and cortical slices prepared from rats.

After the rat striatal slices were loaded with [^{3}H]DA, the average uptake of radioactivity was $763{,}000 \pm 90{,}383$ Bq/g (n = 6), and the average resting release value during a 3 min collection period was $0.53 \pm 0.07\%$ of total radioactivity. Electrical stimulation resulted in the release of radioactivity ($S_1 = 61{,}553 \pm 9724$ Bq/g or $1.70 \pm 0.25\%$ of total radioactivity), and this stimulated release was repeatable (S_2): $FRS_2/FRS_1 = 0.75 \pm 0.05$ (Fig. 1a). Similar control experiments were performed using [^{3}H]NA in cortical slice preparations, where the FRS_2/FRS_1 ratio was 0.75 ± 0.05 (Fig. 1b).

The removal of oxygen and glucose gradually increased resting DA release levels from striatal and cortical slice preparations (Fig. 2a, b), resulting in 23-fold (Fig. 2a) and three-fold (Fig. 2b) increases in the resting release level after 21 min, respectively. In the striatal slices the DA release was forty times higher after 33 min Fig. 2a). The effects of ischemic conditions on the resting release levels were considerably weaker in cortical slice preparations than in the striatal slice preparations (compare Fig. 2a, b). In separate experiments the released levels of both monoamines (DA and NA) from striatal slices were measured during ischemic conditions. The release rates were significantly higher than in the released levels of these monoamines from cortical slices (Fig. 3a, b). When supramaximal

Table 1 Distribution of [^{3}H] activity released from olfactory bulb slices loaded with [^{3}H]DA at different temperatures (no MAO inhibitors were used)

[^{3}H]	Basal release [%]		Electric stimulation (2 Hz, 240 shocks) [%]	
	37 °C	17 °C	37 °C	17 °C
DA	32.50 ± 2.17 $(32.10 \pm 2.14$ kBq)	26.57 ± 0.49 $(22.01 \pm 4.06$ kBq)	31.67 ± 5.18 $(78.01 \pm 12.75$ kBq)$^{\#}$	60.41 ± 2.78*$^{\#}$ $(138.53 \pm 6.37$ kBq)$^{\#}$
DOPAC	37.50 ± 3.13	$52.46 \pm 3.92^{\#}$	24.47 ± 6.20	27.52 ± 2.47
DOPAL	n.d.	n.d.	5.49 ± 0.31*	0.89 ± 0.10*
DOPET	n.d.	n.d.	16.88 ± 3.42*	1.93 ± 0.11*
3-MT	11.91 ± 2.94	12.24 ± 1.72	5.55 ± 2.10	1.67 ± 0.65
HVA	18.10 ± 1.97	8.72 ± 3.83	11.13 ± 3.29	4.28 ± 0.60
Daq	n. d	n.d	4.80 ± 0.82*	3.30 ± 0.11
[^{3}H] activity [kBq]	98.77 ± 5.64	82.87 ± 3.90	$246.30 \pm 42.06^{\#\#}$	$229.32 \pm 24.72^{\#\#}$

DA dopamine, *MAO* monoamine oxidase, *DOPAL* 3,4-dihydroxyphenylacetaldehyde, *DOPET* 3,4-dihydroxyphenilethanol, *3-MT* 3-methoxy, 4-hydroxyphenethylamine, *DOPAC* 3,4-dihydroxyphenylacetic acid, *HVA* homovanillic acid, *Daq* dopamine quinone, *n.d.* not detectable

The statistical significance of the results was determined by the TIBC statistical program. To assess the normality of all the continuous variables measured, the Kolmogorov–Smirnov test was used and performed for each individual repeated measurement. If the measured variables met the normality assumption, two-way factorial measures (FM ANOVA) analysis was performed. *significant difference ($p < 0.05$) between 37 and 17 °C; $^{\#}p < 0.05$ between stimulation evoked and resting release

Note that, at 17 °C, the amount of [^{3}H]DA (60.41% of total radioactivity $= 138.53 \pm 6.37$ kBq) is significantly higher than the amount at 37 °C ($31.67\% = 78.01 \pm 12.75$ kBq). At 17 °C, the stimulation-evoked release of DOPAL and DOPET was inhibited and the evoked release was enhanced. The release is measured in 3 min collection periods. N$=6$

$^{\#\#}$Significant difference ($p < 0.05$) between the basal and stimulation release values obtained at 17 °C
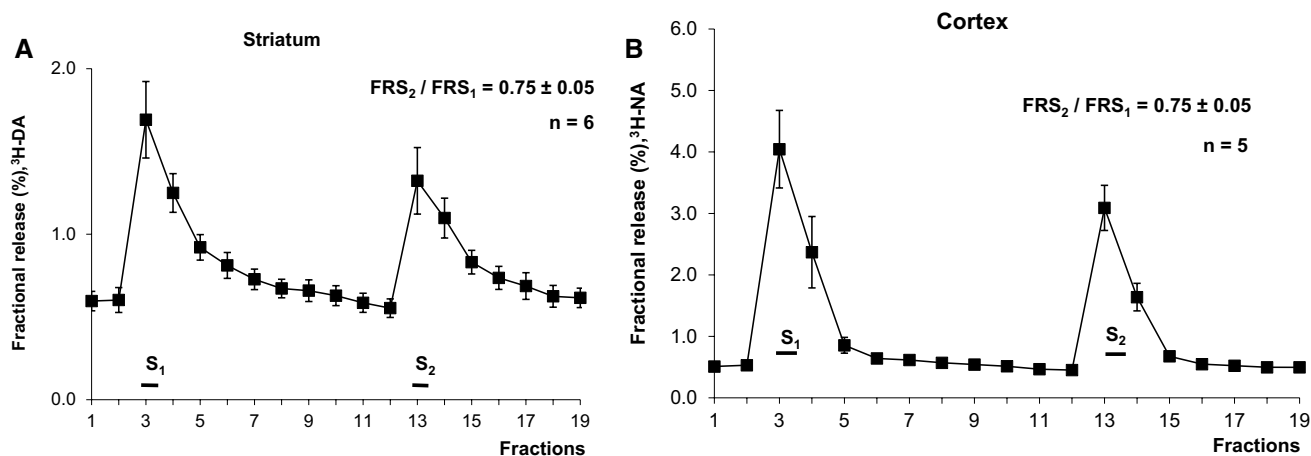

Fig. 1 Release of monoamines (DA and NA). **a** The release of [^{3}H] dopamine (^{3}H-DA) from rat striatal slices in response to stimulation and at rest. The preparations were stimulated during the 3rd (S$_1$) and 13th (S$_2$) fractions (2 Hz, 2 ms, 240 shocks). The release of [^{3}H]DA is expressed as the fractional release (FR). S$_1 = 61{,}553 \pm 9{,}724$ Bq/g ($n=6$). The increase by S$_1$ $p < 0.05$ (t-test for Dependent Samples). **b** The release of [^{3}H]NA from rat cortical slices following stimulation (2 Hz, 2 ms, 360 shocks). S$_1 = 27{,}636 \pm 1{,}554$ Bq/g ($n=5$). The increase by S$_1$ $p < 0.05$ (Wilcoxon Matched Pairs Test). The stimulations are indicated. For further details see "Materials and Methods"

electrical axonal stimulation was applied during the peak resting release level under ischemic conditions, small insignificant additional increases were observed (data not shown). These findings are consistent with those reported by other studies [72, 73], which showed that the disappearance of synaptic activity is the earliest consequence of cerebral ischemia. In both preparations, when Ca^{2+} ions were removed and 1 mM EGTA was added to the Krebs solution, the resting release levels of both [^{3}H]DA (Fig. 4a) and [^{3}H]NA (Fig. 5) increased further in response to the combination of oxygen and glucose deprivation.
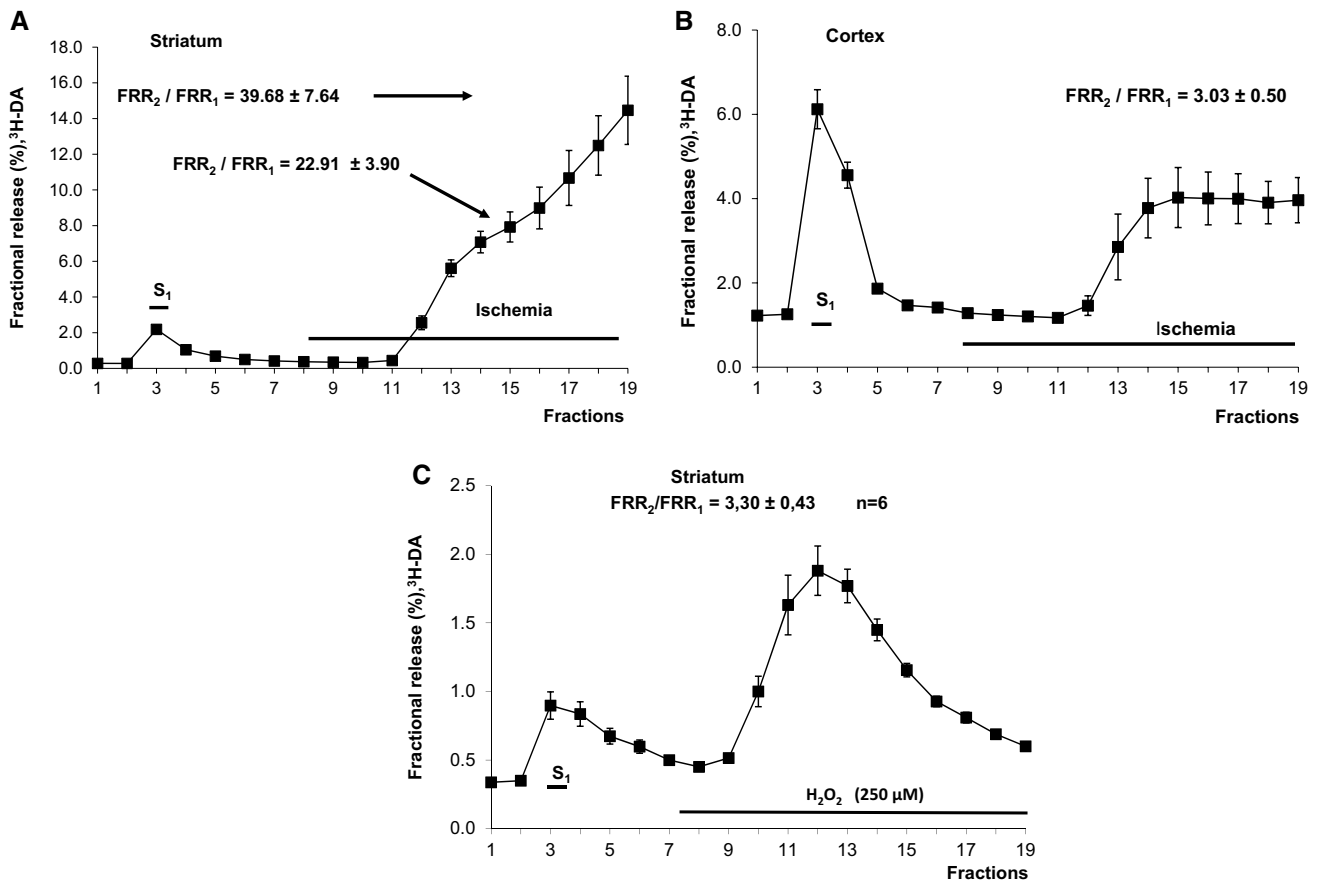
Fig. 2 The effects of oxygen and glucose deprivation on the resting release of [^{3}H]DA from rat striatal (**a**) and cortical (**b**) slices. The removal of oxygen and glucose (ischemia) was introduced, as indicated, during the 8th fraction and continued until the end of the experiment. The striatal (2 Hz, 2 ms, 240 shocks) and cortical (2 Hz, 2 ms, 360 shocks) slices were stimulated as indicated. n = 6–6. The release of radioactivity is expressed as the fractional release (FR). The effect of ischemia on resting release was expressed as the ratio of the FR values measured at the 7th (R_1) and at 15th (R_2)(FRR$_2$/FRR$_1$ = 22.91 ± 3.90) $p < 0.05$ (t-test for Dependent Samples) and at the 7th (R_1) and 19th (R_2) fractions (FRR$_2$/FRR$_1$ = 39.68 ± 7.64) (**a**) (t-test for Dependent Samples). In cortical slices FRR$_2$/FRR$_1$ ratio was calculated measuring FR values at 19th (R_2) and 7th (R_1) collection periods (**b**) (t-test for Dependent Samples). 2 For further information, see the "Materials and Methods" section. **c** The effect of oxidative stress (H$_2$O$_2$, 250 μM) on the resting release of [^{3}H]DA from striatal slice preparations (n = 6). H$_2$O$_2$ was in the Krebs solution as indicated. The administration of oxidative stress started at the 7th fraction and continued until the end of experiments. The release is expressed as fractional release. For experimental conditions, see "Materials and Methods"

It is generally accepted that oxidative stress (through H$_2$O$_2$, a breakdown product of DA metabolism) caused by ischemia is also involved in brain injury [74]. H$_2$O$_2$ is a stable molecule both in the intracellular and extracellular space, which makes this ROS nonsynaptically exert its effect of inhibiting stimulation-induced transmitter release [75]. A previous study showed that endogenous glutamate acting on AMPA receptors generates H$_2$O$_2$ that diffuses and reduces stimulation-evoked release of DA [75]. In our experiments, H$_2$O$_2$ at a 250 μM concentration increased the non-vesicular resting release of [^{3}H]DA (Fig. 2c), producing a threefold increase (FRR$_2$/FRR$_1$ = 3.30 ± 0.43, n = 6). These findings are consistent with our previous observations [19, 20]. Moreover, in the effluent toxic metabolites (DOPAL, DOPET) and quinone were detected in addition to DA and its metabolites (DOPAC, HVA) (Table 3).

Effects of NCX Inhibition

The presence of the reverse NCX inhibitor KB-R7943 [41] at a concentration of 10 μM significantly reduced the effects of ischemia on striatal (Fig. 4a, b) and cortical (Fig. 5) slice preparations, as determined by measuring the resting release levels of [^{3}H]DA and [^{3}H]NA.

When extracellular Ca^{2+} was removed in the presence of KB-R7943, preventing nerve terminals from exchanging
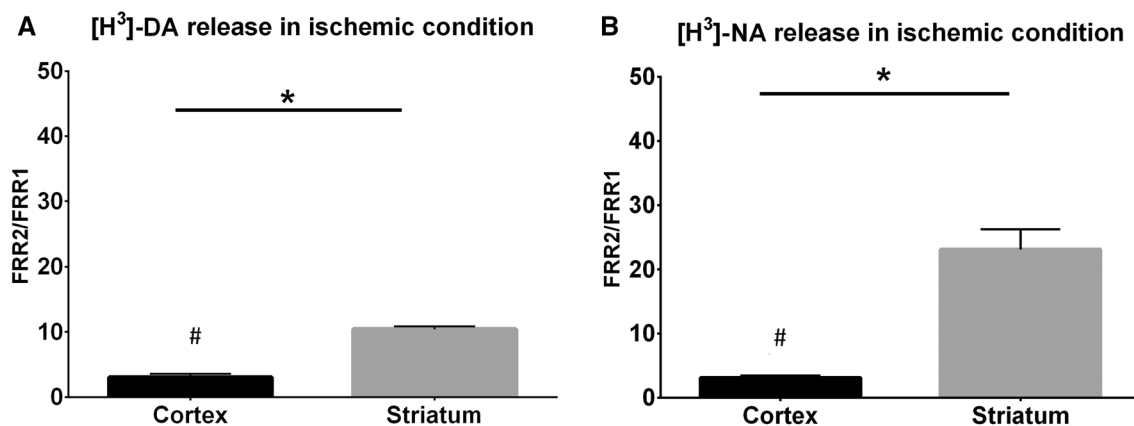
Fig. 3 The resting release of [³H]DA (A) and [³H]NA (B) from rat cortical and striatal slice preparations and the effects of oxygen and glucose deprivation (ischemia) on resting release (n=6–6). Cortical and striatal slice preparations were exposed to ischemia starting with the 8th fraction and rate of release after 18 min during ischemia at 14th fraction was taken into account (R_2). Release in 7th fraction is R_1. For further details, see the "Materials and Methods" section. The effect of ischemia was expressed as FRR_2/FRR_1. FRR_1 was used as an internal control. When either [³H]DA or [³H]NA release was measured, the effects of oxygen and glucose deficiency were more pronounced in striatal slices than in cortical slices. *$p < 0.05$, #$p < 0.05$. Note that these experiments were carried out separately from the experiments shown in Figs. 2, 4 and 5. Repeated measures analysis of variance followed by multiple comparison method of Tukey-test. *$p < 0.05$
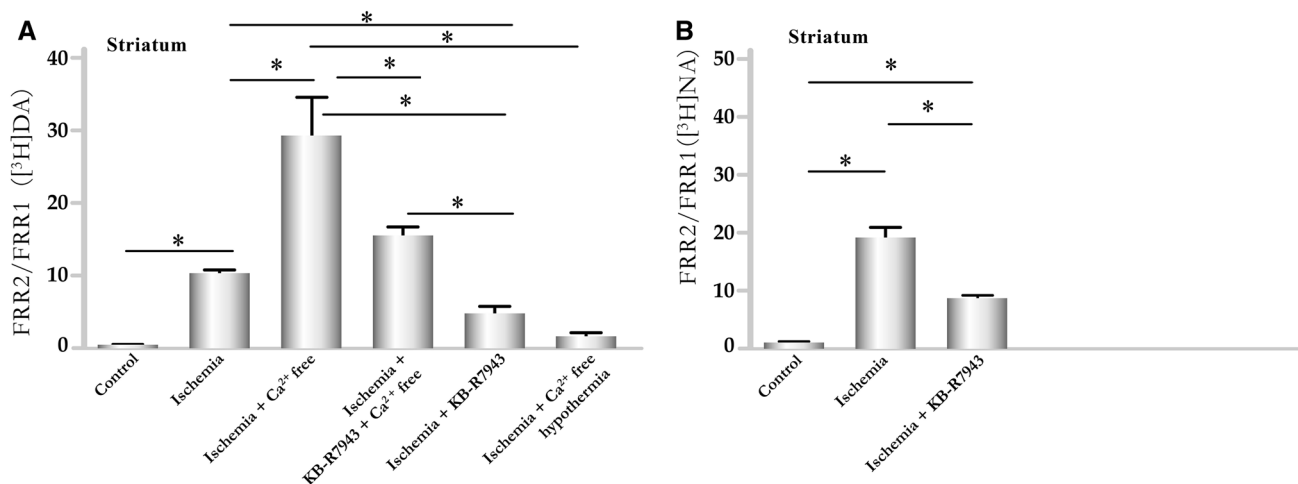
Fig. 4 a The resting release of [³H]DA from rat striatal slices in response to oxygen and glucose deficiency (ischemia) (n=6) and the potentiation of release by Ca^{2+} removal (n=6). In the control experiments, $FRR_2/FRR_1 = 0.91 \pm 0.07$ (n=5). The effect of ischemia on resting release was expressed as the ratio of the FR values between the second FR measurement (FRR_2 noted after 18 min during ischemia) and the first FR measurement (FRR_1 internal control) (FRR_2/FRR_1) of released radioactivity. During hypothermic experiments, the preparations were exposed to a temperature of 17 °C (beginning 30 min before the start of sample collection). KB-R7943 (10 μM) was present in the Krebs solution starting with the 7th collection period and was maintained in the solution throughout the experiments (n=6). Ca^{2+}-free Krebs solution was prepared (Ca^{2+} was removed and 1 mM EGTA was added to the solution starting with the 5th fraction). Note that KB-R7943 significantly reduced the excessive release of [³H]DA in response to both ischemia (starting from the 7th collection period) and fully inhibited the release of [³H]DA. Repeated measures analysis of Variance followed by multiple comparison method of Tukey-test *$p < 0.05$. **b** The effect of ischemia on the resting release of [³H]NA from rat striatal slice preparations. KB-R7943 (10 μM) was added to the Krebs solutions starting with the 7th fraction and was maintained in the solution for the remainder of the experiment. The effect of ischemia on resting release was expressed as the ratio between the FR values of the second measurement (FRR_2, during ischemia) and those of the first measurement (FRR_1, internal control)(FRR_2/FRR_1) of released radioactivity. For condition see legend of **a**. Repeated Measures Analysis of Variance followed by multiple comparison method of Tukey-test). *$p < 0.05$
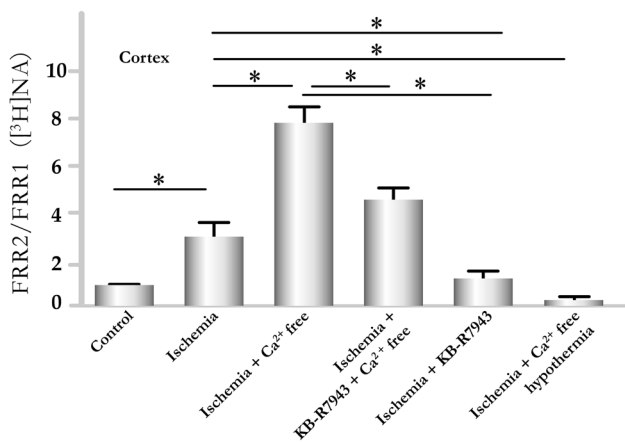
Fig. 5 The resting release of [³H]NA from rat cortical slices in response to oxygen and glucose deficiency (ischemia) at rest, and the potentiation of release by Ca^{2+} removal. For the control experiment, $FRR_2/FRR_1 = 0.89 \pm 0.60$ (n = 5). The preparations were superfused, 3-min fractions were collected, and the effects of ischemia were noted after 18 min (n = 6). KB-R 7943 (10 μM) was present in the Krebs solution starting from the 7th collection and was maintained in the solution throughout the experiments (n = 6). Ca^{2+}-free Krebs solution was used (Ca^{2+} was removed and 1 mM EGTA was added to the solution starting with the 5th fraction). Note that KB-R7943 significantly reduced the excessive release of [³H]NA in response to both ischemia + Ca^{2+}-free solution. Hypothermia (starting from the 7th collection period) also inhibited the $[Ca^{2+}]_o$-independent resting release of [³H]NA. For details, see the legend of Fig. 4. Repeated measures analysis of variance followed by multiple comparison method of Tukey-test. *p < 0.05

Na^+ for Ca^{2+}, the released levels of transmitters induced by ischemia were significantly reduced compared to release under ischemia in Ca^{2+}-free Krebs solution (Figs. 4 and 5).

Effects of Hypothermia

The temperature sensitivity of Na^+/Cl^--dependent monoamine transporters was also demonstrated [76]. The released levels of transmitters evoked by oxygen and glucose removal were significantly reduced in both striatal and cortical slices when the bath temperature was lowered from 37 to 17 °C, even in the absence of extracellular Ca^{2+} (Figs. 4 and 5). This effect is likely due to the temperature dependence of both the uptake process and NCX [77] and provides evidence that the reversed activity of the transporter is responsible for the release of monoamines.

DOPAL is a toxic by-product caused by the oxidation of DA by MAO-B. 3,4-dihydroxyphenilethanol (DOPET) is a subsequent metabolite of DOPAL, produced by aldehyde reductase. No toxic metabolites were detected during resting release, although [³H]DA was present in the perfusate obtained from the olfactory bulb slice preparations (Table 1). Electrical stimulation (2 Hz, 240 shocks) enhanced the release of both [³H]DA and its toxic metabolites (Table 1).

Table 2 Uptake and stimulation-evoked release of [³H]-noradrenaline from human cortical slices (for further information see the "Materials and Methods" section)

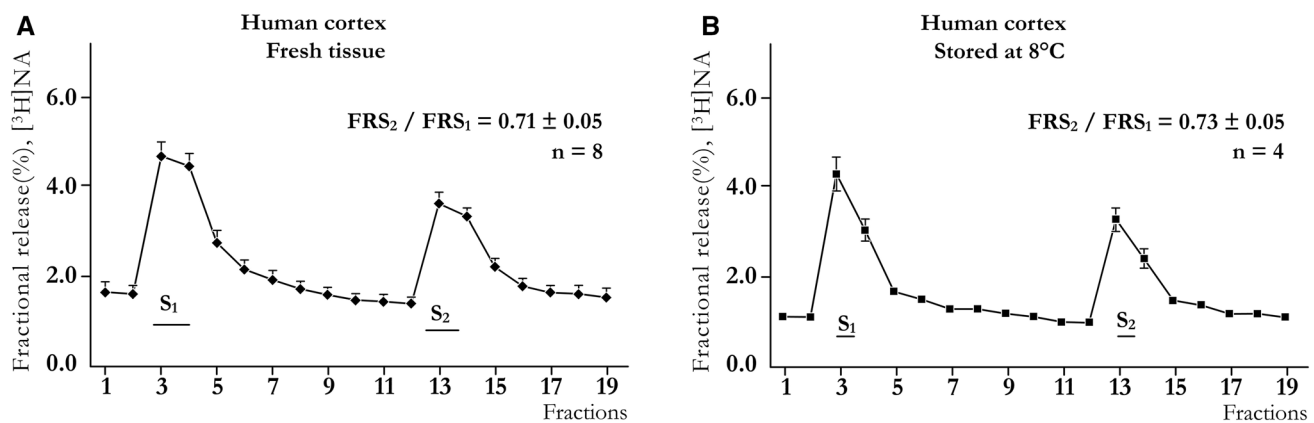|	Fresh tissue	Tissue maintained at 8 °C for 20 h	Significance
Uptake (Bq/g)	104,551 ± 8842	119,479 ± 26,467	n.s
	(8)	(6)	
S_1 (Bq/g)	6991 ± 975	5941 ± 1731	n.s
	(8)	(8)	
S_2 (Bq/g)	3895 ± 461	3148 ± 372	
	(8)	(2)	
FRS_2/FRS_1	0.71 ± 0.04	0.72 ± 0.05	n.s
	(8)	(4)	

Repeated measures analysis of variance followed by multiple comparison method of Tukey-test

When the temperature was reduced from 37 to 17 °C, the proportion of released [³H]DA was increased, and the released levels of [³H]DOPAL and [³H]DOPET were significantly reduced (Table 1). During DA metabolism, the catechol ring of DA undergoes oxidation and forms a quinone, a toxic metabolite of DA [64] and an ROS [40]. Dopamine quinone (Daq) was also detected in the perfusate when the tissue was stimulated. Interestingly, the release of this metabolite in response to stimulation was not affected by hypothermia (Table 1).

Effects of Hypothermia on NA Release from Human Cortical Slices

In our previous study, we showed that the release of transmitters at rest and in response to axonal stimulation can be studied in human cortical slice preparations that are freshly obtained from brain operations [78]. Therefore, to study the preservative effects of hypothermia against brain tissue damage, human frontal cortical slices were maintained at 8 °C for 20 h without oxygen. After rewarming and loading the samples with [³H]NA, the uptake and release values did not differ from those measured in tissues that were not exposed to long-lasting hypoxic conditions at low temperatures (Table 2). The release of transmitters that was evoked by stimulation was repeatable in both freshly prepared slices (Fig. 6a) and in slices prepared after being stored at 8 °C under hypoxic conditions (Fig. 6b); this result indicates that even after 20 h, the uptake and release functions were maintained in stored brain tissues. These findings are in accordance with those reported by others that cooling preserves brain tissue and allows it to function for a limited time [79, 80].

A

B

Fig. 6 The release of [³H]NA from human cortical slices that were either freshly prepared immediately after the operation (**a**) or prepared from tissue maintained at 8 °C for 20 h (**b**) under hypoxic conditions. The release was measured at 37 °C. The slices were stimulated (2 Hz, 2 ms, 240 shocks) twice (S_1 and S_2), as indicated. The fractional release (FR) values measured in the samples obtained from the frontal, occipital and parietal cortices were pooled. Increase of [³H]NA release evoked by S_1 $p < 0.05$ (t-test for Dependent Samples for **a** and **b**)

Discussion

Stroke is a leading cause of disability in adults, resulting in social and economic burdens worldwide [81, 82]. Numerous attempts [1, 2, 13, 83–94] have been made to reveal the pathological mechanisms underlying cerebral ischemia and to design new drugs [2, 95–99] that reduce the effects of complications due to stroke [100–102]. Because all of the pharmacological interventions that have been tested to date have failed, new targets for novel therapies are needed.

The human brain is highly metabolically active and accounts for approximately 20% of the oxygen consumption and 25% of the glucose utilization in the body [103]. During chemical neurotransmission, the exocytosis of each vesicle containing transmitters from the presynaptic nerve terminal requires approximately 12,000 ATP molecules [104]. Therefore, the maintenance of functional communications within and among neuronal circuitries in the brain is very sensitive to energy supply deficits. Synaptic transmission failure is the earliest consequence of cerebral ischemia [72, 73].

Rapid synaptic chemical transmission induced in a quantal form requires synchronous vesicle fusion that is evoked by an action potential-induced Ca^{2+}-influx (Fig. 7a). However, transmitter release also occurs from the cytoplasm, and this release is independent of action potentials and $[Ca^{2+}]_o$ [25]. While quantal release is subject to presynaptic modulation, $[Ca^{2+}]_o$-independent resting release is not affected by presynaptic receptor activation [105]. A prominent feature of cerebral ischemia is the excessive cytoplasmic accumulation of both Ca^{2+} and Na^+ ions and the excessive production of ROS [21, 22, 106], a condition that can also result in cell death [107].

Cytoplasmic Release of Catecholamines

In previous studies, it was shown that hypoxic or hypoglycaemic conditions can increase the $[Ca^{2+}]_o$-independent release of transmitters at rest, including monoamines (DA and NA) [11, 24–26, 108–110] and glutamate [23, 26, 35, 111, 112]. Glutamate released into the extraneuronal space is neurotoxic via the activation of extrasynaptic GluN2B glutamate receptors [113, 114] and the subsequent massive increase in Ca^{2+} influx [115]. Furthermore, H_2O_2 produced by glutamate [75, 76] via activation of AMPA receptors is also involved in excitotoxicity. Convincing pharmacological and clinical evidence has demonstrated that the ability of fluoxetine to inhibit GluN2B receptors may have neuroprotective effects [116]. Despite the validation of glutamate toxicity in pharmacological experiments, clinical trials targeting NMDA receptors have failed to achieve beneficial effects [2].

When ischemia was simulated by the removal of oxygen and glucose, the resting cytoplasmic release of catecholamines was enhanced (Figs. 4, 5, and 7b). Ca^{2+} removal potentiated the ischemia-induced resting release of transmitters (Figs. 4 and 5). Similar to our results, the effects of veratridine, a compound that is able to increase $[Na^+]_i$, on GABA release were also potentiated in a $[Ca^{2+}]_o$-free medium [117]. In both cases, $[Na^+]_i$ is not able to be reduced by NCX operating in reverse, due to the lack of extracellular Ca^{2+} to be exchanged for intracellular Na^+. When extracellular Ca^{2+} is removed, $[Na^+]_i$ increases further, forcing transporters (NET and DAT) to operate in reverse and resulting in the excessive release of transmitters from the cytoplasm into the extracellular space (Fig. 7c). These neurochemical data are in accordance with observations from neocortical cell cultures [115] showing that neuronal swelling and death
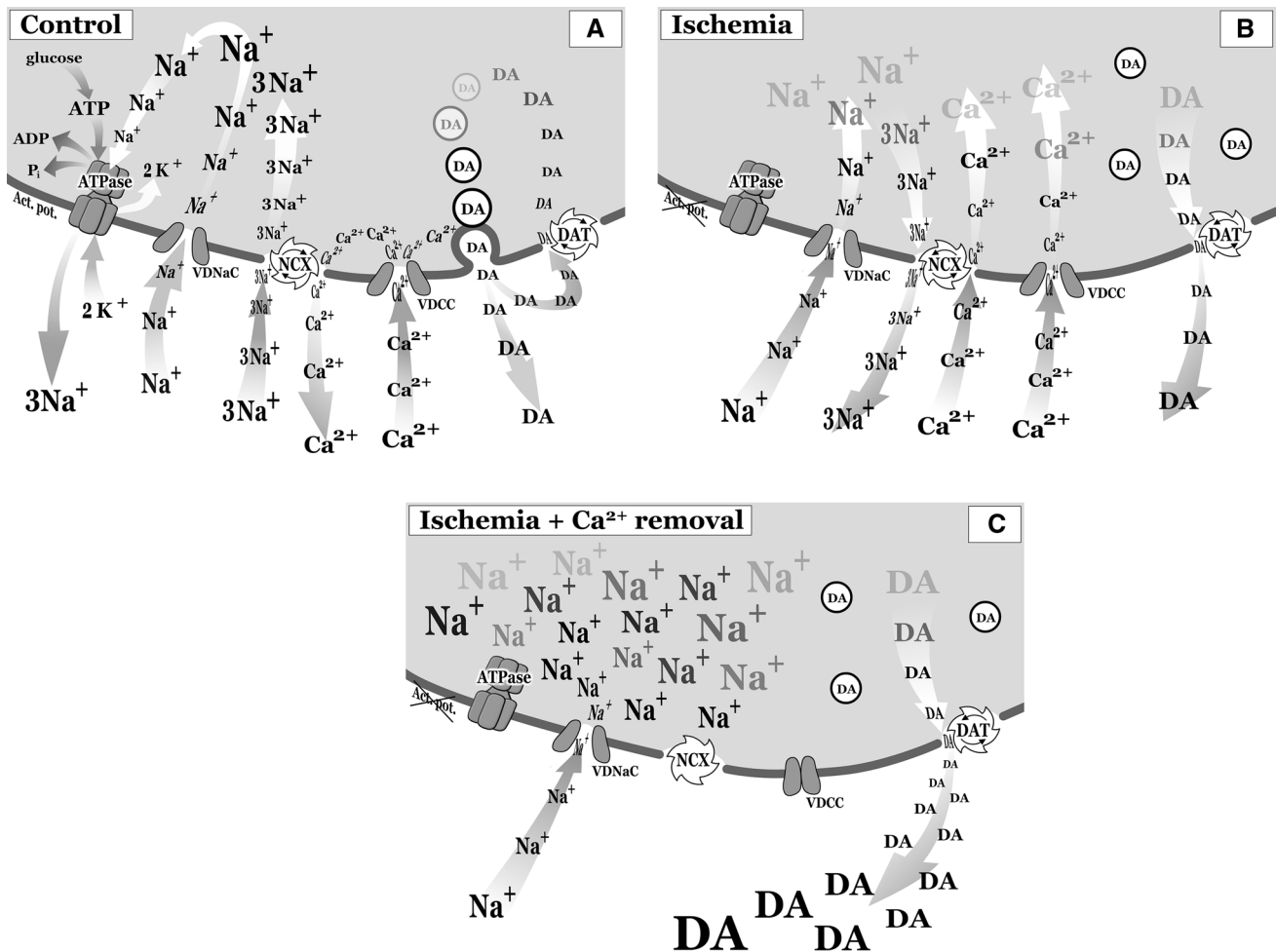
Fig. 7 a The effects of ischemia on transmitter release (dopamine, DA) and the roles played by the Na^+/Cl^- transporter (DAT) and the Na^+/Ca^{2+} exchanger (NCX) in the nonvesicular resting release of DA. Dopamine represents an example of various transmitters (NA, serotonine, glutamate, GABA) which under physiological condition stored in vesicles and could be released in response to depolarization followed by Ca^{2+} influx and taken back by Na^+/Cl^- transporters. **a** In response to an action potential (Act. pot.), Na^+ enters the neuron through voltage dependent sodium channels (VDNaCs), followed by a Ca^{2+} influx through voltage-activated channels (VDCC). The Na^+/Ca^{2+} exchanger (NCX) is activated when $[Ca^{2+}]_i$ is $>$ 100 nM. In response to action potentials, $[Ca^{2+}]_i$ in the nerve terminal can reach concentrations of $>$ 10 µM [32, 33]. The depolarization results in the vesicular release of transmitters (dopamine (DA)). Under physiological conditions, the intraterminal Ca^{2+} levels rise in response to an action potential, and the plasma membrane NCX couples the export of one Ca^{2+} ion to the import of three Na^+ ions ("forward" mode of NCX activity [28, 77]). Monoamine [108] and glutamate [112] transporters, using ATP as an energy source, operate exclusively in the inward mode, rapidly removing exocytotically released transmitters from the extracellular space and returning them to the nerve terminals to be reused during $[Ca^{2+}]_o$-dependent vesicular release. **b** Under ischemic conditions, when ATP synthesis is partially or fully blocked [25, 155], the exchanger (NCX) begins to operate in the reverse mode, producing additional Ca^{2+} influx and resulting in Na^+ efflux. Under this condition, the action potential (Act. pot.) fails, and exocytotic chemical neurotransmission does not occur; the transmit-

ter is released from the cytoplasm by transporters operating in the reverse mode, and the cytoplasmic pool of transmitters (monoamines [23–25], glutamate [23, 26, 156, 157] and GABA [23]) are released. Because ATP is primarily generated by oxidative phosphorylation, the energy supply of the brain depends on a permanent oxygen supply. Any interruption in the oxygen and blood glucose supply, even for only a few minutes, can result in energy delivery failures and cell death [158]. The carrier operates in the reverse mode under the conditions of a reduced Na^+ electrochemical gradient, such as when nerve terminals become energy compromised during the following events: during ischemia; during the selective inhibition of Na^+/K^+-ATPase by ouabain [127, 129, 159]; and after the administration of transporter substrates, such as β-phenylethylamine (β-PEA), mephedrone or amphetamines [46]. The inward transport of substrates by DAT, accompanied by an influx of 2 Na^+ and 1 Cl^- [160], results in a transporter-mediated current [161]. Therefore, in all cases, a high intraterminal $[Na^+]$ results in the Ca^{2+}-independent resting release of catecholamines, their toxic MAO metabolites (aldehydes, DOPAL (3,4-dihydroxyphenylacetaldehyde), quinone and DOPEGAL [133]), in a $[Ca^{2+}]_o$-independent manner. Glutamate is also released and is involved in neurotoxicity, and a depolarization occurs due to the enhanced $[Na^+]_i$. In addition, a Na^+ influx through voltage-dependent Na channels (VDNaCs) occurs. **c** Schematic diagram showing that the exchanger (NCX) plays a crucial role in the $[Ca^{2+}]_o$-independent release of the transmitter DA. When the exchanger is inhibited by a lack of extraneuronal Ca^{2+}, it fails to operate in a reverse mode and is not able to reduce $[Na^+]_i$

Table 3 Effects of oxidative stress, induced by H_2O_2 (250 μM), on the distribution of resting release of [³H]DA and its [³H] metabolites from striatal slices

[³H]	Control resting [%]	Oxidative stress resting H_2O_2, 250 μM [%]	Significance
DA	65.64 ± 2.25	50.66 ± 4.69	n.s.
	63.32 ± 6.11 kBq	82.29 ± 7.61 kBq	
DOPAC	13.99 ± 1.46	10.97 ± 1.12	n.s.
DOPAL	n.d.	3.65 ± 1.07	< 0.05
DOPET	n.d.	8.33 ± 2.73	< 0.05
3-MT	10.49 ± 0.64	10.62 ± 1.30	n.s.
HVA	9.88 ± 0.43	6.07 ± 0.79	< 0.05
Daq	n.d.	9.7 ± 0.97	< 0.05
[³H] activity [kBq]	96.48 ± 8.85	$162.44 \pm 13, 18$ kBq	< 0.05

Note that during ischemia, oxidative stress is constantly occurring

The release and distribution of [³H]DA and its metabolites were measured using HPLC combined with scintillation spectrometry at the 2nd (control) and 13th (oxidative stress) collection points. Note that the data shown in this Table were not obtained from the experiments shown in Fig. 2c. n = 4

see Table 1, *n.s.* not significant, *n.d.* not detectable

The statistical significance of the results was determined by the TIBC statistical program. To assess the normality of all the continuous variables measured, the Kolmogorov–Smirnov test was used and performed for each individual repeated measurement. If the measured variables met the normality assumption, two-way factorial measures (FM ANOVA) analysis was performed

were increased by the removal of Ca^{2+} from the ischemic medium.

Effects of NCX Inhibition

NCX has been shown to be involved in stroke pathophysiology, and its acute and long-term activation reduces brain injury and restores behavioural functions [59]. Furthermore, convincing evidence was obtained demonstrating that KB-R7943 attenuates the elevation of $[Ca^{2+}]_i$ in response to chemical ischemia [107] and protects CA1 neurons in hippocampal slices against hypoxic and hypoglycaemic injury [41]. These effects also imply that under this condition, $[Na^+]_i$ continues to increase [107]. Based on our study with Ca^{2+} removal, it appears likely that excessive Na^+ influx, in the absence of NCX-driven Na^+ efflux results in additional Na^+ loading and further enhances the $[Ca^{2+}]_o$-independent cytoplasmic release of transmitters (Fig. 7).

The present study demonstrated that the inhibition of the reverse activation of NCX using KB-R7943 [118–120] preferentially suppressed the resting release of transmitters, which was enhanced by oxygen and glucose removal in both the presence and absence of external Ca^{2+} (Figs. 4 and 5). These findings reveal the role played by the NCX during resting transmitter release. The restricted localizations of the NCX2 and NCX3 isomers primarily to neurons [30] and of NCX1 to cardiac muscle also clarifies the roles played by NCX2 and NCX3 in regulating $[Ca^{2+}]_i$ and transmitter release in the CNS. Our findings suggest that NCX functioning in reverse mode is involved not only in increasing the level of Ca^{2+} influx but also in increasing the resting release

levels of transmitters during ischemia (Fig. 7b). The depolarization caused by high $[Na^+]_i$ further increases Na^+ influx through the voltage-dependent sodium channel (VDNaC, Fig. 7b, c). Enhanced $[Na^+]_i$ appears likely to trigger transmitter release through a carrier-dependent process. Our findings that hypothermia, a condition that is able to inhibit monoamine transporter function [121], was able to reduce or prevent increased levels of transmitter release (Figs. 4 and 5) strongly supports this mechanism.

Based on our experimental data, the inhibitory effects of KB-R7943 on ischemia-induced transmitter release could easily be ascribed to its inhibitory effects on the reverse operation of NCX. When NCX activity was inhibited by removing Ca^{2+} from the perfusion fluid, KB-R7943 was still able to reduce the ischemia-induced release, a contradiction that requires discussion. Several explanations may explain this discrepancy. KB-R7943 also has several other actions that may be involved in its effects on transmitter release and neuroprotection [28]. The inhibitory effects of KB-R7943 on NMDA channels ($IC_{50} = 0.1–11$ μM) [122] appears to be one logical explanation for this contradictory effect on transmitter release. Recent evidence suggests that NMDA, but not AMPA receptor antagonists prevented the increased release of transmitters, including those mediated by glutamate and neuronal injury [115]. Furthermore, the fact that Na^+ channel blockades reduce or inhibit the effects of ischemia on transmitter release [123] supports this explanation. To confirm that the inhibition of the NCX isomer is indeed responsible for the moderation of ischemia-induced transmitter release, selective antagonists, with and without effects on NMDA and Na^+ channels, are needed that are

capable of inhibiting NCX acting in reverse mode. Furthermore, the findings that KB-R7943 inhibits the nicotine receptors expressed in neurons [124] and the noradrenaline transporter [125] also demonstrate the need to study a more selective NCX inhibitor.

The role played by NCX during cerebral ischemia also depends on the anatomical region injured by the insult [28]. In the penumbral region, where the Na^+/K^+-activated ATPase is not fully blocked and remains operational, the NCX continues to operate in the forward mode. However, in the ischemic core region, which is primarily represented in our experiments involving oxygen and glucose removal (Fig. 7b), ATP synthesis and the Na^+/K^+-activated ATPase are fully blocked; accordingly, $[Na^+]_i$ remains high. When the activity of the Na^+/K^+-activated ATPase was inhibited by ouabain, resting transmitter release increased [126–130].

Protective Effects of Hypothermia on the Release of DA and Its Toxic Metabolites

The beneficial effects of hypothermia on brain function in response to global and local cerebral ischemia have been demonstrated previously [131, 132]. In animal experiments, local short-term cooling after tissue plasminogen activation was shown to protect against side effects, such as oedema, and increased infarct volumes [132]. In our experiments hypothermia inhibited the cytoplasmic release of catecholamines in response to ischemia (Figs. 3, 4, 5) and prevented the release of deaminated toxic DA byproducts (DOPAL) into the extraneuronal space (Tables 1 and 3). These results also provide explanations for the neuroprotective effects of hypothermia.

Under local hypothermic conditions, both the NCX and the uptake transporters become partially or fully inhibited; this inhibition prevents or reduces the excessive carrier-mediated release of transmitters from the cytoplasm and the secondary $[Ca^{2+}]_i$ elevation caused by the accumulation of $[Na^+]_i$. The level of $[^3H]DA$ release at 17 °C was significantly enhanced compared to that at 37 °C, although its metabolites were observed at much higher proportions at the higher temperature (Table 1). Gerkau et al. [107] performed in vivo experiments showing that Na^+ elevation in periinfarct regions results in the reversal of the NCX, triggering a massive secondary $[Ca^{2+}]_I$ influx while promoting the export of Na^+. The sustained elevation of $[Ca^{2+}]_i$ results in the activation of various degradative enzymes, such as phospholipases and proteases. This condition represents a serious risk for neurons. Under this condition, the role of the reversed operation of Na^+/Cl^- transporters is crucial. Hypothermia reduces their operation and prevents transmitters (monoamines and glutamate) from being released into the extracellular space, where they can exert neurotoxic activities, allowing the tissue to recover from an energy-compromised state. Many stroke patients may still fall outside of the clinical time windows for effective treatment. The administration of local hypothermia by emergency services may be an important step, as hypothermia with thrombolysis represents a chance to improve our neuroprotective strategies.

Toxic Metabolites of Catecholamine Metabolism

Toxic MAO-B metabolites of catecholamines (DOPAL and 3,4-dihydroxyphenylglycolaldehyde, DOPEGAL) can also cause neuronal injury [37, 133]. Dopamine is also metabolized into H_2O_2 by MAO-B, and if dopamine is not reduced by cellular antioxidants (GSH and GSH peroxidase), it will react with iron and form hydroxy radicals [134]. In addition, the catechol ring of DA can undergo oxidation and form quinone [64] and H_2O_2 + superoxide anions, which are neurotoxic and alter mitochondrial respiration [135]. The energy depletion of mitochondria evoked by ischemic insult results in excessive production of ROS. Mitochondrial oxidative stress is one of the mediators of ischemic brain injury [74].

DOPAL, the toxic metabolite of DA, was detected in the superfusate when $[^3H]DA$ release was induced by electrical stimulation (Table 1). DOPET, the product of the subsequent metabolism DOPAL by aldehyde reductase, was also enhanced (Table 1). Under these conditions, DA was released from the vesicles. In contrast in our study, we also observed the presence of DOPAL and quinone when DA was released from the cytoplasm under oxidative stress (H_2O_2) conditions (Tables 1 and 3). Thus, during ischemia, when dopaminergic axon terminals are energy compromised the resting, non-vesicular release of DA is accelerated. The occurence of neurotoxic DA metabolites (DOPAL and quinone) in the perfusate indicates that they play roles in neuronal toxicity [133, 136, 137].

In our experiments (Fig. 2c), oxidative stress induced by H_2O_2, a side product of catecholamine breakdown, resulted in the excessive cytoplasmic release of catecholamines [106] and increased levels of toxic DA metabolites (DOPAL) (Table 3). Furthermore, evidence was obtained that oxidative stress (H_2O_2) combined with Na^+ load drains energy sources, ATP level decreases in the nerve terminal [138, 139]. The importance of our observation with H_2O_2 is further supported by findings that the production of ROS is increased in response to stroke and reperfusion [134] and that the presence of H_2O_2 at a concentration of 0.1 mM could be detected in the striatum [140]. These findings suggest that ischemia-associated oxidative stress and the toxic metabolites of catecholamines may also be responsible for neurotoxicity.

Extracellular Space as the Route for Non-synaptic Communication and the Location of Toxic Dopamine Metabolites During Ischemia

Recently, we have provided neurochemical and pharmacological evidence [46] that amphetamine-like drugs of abuse and the trace amine β-phenylethylamine (β-PEA) excessively increase the $[Ca^{2+}]_o$-independent, non-vesicular release of DA from the cytoplasm into the extracellular space and inhibit the high-affinity transporter. Increases in the DA concentration in the extrasynaptic space tonically control the activity of neurons equipped with DA receptors and are likely to be associated with the reinforcing effects and abusive potential of amphetamines. An overwhelming number of varicosities within the central nervous system are non-synaptically localized [6, 141–143], and they directly release their transmitters into the extracellular space [12, 127, 144]. Therefore, the volume of the extracellular space [145, 146], as an important factor that influences the ambient concentrations of transmitters and their toxic metabolites, and the drugs used in practice that inhabit the extracellular space [6] must also be discussed.

After monoamine transmitters (DA and NA), their toxic metabolites or glutamate have been released into the extracellular space, their diffusion and concentrations are affected by the volume of the space they are released into and their tortuosity [146–150]. During ischemia, a rapid decrease in the volume of the extracellular space and an increase in tortuosity have been reported [151]. These ischemic effects result in the increased concentrations of transmitters released into the extracellular space, augmenting their toxic effects [147]. In addition, Dr. Sykova and her colleagues provided evidence of the roles played by the volume of the extracellular space in various central nervous system diseases [147, 152, 153].

Summary

The mechanism underlying the ischemia-induced release of transmitters from the cytoplasm involves the inhibition of the Na^+/K^+–ATPase enzyme due to energy depletion, the subsequent intracellular Na^+ accumulation, and the sodium-dependent reversal of the Na^+/Ca^{2+} exchanger (Fig. 7b) and monoamine uptake carrier [5]. Moreover, the auto-oxidation of catecholamines by the cytoplasmic monoaminoxidase enzyme results in the formation of H_2O_2 and toxic metabolites (DOPAL), which are then released into the extracellular space.

Our findings not only suggest the role played by NCX in the reduction of $[Na^+]_i$ under ischemic conditions but also suggest that the brain NCX isoforms, which are able

to operate in reverse mode and produce Ca^{2+} overload in neurons, may represent therapeutic targets for the development of new drugs [154]. Moreover, in ischemic insults, local cooling might be a potential treatment for acute stroke intervention, preventing the excessive release of transmitters and their toxic metabolites into the extraneuronal space and lengthening the clinical time windows for effective treatment.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Nagy Z, Nardai S (2017) Cerebral ischemia/reperfusion injury: from bench space to bedside. Brain Res Bull 134:30–37
2. Moskowitz MA, Lo EH, Iadecola C (2010) The science of stroke: mechanisms in search of treatments. Neuron 67:181–198
3. Feigin VL, Lawes CM, Bennett DA, Anderson CS (2003) Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. Lancet Neurol 2:43–53
4. Vizi ES (1984) Physiological role of cytoplasmic and non-synaptic release of transmitter. Neurochem Int 6:435–440
5. Vizi ES (2000) Role of high-affinity receptors and membrane transporters in nonsynaptic communication and drug action in the central nervous system. Pharmacol Rev 52:63–89
6. Vizi ES, Fekete A, Karoly R, Mike A (2010) Non-synaptic receptors and transporters involved in brain functions and targets of drug treatment. Br J Pharmacol 160:785–809
7. Gerevich Z, Tretter L, Adam-Vizi V, Baranyi M, Kiss JP, Zelles T, Vizi ES (2001) Analysis of high intracellular [Na+]-induced release of [3H]noradrenaline in rat hippocampal slices. Neuroscience 104:761–768
8. Vizi ES (1990) Synaptic and nonsynaptic cross talk between neurons. Role of presynaptic alpha 2-receptors in mental disorders. Ann N Y Acad Sci 604:344–352
9. Vizi ES (1980) Modulation of cortical release of acetylcholine by noradrenaline released from nerves arising from the rat locus coeruleus. Neuroscience 5:2139–2144
10. Vizi ES, Labos E (1991) Non-synaptic interactions at presynaptic level. Prog Neurobiol 37:145–163

11. Kiss JP, Vizi ES (2001) Nitric oxide: a novel link between synaptic and nonsynaptic transmission. Trends Neurosci 24:211–215

12. Vizi ES (1984) Non-synaptic interactions between neurons: modulation of neurochemical transmission. Pharmacological and Clinical Aspects, Wiley, Chichester, New York

13. Huang L, Otrokocsi L, Sperlagh B (2019) Role of P2 receptors in normal brain development and in neurodevelopmental psychiatric disorders. Brain Res Bull 150:201–206

14. Agnati LF, Zoli M, Stromberg I, Fuxe K (1995) Intercellular communication in the brain: wiring versus volume transmission. Neuroscience 69:711–726

15. Fuxe K, Agnati LF (1991) Two principal modes of electrochemical communication in the brain: volume versus wiring transmission. Raven Press, New York.

16. Szatkowski M, Attwell D (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. Trends Neurosci 17:359–365

17. Raiteri M, Cerrito F, Cervoni AM, Levi G (1979) Dopamine can be released by two mechanisms differentially affected by the dopamine transport inhibitor nomifensine. J Pharmacol Exp Ther 208:195–202

18. Raiteri L, Raiteri M (2015) Multiple functions of neuronal plasma membrane neurotransmitter transporters. Prog Neurobiol 134:1–16

19. Milusheva E, Sperlagh B, Shikova L, Baranyi M, Tretter L, Adam-Vizi V, Vizi ES (2003) Non-synaptic release of [3H]noradrenaline in response to oxidative stress combined with mitochondrial dysfunction in rat hippocampal slices. Neuroscience 120:771–781

20. Milusheva E, Baranyi M, Kittel A, Sperlagh B, Vizi ES (2005) Increased sensitivity of striatal dopamine release to H_2O_2 upon chronic rotenone treatment. Free Radic Biol Med 39:133–142

21. Nicholson C, Phillips JM (1981) Ion diffusion modified by tortuosity and volume fraction in the extracellular microenvironment of the rat cerebellum. J Physiol 321:225–257

22. Nicholson C, Sykova E (1998) Extracellular space structure revealed by diffusion analysis. Trends Neurosci 21:207–215

23. Globus MY, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD (1988) Effect of ischemia on the in vivo release of striatal dopamine, glutamate, and gamma-aminobutyric acid studied by intracerebral microdialysis. J Neurochem 51:1455–1464

24. Milusheva E, Doda M, Pasztor E, Lajtha A, Sershen H, Vizi ES (1992) Regulatory interactions among axon terminals affecting the release of different transmitters from rat striatal slices under hypoxic and hypoglycemic conditions. J Neurochem 59:946–952

25. Milusheva EA, Doda M, Baranyi M, Vizi ES (1996) Effect of hypoxia and glucose deprivation on ATP level, adenylate energy charge and [Ca2+]o-dependent and independent release of [3H]dopamine in rat striatal slices. Neurochem Int 28:501–507

26. Nakai T, Milusheva E, Baranyi M, Uchihashi Y, Satoh T, Vizi ES (1999) Excessive release of [3H]noradrenaline and glutamate in response to simulation of ischemic conditions in rat spinal cord slice preparation: effect of NMDA and AMPA receptor antagonists. Eur J Pharmacol 366:143–150

27. Blaustein MP, Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. Physiol Rev 79:763–854

28. Annunziato L, Pignataro G, Di Renzo GF (2004) Pharmacology of brain Na^+/Ca^{2+} exchanger: from molecular biology to therapeutic perspectives. Pharmacol Rev 56:633–654

29. Luther PW, Yip RK, Bloch RJ, Ambesi A, Lindenmayer GE, Blaustein MP (1992) Presynaptic localization of sodium/calcium exchangers in neuromuscular preparations. J Neurosci 12:4898–4904

30. Nicoll DA, Quednau BD, Qui Z, Xia YR, Lusis AJ, Philipson KD (1996) Cloning of a third mammalian Na^+-Ca^{2+} exchanger, NCX3. J Biol Chem 271:24914–24921

31. Juhaszova M, Church P, Blaustein MP, Stanley EF (2000) Location of calcium transporters at presynaptic terminals. Eur J Neurosci 12:839–846

32. Lorincz T, Kisfali M, Lendvai B, Sylvester Vizi E (2016) Phenotype-dependent Ca(2+) dynamics in single boutons of various anatomically identified GABAergic interneurons in the rat hippocampus. Eur J Neurosci 43:536–547

33. Kisfali M, Lőrincz T, Vizi ES (2013) Comparison of Ca^{2+} transients and $[Ca^{2+}]_i$ in the dendrites and boutons of non-fast-spiking GABAergic hippocampal interneurons using two-photon laser microscopy and high- and low-affinity dyes. J Physiol 591:5541–5553

34. Hoyt KR, Arden SR, Aizenman E, Reynolds IJ (1998) Reverse Na^+/Ca^{2+} exchange contributes to glutamate-induced intracellular Ca^{2+} concentration increases in cultured rat forebrain neurons. Mol Pharmacol 53:742–749

35. Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403:316–321

36. Sibarov DA, Abushik PA, Poguzhelskaya EE, Bolshakov KV, Antonov SM (2015) Inhibition of plasma membrane Na/Ca-exchanger by KB-R7943 or lithium reveals its role in Ca-dependent N-methyl-d-aspartate receptor inactivation. J Pharmacol Exp Ther 355:484–495

37. Goldstein DS, Sullivan P, Holmes C, Miller GW, Alter S, Strong R, Mash DC, Kopin IJ, Sharabi Y (2013) Determinants of buildup of the toxic dopamine metabolite DOPAL in Parkinson's disease. J Neurochem 126:591–603

38. Wey MC, Fernandez E, Martinez PA, Sullivan P, Goldstein DS, Strong R (2012) Neurodegeneration and motor dysfunction in mice lacking cytosolic and mitochondrial aldehyde dehydrogenases: implications for Parkinson's disease. PLoS ONE 7:e31522

39. Rees JN, Florang VR, Eckert LL, Doorn JA (2009) Protein reactivity of 3,4-dihydroxyphenylacetaldehyde, a toxic dopamine metabolite, is dependent on both the aldehyde and the catechol. Chem Res Toxicol 22:1256–1263

40. Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53(Suppl 3):S26–S36 **(discussion S36-8)**

41. Schroder UH, Breder J, Sabelhaus CF, Reymann KG (1999) The novel Na^+/Ca^{2+} exchange inhibitor KB-R7943 protects CA1 neurons in rat hippocampal slices against hypoxic/hypoglycemic injury. Neuropharmacology 38:319–321

42. Abramochkin DV, Alekseeva EI, Vornanen M (2013) Inhibition of the cardiac inward rectifier potassium currents by KB-R7943. Comp Biochem Physiol C Toxicol Pharmacol 158:181–186

43. Hagerdal M, Harp J, Nilsson L, Siesjo BK (1975) The effect of induced hypothermia upon oxygen consumption in the rat brain. J Neurochem 24:311–316

44. Vizi ES (1998) Different temperature dependence of carrier-mediated (cytoplasmic) and stimulus-evoked (exocytotic) release of transmitter: a simple method to separate the two types of release. Neurochem Int 33:359–366

45. Vizi ES, Sperlagh B (1999) Separation of carrier mediated and vesicular release of GABA from rat brain slices. Neurochem Int 34:407–413

46. Zsilla G, Hegyi DE, Baranyi M, Sylvester Vizi E (2018) 3,4-Methylenedioxymethamphetamine, mephedrone, and beta-phenylethylamine release dopamine from the cytoplasm by means of transporters and keep the concentration high and constant by blocking reuptake. Eur J Pharmacol 837:72–80

47. Shankaran S, Pappas A, McDonald SA, Vohr BR, Hintz SR, Yolton K, Gustafson KE, Leach TM, Green C, Bara R, Petrie Huitema CM, Ehrenkranz RA, Tyson JE, Das A, Hammond J, Peralta-Carcelen M, Evans PW, Heyne RJ, Wilson-Costello DE, Vaucher YE, Bauer CR, Dusick AM, Adams-Chapman I, Goldstein RF, Guillet R, Papile LA (2012) Childhood outcomes

after hypothermia for neonatal encephalopathy. N Engl J Med 366:2085–2092

48. Gunn AJ, Laptook AR, Robertson NJ, Barks JD, Thoresen M, Wassink G, Bennet L (2017) Therapeutic hypothermia translates from ancient history in to practice. Pediatr Res 81:202–209

49. Yenari MA, Han HS (2012) Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 13:267–278

50. Kim JH, Seo M, Han HS, Park J, Suk K (2013) The neurovascular protection afforded by delayed local hypothermia after transient middle cerebral artery occlusion. Curr Neurovasc Res 10:134–143

51. Davidson JO, Wassink G, van den Heuij LG, Bennet L, Gunn AJ (2015) Therapeutic hypothermia for neonatal hypoxic-ischemic encephalopathy—where to from here? Front Neurol 6:198

52. Carlsson C, Hagerdal M, Siesjo BK (1976) Protective effect of hypothermia in cerebral oxygen deficiency caused by arterial hypoxia. Anesthesiology 44:27–35

53. Berntman L, Welsh FA, Harp JR (1981) Cerebral protective effect of low-grade hypothermia. Anesthesiology 55:495–498

54. Silveira RC, Procianoy RS (2015) Hypothermia therapy for newborns with hypoxic ischemic encephalopathy. J Pediatr (Rio J) 91:S78–83

55. Volbers B, Herrmann S, Willfarth W, Lucking H, Kloska SP, Doerfler A, Huttner HB, Kuramatsu JB, Schwab S, Staykov D (2016) Impact of hypothermia initiation and duration on peri-hemorrhagic edema evolution after intracerebral hemorrhage. Stroke 47:2249–2255

56. Laptook AR (2017) Therapeutic hypothermia for preterm infants with hypoxic-ischemic encephalopathy: how do we move forward? J Pediatr 183:8–9

57. Ohta H, Terao Y, Shintani Y, Kiyota Y (2007) Therapeutic time window of post-ischemic mild hypothermia and the gene expression associated with the neuroprotection in rat focal cerebral ischemia. Neurosci Res 57:424–433

58. Diaz J, Abiola S, Kim N, Avaritt O, Flock D, Yu J, Northington FJ, Chavez-Valdez R (2017) Therapeutic hypothermia provides variable protection against behavioral deficits after neonatal hypoxia-ischemia: a potential role for brain-derived neurotrophic factor. Dev Neurosci 39:257–272

59. Cerullo P, Brancaccio P, Anzilotti S, Vinciguerra A, Cuomo O, Fiorino F, Severino B, Di Vaio P, Di Renzo G, Annunziato L, Pignataro G (2018) Acute and long-term NCX activation reduces brain injury and restores behavioral functions in mice subjected to neonatal brain ischemia. Neuropharmacology 135:180–191

60. Borbely Z, Csomo BK, Kittel A, Gerber G, Varga G, Vizi ES (2017) Effect of rat spinal cord injury (hemisection) on the ex vivo uptake and release of [(3)H]noradrenaline from a slice preparation. Brain Res Bull 131:150–155

61. Harsing LG Jr, Vizi ES (1984) Release of endogenous dopamine from rat isolated striatum: effect of clorgyline and (-)-deprenyl. Br J Pharmacol 83:741–749

62. Vizi ES, Palkovits M, Lendvai B, Baranyi M, Kovacs KJ, Zelles T (2004) Distinct temperature-dependent dopamine-releasing effect of drugs of abuse in the olfactory bulb. Neurochem Int 45:63–71

63. Vizi ES, Harsing LG Jr, Zimanvi I, Gaal G (1985) Release and turnover of noradrenaline in isolated median eminence: lack of negative feedback modulation. Neuroscience 16:907–916

64. Baranyi M, Milusheva E, Vizi ES, Sperlagh B (2006) Chromatographic analysis of dopamine metabolism in a Parkinsonian model. J Chromatogr A 1120:13–20

65. Whittingham TS, Lust WD, Passonneau JV (1984) An in vitro model of ischemia: metabolic and electrical alterations in the hippocampal slice. J Neurosci 4:793–802

66. Ramamoorthy S, Bauman AL, Moore KR, Han H, Yang-Feng T, Chang AS, Ganapathy V, Blakely RD (1993) Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. Proc Natl Acad Sci USA 90:2542–2546

67. Vizi ES, Zsilla G, Caron MG, Kiss JP (2004) Uptake and release of norepinephrine by serotonergic terminals in norepinephrine transporter knock-out mice: implications for the action of selective serotonin reuptake inhibitors. J Neurosci 24:7888–7894

68. Moron JA, Brockington A, Wise RA, Rocha BA, Hope BT (2002) Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines. J Neurosci 22:389–395

69. Uhl GR, Hall FS, Sora I (2002) Cocaine, reward, movement and monoamine transporters. Mol Psychiatry 7:21–26

70. Giros B, el Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T, Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol Pharmacol 42:383–390

71. Dahlin A, Xia L, Kong W, Hevner R, Wang J (2007) Expression and immunolocalization of the plasma membrane monoamine transporter in the brain. Neuroscience 146:1193–1211

72. Eccles RM, Loyning Y, Oshima T (1966) Effects of hypoxia on the monosynaptic reflex pathway in the cat spinal cord. J Neurophysiol 29:315–331

73. Hofmeijer J, van Putten MJ (2012) Ischemic cerebral damage: an appraisal of synaptic failure. Stroke 43:607–615

74. Starkov AA, Chinopoulos C, Fiskum G (2004) Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. Cell Calcium 36:257–264

75. Chen BT, Avshalumov MV, Rice ME (2001) H(2)O(2) is a novel, endogenous modulator of synaptic dopamine release. J Neurophysiol 85:2468–2476

76. Lindmar R, Loffelholz K (1972) Differential effects of hypothemia on neuronal efflux, release and uptake of noradrenaline. Naunyn Schmiedebergs Arch Pharmacol 274:410–414

77. Torok TL (2007) Electrogenic Na$^+$/Ca^{2+}-exchange of nerve and muscle cells. Prog Neurobiol 82:287–347

78. Vizi ES, Pasztor E (1981) Release of acetylcholine from isolated human cortical slices: inhibitory effect of norepinephrine and phenytoin. Exp Neurol 73:144–153

79. Buskila Y, Breen PP, Tapson J, van Schaik A, Barton M, Morley JW (2014) Extending the viability of acute brain slices. Sci Rep 4:5309

80. Komlosi G, Molnar G, Rozsa M, Olah S, Barzo P, Tamas G (2012) Fluoxetine (prozac) and serotonin act on excitatory synaptic transmission to suppress single layer 2/3 pyramidal neuron-triggered cell assemblies in the human prefrontal cortex. J Neurosci 32:16369–16378

81. Hankey GJ (2017) Stroke. Lancet 389:641–654

82. Marsh JD, Keyrouz SG (2010) Stroke prevention and treatment. J Am Coll Cardiol 56:683–691

83. Deng Q, Chang Y, Cheng X, Luo X, Zhang J, Tang X (2018) Postconditioning with repeated mild hypoxia protects neonatal hypoxia-ischemic rats against brain damage and promotes rehabilitation of brain function. Brain Res Bull 139:31–37

84. Guo H, Zhao L, Wang B, Li X, Bai H, Liu H, Yue L, Guo W, Bian Z, Gao L, Feng D, Qu Y (2018) Remote limb ischemic postconditioning protects against cerebral ischemia-reperfusion injury by activating AMPK-dependent autophagy. Brain Res Bull 139:105–113

85. Huang L, Wang C, Ge R, Ni H, Zhao S (2017) Ischemia deteriorates spike encoding at cortical GABAergic neurons and cerebellar Purkinje cells by increasing the intracellular Ca(2). Brain Res Bull 131:55–61

86. Kaidonis G, Rao AN, Ouyang YB, Stary CM (2018) Elucidating sex differences in response to cerebral ischemia: immunoregulatory mechanisms and the role of microRNAs. Prog Neurobiol 176:73–85

87. Lan R, Wu JT, Wu T, Ma YZ, Wang BQ, Zheng HZ, Li YN, Wang Y, Gu CQ, Zhang Y (2018) Mitophagy is activated in brain damage induced by cerebral ischemia and reperfusion via the PINK1/Parkin/p62 signalling pathway. Brain Res Bull 142:63–77

88. Li Z, Chen H, Lv J, Zhao R (2017) The application and neuroprotective mechanisms of cerebral ischemic post-conditioning: a review. Brain Res Bull 131:39–46

89. Liu JJ, Ding XY, Xiang L, Zhao F, Huang SL (2017) A novel method for oxygen glucose deprivation model in organotypic spinal cord slices. Brain Res Bull 135:163–169

90. Marin MA, Carmichael ST (2018) Stroke in CNS white matter: models and mechanisms. Neurosci Lett 684:193–199

91. Naderi Y, Sabetkasaei M, Parvardeh S, Zanjani TM (2017) Neuroprotective effect of minocycline on cognitive impairments induced by transient cerebral ischemia/reperfusion through its anti-inflammatory and anti-oxidant properties in male rat. Brain Res Bull 131:207–213

92. Rekling JC (2003) Neuroprotective effects of anticonvulsants in rat hippocampal slice cultures exposed to oxygen/glucose deprivation. Neurosci Lett 335:167–170

93. Xie Z, Cao BQ, Wang T, Lei Q, Kang T, Ge CY, Gao WJ, Hui H (2018) LanCL1 attenuates ischemia-induced oxidative stress by Sirt3-mediated preservation of mitochondrial function. Brain Res Bull 142:216–223

94. Zhang LY, Hu YY, Zhao CC, Qi J, Su AC, Lou N, Zhang MY, Li L, Xian XH, Gong JX, Zhao H, Zhang JG, Li WB, Zhang M (2019) The mechanism of GLT-1 mediating cerebral ischemic injury depends on the activation of p38 MAPK. Brain Res Bull 147:1–13

95. Wu J, Chen Y, Yu S, Li L, Zhao X, Li Q, Zhao J, Zhao Y (2017) Neuroprotective effects of sulfiredoxin-1 during cerebral ischemia/reperfusion oxidative stress injury in rats. Brain Res Bull 132:99–108

96. Xue Y, Qu Z, Fu J, Zhen J, Wang W, Cai Y, Wang W (2017) The protective effect of astaxanthin on learning and memory deficits and oxidative stress in a mouse model of repeated cerebral ischemia/reperfusion. Brain Res Bull 131:221–228

97. Sebastian-Serrano A, de Diego-Garcia L, di Lauro C, Bianchi C, Diaz-Hernandez M (2019) Nucleotides regulate the common molecular mechanisms that underlie neurodegenerative diseases; therapeutic implications. Brain Res Bull 18:30768–30778. **(Epub ahead of print)**https://doi.org/10.1016/j.brainresbull.2019.01.031

98. Jimenez-Mateos EM, Smith J, Nicke A, Engel T (2018) Regulation of P2X7 receptor expression and function in the brain. Brain Res Bull pii S0361–9230(18):30734–30742. https://doi.org/10.1016/j.brainresbull.2018.12.008

99. Cheng HY, Wang YS, Hsu PY, Chen CY, Liao YC, Juo SH (2019) miR-195 Has a potential to treat ischemic and hemorrhagic stroke through neurovascular protection and neurogenesis. Mol Ther Methods Clin Dev 13:121–132

100. Rodrigo R, Fernandez-Gajardo R, Gutierrez R, Matamala JM, Carrasco R, Miranda-Merchak A, Feuerhake W (2013) Oxidative stress and pathophysiology of ischemic stroke: novel therapeutic opportunities. CNS Neurol Disord Drug Targets 12:698–714

101. Meschia JF, Brott T (2018) Ischaemic stroke. Eur J Neurol 25:35–40

102. McDermott M, Jacobs T, Morgenstern L (2017) Critical care in acute ischemic stroke. Handb Clin Neurol 140:153–176

103. Sokoloff L, Reivich M, Kennedy C, Des Rosiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M (1977) The [14C] deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. J Neurochem 28:897–916

104. Helmchen F, Borst JG, Sakmann B (1997) Calcium dynamics associated with a single action potential in a CNS presynaptic terminal. Biophys J 72:1458–1471

105. Vizi ES, Bernath S, Kapocsi J, Serfozo P (1986) Transmitter release from the cytoplasm is of physiological importance but not subject to presynaptic modulation. J Physiol (Paris) 81:283–288

106. Langeveld CH, Schepens E, Stoof JC, Bast A, Drukarch B (1995) Differential sensitivity to hydrogen peroxide of dopaminergic and noradrenergic neurotransmission in rat brain slices. Free Radic Biol Med 19:209–217

107. Gerkau NJ, Rakers C, Durry S, Petzold GC, Rose CR (2017) Reverse NCX attenuates cellular sodium loading in metabolically compromised cortex. Cereb Cortex 28:1–17

108. Lohr KM, Masoud ST, Salahpour A, Miller GW (2017) Membrane transporters as mediators of synaptic dopamine dynamics: implications for disease. Eur J Neurosci 45:20–33

109. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000) The sympathetic nerve–an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev 52:595–638

110. Sumiya Y, Torigoe K, Gerevich Z, Kofalvi A, Vizi ES (2001) Excessive release of [3H] noradrenaline by veratridine and ischemia in spinal cord. Neurochem Int 39:59–63

111. Brassai A, Suvanjeiev RG, Ban EG, Lakatos M (2015) Role of synaptic and nonsynaptic glutamate receptors in ischaemia induced neurotoxicity. Brain Res Bull 112:1–6

112. Sheldon AL, Robinson MB (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int 51:333–355

113. Chen Q, He S, Hu XL, Yu J, Zhou Y, Zheng J, Zhang S, Zhang C, Duan WH, Xiong ZQ (2007) Differential roles of NR2A- and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. J Neurosci 27:542–552

114. Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405–414

115. Goldberg MP, Choi DW (1993) Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. J Neurosci 13:3510–3524

116. Vizi ES, Kisfali M, Lorincz T (2013) Role of nonsynaptic GluN2B-containing NMDA receptors in excitotoxicity: evidence that fluoxetine selectively inhibits these receptors and may have neuroprotective effects. Brain Res Bull 93:32–38

117. Levi G, Gallo V, Raiteri M (1980) A reevaluation of veratridine as a tool for studying the depolarization-induced release of neurotransmitters from nerve endings. Neurochem Res 5:281–295

118. Watano T, Kimura J, Morita T, Nakanishi H (1996) A novel antagonist, No. 7943, of the Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular cells. Br J Pharmacol 119:555–563

119. Iwamoto T, Watano T, Shigekawa M (1996) A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. J Biol Chem 271:22391–22397

120. Sibarov DA, Bolshakov AE, Abushik PA, Krivoi II, Antonov SM (2012) Na^+, K^+-ATPase functionally interacts with the plasma membrane Na^+, Ca^{2+} exchanger to prevent Ca^{2+} overload and neuronal apoptosis in excitotoxic stress. J Pharmacol Exp Ther 343:596–607

121. Blaustein MP, King AC (1976) Influence of membrane potential on the sodium-dependent uptake of gamma-aminobutyric acid

by presynaptic nerve terminals: experimental observations and theoretical considerations. J Membr Biol 30:153–173

122. Sobolevsky AI, Khodorov BI (1999) Blockade of NMDA channels in acutely isolated rat hippocampal neurons by the Na⁺/Ca²⁺ exchange inhibitor KB-R7943. Neuropharmacology 38:1235–1242

123. Uchihashi Y, Bencsics A, Umeda E, Nakai T, Sato T, Vizi ES (1998) Na+ channel block prevents the ischemia-induced release of norepinephrine from spinal cord slices. Eur J Pharmacol 346:145–150

124. Pintado AJ, Herrero CJ, Garcia AG, Montiel C (2000) The novel Na(+)/Ca(2+) exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. Br J Pharmacol 130:1893–1902

125. Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, Takahashi K, Takahashi T, Suzuki T, Ota T, Hamano-Takahashi A, Onishi M, Tanaka Y, Kameo K, Baba A (2001) SEA0400, a novel and selective inhibitor of the Na⁺-Ca²⁺ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J Pharmacol Exp Ther 298:249–256

126. Paton WD, Vizi ES, Zar MA (1971) The mechanism of acetylcholine release from parasympathetic nerves. J Physiol 215:819–848

127. Vizi ES (1972) Stimulation, by inhibition of (Na⁺-K⁺-Mg²⁺)-activated ATP-ase, of acetylcholine release in cortical slices from rat brain. J Physiol 226:95–117

128. Vizi ES (1974) Possible connection between the release of acetylcholine and the activity of Na⁺K⁺-activated ATPase. Ergeb Exp Med 17:96–116

129. Vizi ES (1977) Termination of transmitter release by stimulation of sodium-potassium activated ATPase. J Physiol 267:261–280

130. Vizi ES (1978) Na⁺-K⁺-activated adenosinetriphosphatase as a trigger in transmitter release. Neuroscience 3:367–384

131. Kim JH, Yun SH, Jang KH, Park J, Han HS, Rhee D, Suk K (2014) Delayed and prolonged local brain hypothermia combined with decompressive craniectomy: a novel therapeutic strategy that modulates glial dynamics. Exp Neurobiol 23:115–123

132. Zarisfi M, Allahtavakoli F, Hassanipour M, Khaksari M, Rezazadeh H, Allahtavakoli M, Taghavi MM (2017) Transient brain hypothermia reduces the reperfusion injury of delayed tissue plasminogen activator and extends its therapeutic time window in a focal embolic stroke model. Brain Res Bull 134:85–90

133. Burke WJ, Li SW, Chung HD, Ruggiero DA, Kristal BS, Johnson EM, Lampe P, Kumar VB, Franko M, Williams EA, Zahm DS (2004) Neurotoxicity of MAO metabolites of catecholamine neurotransmitters: role in neurodegenerative diseases. Neurotoxicology 25:101–115

134. Halliwell B (1992) Reactive oxygen species and the central nervous system. J Neurochem 59:1609–1623

135. Berman SB, Hastings TG (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. J Neurochem 73:1127–1137

136. Asanuma M, Miyazaki I, Ogawa N (2003) Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox Res 5:165–176

137. Baranyi M, Porceddu PF, Goloncser F, Kulcsar S, Otrokocsi L, Kittel A, Pinna A, Frau L, Huleatt PB, Khoo ML, Chai CL, Dunkel P, Matyus P, Morelli M, Sperlagh B (2016) Novel (Hetero) arylalkenyl propargylamine compounds are protective in toxin-induced models of Parkinson's disease. Mol Neurodegener 11:6

138. Chinopoulos C, Tretter L, Rozsa A, Adam-Vizi V (2000) Exacerbated responses to oxidative stress by an Na(+) load in isolated

nerve terminals: the role of ATP depletion and rise of [Ca(2+)] (i). J Neurosci 20:2094–2103

139. Adam-Vizi V, Ligeti E (1984) Release of acetylcholine from rat brain synaptosomes by various agents in the absence of external calcium ions. J Physiol 353:505–521

140. Hyslop PA, Zhang Z, Pearson DV, Phebus LA (1995) Measurement of striatal H2O2 by microdialysis following global forebrain ischemia and reperfusion in the rat: correlation with the cytotoxic potential of H2O2 in vitro. Brain Res 671:181–186

141. Descarries L, Watkins KC, Garcia S, Bosler O, Doucet G (1996) Dual character, asynaptic and synaptic, of the dopamine innervation in adult rat neostriatum: a quantitative autoradiographic and immunocytochemical analysis. J Comp Neurol 375:167–186

142. Hokfelt T (1968) In vitro studies on central and peripheral monoamine neurons at the ultrastructural level. Z Zellforsch Mikrosk Anat 91:1–74

143. Umbriaco D, Garcia S, Beaulieu C, Descarries L (1995) Relational features of acetylcholine, noradrenaline, serotonin and GABA axon terminals in the stratum radiatum of adult rat hippocampus (CA1). Hippocampus 5:605–620

144. Agnati LF, Fuxe K, Zoli M, Ozini I, Toffano G, Ferraguti F (1986) A correlation analysis of the regional distribution of central enkephalin and beta-endorphin immunoreactive terminals and of opiate receptors in adult and old male rats. Evidence for the existence of two main types of communication in the central nervous system: the volume transmission and the wiring transmission. Acta Physiol Scand 128:201–207

145. Nicholson C, Kamali-Zare P, Tao L (2011) Brain extracellular space as a diffusion barrier. Comput Vis Sci 14:309–325

146. Sykova E, Nicholson C (2008) Diffusion in brain extracellular space. Physiol Rev 88:1277–1340

147. Sykova E, Vargova L (2008) Extrasynaptic transmission and the diffusion parameters of the extracellular space. Neurochem Int 52:5–13

148. Sykova E (2004) Extrasynaptic volume transmission and diffusion parameters of the extracellular space. Neuroscience 129:861–876

149. Sykova E (2001) Glial diffusion barriers during aging and pathological states. Prog Brain Res 132:339–363

150. Sykova E, Roitbak T, Mazel T, Simonova Z, Harvey AR (1999) Astrocytes, oligodendroglia, extracellular space volume and geometry in rat fetal brain grafts. Neuroscience 91:783–798

151. Vorisek I, Sykova E (1997) Ischemia-induced changes in the extracellular space diffusion parameters, K⁺, and pH in the developing rat cortex and corpus callosum. J Cereb Blood Flow Metab 17:191–203

152. Lehmenkuhler A, Sykova E, Svoboda J, Zilles K, Nicholson C (1993) Extracellular space parameters in the rat neocortex and subcortical white matter during postnatal development determined by diffusion analysis. Neuroscience 55:339–351

153. Kilb W, Dierkes PW, Sykova E, Vargova L, Luhmann HJ (2006) Hypoosmolar conditions reduce extracellular volume fraction and enhance epileptiform activity in the CA3 region of the immature rat hippocampus. J Neurosci Res 84:119–129

154. Pignataro G, Sirabella R, Anzilotti S, Di Renzo G, Annunziato L (2014) Does Na(+)/Ca(2)(+) exchanger, NCX, represent a new druggable target in stroke intervention? Transl Stroke Res 5:145–155

155. Okada Y (1974) Recovery of neuronal activity and high-energy compound level after complete and prolonged brain ischemia. Brain Res 72:346–349

156. Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate

and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43:1369–1374

157. Chiu DN, Jahr CE (2017) Extracellular glutamate in the nucleus accumbens is nanomolar in both synaptic and non-synaptic compartments. Cell Rep 18:2576–2583

158. Jeffs GJ, Meloni BP, Bakker AJ, Knuckey NW (2007) The role of the Na(+)/Ca(2+) exchanger (NCX) in neurons following ischaemia. J Clin Neurosci 14:507–514

159. Adam-Vizi V (1992) External Ca(2+)-independent release of neurotransmitters. J Neurochem 58:395–405

160. Gu H, Wall SC, Rudnick G (1994) Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. J Biol Chem 269:7124–7130

161. Pifl C, Rebernik P, Kattinger A, Reither H (2004) Zn2+ modulates currents generated by the dopamine transporter: parallel effects on amphetamine-induced charge transfer and release. Neuropharmacology 46:223–231

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