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Release, neuronal effects and removal of extracellular β -nicotinamide adenine dinucleotide (β -NAD⁺) in the rat brain

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Abstract

Recent evidence supports an emerging role of β -nicotinamide adenine dinucleotide (β -NAD⁺) as a novel neurotransmitter and neuromodulator in the peripheral nervous system: β -NAD⁺ is released in nerve-smooth muscle preparations and adrenal chromaffin cells in a manner characteristic of a neurotransmitter. It is currently unclear whether this holds true for the central nervous system. Using a small-chamber superfusion assay and high-sensitivity HPLC techniques we demonstrate that high K⁺-stimulation of rat forebrain synaptosomes evokes overflow of β -NAD⁺, adenosine 5'-triphosphate (ATP), and their metabolites adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine, ADP-ribose (ADPR), and cyclic ADPR. The high K⁺-evoked overflow of β -NAD⁺ is attenuated by cleavage of SNAP-25 with botulinum neurotoxin A (BoNT/A), by inhibition of N-type voltage-dependent Ca²⁺ channels with ω -conotoxin GVIA, and by inhibition of the proton gradient of synaptic vesicles with bafilomycin A1, suggesting that β -NAD⁺ is likely released via vesicle exocytosis. Western analysis demonstrates that CD38, a multifunctional protein that metabolizes β -NAD⁺, is present on synaptosomal membranes and in the cytosol. Intact synaptosomes degrade β -NAD⁺. 1,*N*⁶-etheno-NAD, a fluorescent analogue of β -NAD⁺, is taken by synaptosomes and this uptake is attenuated by authentic β -NAD⁺, but not by the connexin 43 inhibitor Gap 27. In cortical neurons local applications of β -NAD⁺ cause rapid Ca²⁺ transients likely due to influx of extracellular Ca²⁺. Therefore, rat brain synaptosomes can actively release, degrade and uptake β -NAD⁺, and β -NAD⁺ can stimulate postsynaptic neurons, all criteria needed for a substance to be considered a candidate neurotransmitter in the brain.

Keywords

ATP; brain synaptosomes; ADP-ribose; neurotransmitter release; calcium transients

Introduction

Intracellular β -nicotinamide adenine dinucleotide (β -NAD⁺) serves as a coenzyme for cellular oxidation-reduction reactions, a donor of adenosine 5'-diphospho-ribose (ADPR) in

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posttranslational modifications of proteins, and a precursor to cyclic ADPR (cADPR) and other second messengers with Ca^{2+} -releasing activity (Lee, 2001). In recent years $\beta\text{-NAD}^+$ has also been recognized as an extracellular signaling molecule involved in cell-to-cell communication (Ziegler and Niere, 2004, Billington et al., 2006). In the peripheral nervous system $\beta\text{-NAD}^+$ appears to serve as a novel neurotransmitter and neuromodulator (Smyth et al., 2004, Breen et al., 2006, Mutafova-Yambolieva et al., 2007, Hwang et al., 2011). $\beta\text{-NAD}^+$ is released from neurons in blood vessels, urinary bladder, and colon (Breen et al., 2006, Smyth et al., 2004, Smyth et al., 2006b, Mutafova-Yambolieva et al., 2007, Hwang et al., 2011), and from nerve growth factor-differentiated rat PC12 cells (Yamboliev et al., 2009). Release of $\beta\text{-NAD}^+$ correlates with neuronal activity and requires intact fast Na^+ channels, N-type voltage-dependent Ca^{2+} channels, and the 25-kDa synaptosomal associated protein SNAP-25 (Smyth et al., 2004, Smyth et al., 2006b, Breen et al., 2006, Mutafova-Yambolieva et al., 2007, Smyth et al., 2009). $\beta\text{-NAD}^+$ modulates the release of other neurotransmitters in blood vessels (Smyth et al., 2004), inhibits spontaneous smooth muscle contractions in urinary bladder (Breen et al., 2006) and has shown vasoactive effects in the mesenteric vasculature (Ralevic, 1995, Smyth et al., 2009). $\beta\text{-NAD}^+$ also effectively causes membrane hyperpolarization and relaxation in the colon, consistent with an inhibitory neurotransmitter role in this tissue (Mutafova-Yambolieva et al., 2007, Hwang et al., 2011). Therefore, evidence has been accumulating that $\beta\text{-NAD}^+$ is a novel neurotransmitter in the peripheral nervous system and it remains to be determined whether this also holds true for the central nervous system.

In the brain $\beta\text{-NAD}^+$ contributes to intracellular Ca^{2+} homeostasis, mitochondrial function, energy metabolism and complex processes such as aging and neuronal death (Ying, 2007). In addition, $\beta\text{-NAD}^+$ may participate in intercellular communication (De Flora et al., 2004). For example, cultured hippocampal astrocytes release NAD^+ and respond to extracellular NAD^+ with increased intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (Verderio et al., 2001). Specific binding sites for NAD have been described and a role of NAD as a neuromodulator in rat and guinea-pig hippocampus has been suggested (Richards et al., 1983, Snell et al., 1985, Galarreta et al., 1993). To the best of our knowledge, however, storage and release of $\beta\text{-NAD}^+$ from central nervous system (CNS) neurons have not been reported. In the present study we have utilized high-sensitivity high pressure liquid chromatography (HPLC) methods to characterize candidate purine nucleotide transmitters released by depolarization of rat brain synaptosomes. We demonstrate that brain synaptosomes can actively release, degrade and uptake $\beta\text{-NAD}^+$ and that exogenous application of $\beta\text{-NAD}^+$ can generate robust Ca^{2+} transients in isolated cortical neurons. Taken together, these findings suggest that $\beta\text{-NAD}^+$ is a *candidate* neurotransmitter in the central nervous system.

Methods

Animal

For preparation of brain synaptosomes male Wistar-Kyoto or Wistar rats, 12–15 weeks of age, were euthanized by isoflurane inhalation. This method is approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nevada. After opening the skull the brain was quickly removed and placed in oxygenated Krebs-bicarbonate-HEPES (KBH) buffer (10°C) with the following composition (mmol/L): NaCl 118, KCl 3.5, CaCl_2 1.25, MgSO_4 1.2, KH_2PO_4 1.2, NaCO_3 25, HEPES-NaOH 10, and D-glucose 11.5, pH 7.4 (Lonart and Sudhof, 2000). The forebrain was processed for preparation of synaptosomes as described below. For preparation of neuronal cultures, pregnant female Sprague–Dawley rats (16-day gestation) were obtained from Harlan Laboratories (Livermore, CA, USA). Primary neuronal cultures were established from day 16–17 embryos, as described below. The IACUC of the University of New Mexico approved the experimental protocols involved in culture preparation.

Preparation of synaptosomes

Brain synaptosomes were prepared by differential centrifugation followed by Percoll-gradient centrifugation as described in previous studies (Lonart and Sudhof, 2000, Gualix et al., 2003, Gomez-Villafuertes et al., 2007, Wang, 2007) and shown in Figure 1. Brain tissue was homogenized in brain homogenizing buffer (mmol/L): HEPES 10, sucrose 320, EDTA 2, EGTA 2 (Gomez-Villafuertes et al., 2007, Wang, 2007) using a glass-teflon homogenizer (0.25 mm clearance). Homogenate was then centrifuged for 5 min at $1,000 \times g$ and $4^{\circ}C$ to obtain pellet P1 (nuclei and cell debris) and supernatant S1 (containing synaptosomes). P1 was rewashed with homogenizing buffer, then centrifuged at $1,000 \times g$ for 5 min and supernatant was combined with S1. Pooled S1 fraction was centrifuged at $17,000 \times g$ and $4^{\circ}C$ for 10 min to obtain P2 (synaptosome-rich pellet). P2 was resuspended in Percoll gradient buffer, prepared by dissolving 0.25 mmol/L dithiothreitol in brain homogenizing buffer (Gomez-Villafuertes et al., 2007, Wang, 2007). Resuspended P2 was applied onto a gradient of 23%, 10%, and 3% Percoll. Gradient tubes were centrifuged at $25,000 \times g$ for 10 min and $4^{\circ}C$, and synaptosomes were recovered from the 10%/23% Percoll interface. To remove residual Percoll, synaptosomes were diluted with 10 volumes of aerated (95% O_2 , 5% CO_2) ice-cold KBH buffer and centrifuged at $20,000 \times g$ for 5 min at $4^{\circ}C$. Synaptosome-rich pellet P3 was resuspended on ice in KBH buffer and aliquots of the P3 synaptosomal suspension were used for experiments as described below. In some experiments P3 suspension was centrifuged at $20,000 \times g$ for 5 min and synaptosomes in pellet P4 were disrupted by placing in ice-cold hypotonic solution for 30 min. Supernatant S5 (intrasynaptosomal content) was obtained by a 5-min centrifugation at $20,000 \times g$ and used to measure intrasynaptosomal concentrations of purines and catecholamines and uptake of 1, N^6 -etheno-NAD (eNAD).

HPLC assay of etheno-purines

Endogenous purines (e.g., ATP, ADP, AMP, ADO, β -NAD⁺, ADPR, and cADPR) in superfusates were measured after derivatization to 1, N^6 -etheno-derivatives to increase detection sensitivity as described previously (Levitt et al., 1984, Bobalova et al., 2002). One hundred μ l of a citrate phosphate buffer (pH 4.0) was added to 200 μ l of superfusate sample and purines were etheno-derivatized by adding 10 μ l of 2-chloroacetaldehyde for 40 min at $80^{\circ}C$. Endogenous ATP, ADP, AMP, and ADO were derivatized to the 1, N^6 -derivatives eATP, eADP, eAMP, and eADO, respectively (Bobalova et al., 2002). β -NAD⁺, ADPR and cADPR are all derivatized to eADPR (Smyth et al., 2004). Etheno-purines were assayed by HPLC with fluorescence detection (HPLC-FLD) as described previously (Bobalova et al., 2002). Agilent 1100 and 1200 liquid chromatography module systems (Agilent Technologies, Wilmington, DE) were used throughout this study. The mobile phase consisted of 0.1 mol/L KH_2PO_4 (pH 6.0) as eluent A; eluent B contained of 35 % methanol and 65 % eluent A. Gradient elution was employed according to the following linear program: time 0, 0 % eluent B; 18 min, 100 % eluent B at a flow rate of 1 ml/min and run time 20 min. Chromatography signals were recorded at Ex of 230 nm and Em of 420 nm. The amounts of purines in each sample were calculated from calibration curves of purine standards run simultaneously with every set of experimental samples. Results were normalized for sample volume and amount of protein loaded. The overflow of purines was expressed in nmol/mg protein.

Intrasynaptosomal contents of purines

One hundred μ l of intrasynaptosomal fraction (described in *Preparation of synaptosomes*) were derivatized to 1, N^6 -etheno-purines and etheno purines were assayed by HPLC-FLD (Bobalova et al. 2002, Smyth et al., 2004).

Spontaneous secretion of purines by synaptosomes

Contents of purines were measured in samples collected after placing P3 synaptosomal suspension in a small-chamber superfusion system (described in *High K⁺-evoked overflow*) and superfused with KBH solution as described below. After 15-minute equilibration eight hundred μ l of superfusate were collected in each protocol. Samples were analyzed for purines by a HPLC-FLD technique.

High K⁺-evoked overflow

Synaptosomes (600–800 μ g protein) were applied under gentle suction onto Whatman GF/B filter paper (3 mm diameter) which was placed in a 0.45 μ m Cameo 3N syringe filter serving as a perfusion chamber as described previously (Yamboliev et al., 2009). The chamber was perfused with a KBH solution (37°C) at 0.8 ml/min. After equilibration, 800 μ l of superfusate were collected as pre-stimulation samples containing spontaneously released substances. Synaptosomes were then stimulated with KCl (25 mmol/L or 65 mmol/L). The high K⁺-solutions were prepared by substituting NaCl for equal amounts of KCl to maintain isotonicity. Eight hundred μ l samples of superfusate were collected at 1, 2, 5 or 10 minutes of superfusion in ice-cold Eppendorf tubes. In some experiments synaptosomes were incubated with 300 nmol/L BoNT/A for 2 hours at 37°C and then high K⁺ overflow experiments were performed. In some experiments synaptosomes were superfused for 30 min with either bafilomycin A1 (0.1 μ mol/L) or ω -conotoxin GVIA (ω -Ctx GVIA, 20–50 nmol/L) prior to treatment with a high-K⁺ (25 mmol/L) KBH solution.

HPLC fraction analysis

To identify the ratio of β -NAD⁺:ADPR:cADPR forming eADPR at 11.2 min we carried out an HPLC fraction analysis (Smyth et al., 2004) on samples from the intrasynaptosomal compartment as well as samples collected before and during high-K⁺ stimulation. Briefly, in overflow experiments 2.4-ml synaptosome superfusate samples were collected in tubes and immersed in liquid N₂. Control samples from KBH or high K⁺ solutions (no contact with synaptosomes) were also collected. The samples were concentrated by Speed Vacuum (Savant SVC100, Thermo Electron Corp., Westmont, IL) to 1 ml. Nine hundred μ l of each concentrated sample were injected into the HPLC system and 400 μ l-fractions were collected according to retention time (cADPR 7.0–7.4 min, ADPR 8.3–8.7 min, and β -NAD⁺ 10.3–10.7 min). Fractions were subjected to etheno-derivatization with 2-chloroacetaldehyde and were analyzed for eADPR content by HPLC-FLD.

Degradation of β -NAD⁺ and ATP

Figure 2 depicts enzymatic pathways for the degradation of β -NAD⁺ and ATP. Six hundred μ g of synaptosomes were loaded in a small-chamber superfusion system and superfused with KBH solution (see *High K⁺-evoked overflow*). Following equilibration, chambers were superfused with substrates eATP (50 nmol/L), eNAD (0.2 μ mol/L) or NGD (0.2 mmol/L) in KBH solution (Bobalova and Mutafova-Yambolieva, 2003, Smyth et al., 2006a) to assay ATP-ase, NAD-glycohydrolase, and ADP-ribosyl cyclase activities, respectively. Eight hundred μ l-samples from substrate solution (no synaptosomes present, *S1*), and from superfusate after 1-min contact of synaptosomes with substrate (*S2*) were collected in ice-cold tubes and immersed in liquid N₂. Purines in samples were assayed by HPLC-FLD and enzymatic activities were determined by disappearance of substrate and appearance of products. Each purine was quantified against known standards and data were normalized for protein amount. Derivatized purines were detected at Ex of 230 nm and Em of 420 nm, whereas the non-modified compounds (i.e., NGD and cGDPR) were detected at Ex of 270 nm and Em of 400 nm (Bobalova et al., 2002). cGDPR standard was prepared by incubation of NGD with *Aplysia californica* as described previously (Smyth et al., 2006a).

Uptake of NAD

P3 synaptosomes were incubated with eNAD (1 mmol/L) at 37°C for 1, 5 or 60 minutes to measure the uptake of NAD. The reactions were stopped by inserting the tubes on ice and the tubes were centrifuged at $22,000 \times g$ for 5 minutes at 4°C to produce synaptosome-containing pellet. The pellet fraction was then washed twice in 0.5–1 ml ice-cold KBH buffer and synaptosomes were hypotonically disrupted for 30 min on ice. Suspension was then centrifuged again at $22,000 \times g$ for 5 min at 4°C to produce supernatant (S5) as intrasynaptosomal fraction. Intrasynaptosomal samples were processed for purine detection by HPLC-FLD. Appearance of eNAD and its metabolites eADPR and eADO in intrasynaptosomal fractions was used as a measure of eNAD uptake. In some experiments synaptosomes were incubated with a mixture of eNAD (1 mmol/L) and β -NAD⁺ (10 mmol/L) to examine whether authentic β -NAD⁺ competes with eNAD for uptake mechanisms. In some experiments synaptosomes were pre-incubated with Gap 27 (100 μ mol/L) to examine whether connexin hemichannels are involved in the uptake of eNAD.

Western immunoblot analysis

Equal amounts of total protein from synaptosomal extracts or intrasynaptosomal fractions were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for 1.5 hours at 24V and 4°C. Membranes were blocked for 1 hour with LI-COR blocking buffer (LI-COR, Inc., Lincoln, NE) and probed with the following primary antibodies diluted in LI-COR buffer: anti-synaptophysin (mouse monoclonal, Chemicon, dilution 1:1000), anti-chromogranin B (rabbit polyclonal, Abcam, dilution 1:300), anti-CD38 (goat polyclonal, Santa Cruz Biotechnology, dilution 1:200), and anti-SNAP25 (mouse monoclonal, Sternberger, dilution 1:1000). After removal of excess primary antibody, membranes were incubated for 45 min at room temperature with secondary antibodies coupled to infrared fluorescence markers with Em of 800 nm (IR800, Rockland Immunochemicals, PA) or 680 nm (Alexa Fluor 680, Molecular Probes, OR) diluted 1:100,000 in LI-COR buffer. Images were obtained with an infrared Odyssey scanner (LI-COR, Inc., Lincoln, NE).

Cell culture

Primary neuronal cultures were established from 16–17 day old rat embryos as described (Paul et al., 2003). Briefly, the cortex with the adjoining striatum were dissected out, dissociated mechanically and re-suspended in Dulbecco's minimum essential medium/F12 (1:1) supplemented with 5% fetal calf serum. Cells (8×10^6 cells/60 mm dish) were plated on poly-d-lysine-coated tissue coverslips and grown for 12–14 days in vitro (DIV) at 37°C in a humidified atmosphere consisting of 5% CO₂. To inhibit proliferation of non-neuronal cells, 10 μ M cytosine d-arabinofuranoside was added to the cultures 72 h after plating. Thereafter neurons were maintained in Minimal Essential Medium containing 5% fetal calf serum until the day of experiment.

Imaging of intracellular Ca²⁺ transients

Cytosolic Ca²⁺ levels were assessed using the high-affinity ratiometric indicator Fura-2 as described previously (Tafoya et al., 2008). Cultures were loaded at room temperature with 3 μ M Fura-AM for 20 min in HEPES buffer (mmol/L): 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 11 Glucose, 10 HEPES, pH 7.6 and then rinsed for 20 min in HEPES to allow for indicator de-esterification. Coverslips were then transferred to the recording chamber and superfused with HEPES (2 ml/min) at room temperature. β -NAD⁺, ADPR or ATP (50 mmol/L loaded in picospritz pipettes) were applied locally using pressure ejection (500 ms, 40 psi, Picospritzer II, General Valve Corp.) from a conventional patch recording electrode (5M Ω). This method allows rapid and reproducible ejections of very small volumes while avoiding the inherent desensitization of nerve cells which accompanies other methodologies (i.e., bath

application or superfusion). Due to the very small volume of ejections (calculated at fractions of a nanoliter in the present study) the final concentration at neurons is likely very much lower than the agonist concentration loaded in the picospritz pipette. In some experiments the recording chamber was superfused with α,β -methylene-ATP (α,β -MeATP, 50 $\mu\text{mol/L}$) for 10 minutes prior to local application of $\beta\text{-NAD}^+$. Cultures were allowed to equilibrate for 20 min before recording began. Fura-2 excitation was achieved using 350/380 nm excitation pairs (40 ms each) delivered from a monochromator (TiLL Photonics GmbH, Grafeling, Germany) via a 40 $\times$ WI objective (Olympus, N.A. 0.8). Fluorescence emission (510 nm) was detected using an interline transfer cooled CCD (TiLL Imago). Image pairs were background-subtracted and then ratioed (Till Vision v 4.0). Uncertainties regarding final intracellular concentrations following loading of the AM form of Fura-2 and Kd in the intracellular compartment limits reliable conversions to Ca^{2+} concentrations, and measurements of Ca^{2+} are expressed as ratios of background-subtracted ratios of the 350/380nm excited fluorescence. Responses in each cell were compared with depolarization responses to 40 mmol/L KCl-containing buffer, obtained at the end of each experiment.

Statistics

Data are presented as means $\pm$ SE. One-way ANOVA with a *post hoc* Bonferroni's multiple comparison test was performed using GraphPadPrism v. 3 for Windows (GraphPad Software, San Diego, CA) when three or more groups of data were compared. Paired or unpaired two-tailed Student's *t*-test was performed using the same software when two groups of data were compared. A probability value of less than 0.05 was considered significant.

Drugs

ATP, ADP, α,β MeATP, AMP, ADO, ADPR, $\beta\text{-NAD}^+$, NGD, and ω -conotoxin GVIA were purchased from Sigma-Aldrich (St. Louis, MO). eNAD and cADPR came from Biolog (Bremen, Germany). Bafilomycin A1 and Gap 27 came from Tocris Bioscience (Ellisville, MO). BoNT/A was purchased from List Biological Laboratories (Campbell, CA).

Results

Intra-synaptosomal content of purines and spontaneous release of endogenous purines from rat brain synaptosomes

To determine substances that have the potential to be released from synaptosomes upon membrane depolarization, we first assayed the intrasynaptosomal content of endogenous purines (Table 1). Note that the absolute amounts of individual purines might have been underestimated due to sample dilution and possible *in vitro* degradation during sample preparation. However, the use of identical methodologies to simultaneously measure intrasynaptosomal and extrasynaptosomal contents of multiple endogenous purine nucleotides allowed comparisons of the amounts of purines potentially available for release and purines released at rest and upon cell membrane depolarization. The amounts of purines spontaneously released from synaptosomes superfused with normal KBH buffer (5 mmol/L KCl) are shown in Table 1; approximately 0.2–0.5% of the intrasynaptosomal content of purines appeared to be released spontaneously from isolated synaptosomes.

An HPLC fraction analysis demonstrated that the $\beta\text{-NAD}^+$ +ADPR+cADPR cocktail in the intra-synaptosomal compartment comprised about 71% $\beta\text{-NAD}^+$, 20% ADPR, and 9% cADPR. Identical analysis of the eADPR peak in superfusate samples collected from unchallenged synaptosomes revealed $66.45\pm3.04\%$ $\beta\text{-NAD}^+$, $29.67\pm3.86\%$ ADPR and $3.91\pm2.18\%$ cADPR, $n=7$. Since $\beta\text{-NAD}^+$ was the primary purine nucleotide in the mixture, the amounts of $\beta\text{-NAD}^+$ +ADPR+cADPR were calculated based on $\beta\text{-NAD}^+$ standards.

High-K⁺-evoked release of purines

Regulated vesicular release following membrane depolarization would be consistent with a neurotransmitter role of β -NAD⁺. We therefore examined whether depolarization with high K⁺ concentrations evoked additional release of purine in the superfusate. Superfusion with 25 mmol/L or 65 mmol/L KCl caused additional release of purines including β -NAD⁺. The release of most purines (e.g., β -NAD⁺, AMP and adenosine) appeared to be concentration-dependent (Fig. 3). Twenty five mmol/L KCl produced the highest overflow of ATP at 5 min ($P<0.05$, $t=3.52$ vs. 0 min control), whereas the overflow of β -NAD⁺+ADPR+cADPR was significantly enhanced at 2 min ($P<0.05$, $t=3.488$), 5 min ($P<0.05$, $t=3.297$), and 10 min ($P<0.05$, $t=3.742$), $F=5.103$ after stimulation with KCl. Likewise, the overflow of total purines was significantly increased at 5 min ($P<0.05$, $t=3.153$) and 10 min ($P<0.05$, $t=3.637$), $F=4.431$. The overflow of ADP, AMP and ADO did not reach statistical significance at any time point. At 65 mmol/L KCl the overflow of AMP and ADO was increased at 5 min ($P<0.05$, $t=3.026$, $F=2.952$ for AMP and $P<0.05$, $t=3.048$, $F=2.883$ for ADO). The overflow of β -NAD⁺+ADPR+cADPR and total purines was enhanced at 2 min ($P<0.05$, $t=2.954$, $F=2.94$ for β -NAD⁺+ADPR+cADPR and $P<0.05$, $t=3.128$, $F=4.132$ for total purines) and 5 min ($P<0.05$, $t=2.974$, $F=2.94$ for β -NAD⁺+ADPR+cADPR and $P<0.05$, $t=3.625$, $F=4.132$ for total purines) of stimulation. The overflow of ATP and ADP did not reach statistical significance from 0 time; one-way analysis of variance and Bonferroni's multiple comparison *post hoc* test. Time control experiments showed a fairly consistent evoked release of purines within the first two hours after isolation of synaptosomes. Samples collected during 2-min stimulation with 65 mmol/L KCl contained 41.02 ± 11.21 pmol/mg protein β -NAD⁺, 28.69 ± 10.12 pmol/mg protein ADPR and 0.495 ± 0.17 pmol/mg protein cADPR, which represents $62.72\pm3.38\%$, $36.42\pm3.52\%$ and $0.862\pm0.317\%$ of the pool, respectively, $n=7$.

We next examined whether disruption of synaptic vesicle release machinery affects spontaneous and K⁺ depolarization-evoked release of purines. Treatment with clostridium botulinum neurotoxin A (BoNT/A, 300 nmol/L), an inhibitor of the SNARE protein SNAP-25 (Blasi et al., 1993), significantly attenuated the additional release of purines evoked by high-K⁺ solution (25 mmol/L) (Fig. 4B,E): the overflow of β -NAD⁺+ADPR+cADPR was increased in controls at 2 min ($P<0.05$, $t=2.919$) and 5 min ($P<0.05$, $t=3.275$), $F=4.365$ after KCl stimulation, whereas in the BoNT/A-treated preparations the overflow of β -NAD⁺+ADPR+cADPR remained unchanged by KCl ($P>0.05$). Total purines were also increased in controls at 2 min ($P<0.05$, $t=2.769$) and 5 min ($P<0.05$, $t=2.820$), $F=3.556$ after KCl stimulation, but were not significantly increased in BoNT/A-treated preparations ($P>0.05$). Likewise bafilomycin A1 (0.1 μ mol/L for 30 min), a drug blocking vesicular uptake of neurotransmitters by inhibiting membrane H⁺-ATPase (Moriyama and Futai, 1990) or ω -Ctx GVIA (0.02–0.05 μ mol/L for 30 min), a specific inhibitor of N-type (CaV2.2) neuronal voltage-gated Ca²⁺ channels (Olivera et al., 1984), attenuated the high K⁺ (25 mmol/L)-evoked release of β -NAD⁺ (Fig. 4C,D,E), at 1, 2, and 5 min of stimulation $P>0.05$ vs. 0 min. The changes in total purine overflow followed closely the changes in β -NAD⁺ overflow (Fig. 4F).

Degradation of ATP and NAD

To evaluate whether enzymatic activities for termination of neurotransmitter action are present in isolated synaptosomes, synaptosomes were either superfused or incubated with etheno-derivatized purines as substrates (Jamal et al., 1988, Todorov et al., 1997, Bobalova and Mutafova-Yambolieva, 2003). Use of etheno-purines ensures ~1,000,000 times higher sensitivity (Bobalova et al., 2002) and allows for the detection of small changes in substrate or product concentrations. Importantly, spontaneously released endogenous purines collected in the superfusates were not subjected to derivatization prior to HPLC analysis and

hence their fluorescence remained subthreshold. Superfusion of synaptosomes with eATP caused a decrease in levels of the substrate eATP and an increased levels of product eADP + eAMP + eADO (Fig. 5A, $P=0.0071$, $t=6.586$, $df=3$, paired t test, two-tailed), suggesting the presence of enzymes such as E-NTPDases, NPPs and ecto-5'-nucleotidase that degrade ATP, ADP and AMP, respectively (see Fig. 2). Likewise, superfusion (Fig. 5B) or incubation (Fig. 6A) of synaptosomes with eNAD led to an increase of the eNAD metabolites eADPR and eADO ($P=0.0177$, $t=4.753$, $df=3$, paired t test, two-tailed), indicating the presence of NAD-glycohydrolase, NPPs, and 5'-nucleotidase on synaptosome membranes. ADP-ribosyl cyclase was also present and responsible for the increased production of cGDPR from NGD (Fig. 5C, $P=0.0022$, $t=9.870$, $df=3$, paired t test, two-tailed). NGD was used as a substrate instead of β -NAD⁺, because ADP-ribosyl cyclase also converts NGD into cGDPR which is the end product of this reaction, whereas cADPR formed from β -NAD⁺ by ADP-ribosyl cyclase can be further processed to ADPR and other products (Graeff et al., 1994).

Expression of CD38 and synapse-associated proteins

We next examined whether CD38, a protein involved in the degradation of β -NAD⁺, is expressed on intact synaptosomes and in intra-synaptosomal fractions. Western analysis demonstrated CD38 expression on synaptosomes, P3 (Fig. 6B) and in the intra-synaptosomal compartment, S5 (Fig. 6B), which also contained the markers for synaptic vesicles synaptophysin and chromogranin B (Fischer-Colbrie et al., 1987, Jahn and Sudhof, 1994).

Uptake of NAD

To evaluate whether extracellular β -NAD⁺ can be taken up in brain synaptosomes, we incubated synaptosomes with 1 mmol/L eNAD, a highly fluorescent analogue of β -NAD⁺. eNAD is not an endogenous substance and hence, is not present in the intrasynaptosomal compartment under control conditions (Fig. 7A,B 0'). Thus under these conditions, any appearance of eNAD in the intrasynaptosomal compartment after incubation of synaptosomes with eNAD would indicate eNAD uptake. One-, 5- and 60-minute incubation with eNAD led to the appearance of eNAD in the intrasynaptosomal compartment (Fig. 7A). The eNAD products eADPR and eADO also appeared intrasynaptosomally. The appearance of eNAD in the intrasynaptosomal compartment was not affected by incubation of synaptosomes with the connexin 43 hemichannel inhibitor Gap 27, 100 μ mol/L, (Fig. 7B, C), but was significantly reduced by incubation of synaptosomes with 10 mmol/L β -NAD⁺ (Fig. 7E, $P=0.046$, $t=4.48$, $df=2$, paired t test, two-tailed). Likewise, the appearance of eNAD + eADPR + eADO in the intrasynaptosomal compartment remained unchanged by Gap 27 (Fig. 7D), but was reduced by authentic β -NAD⁺ (Fig. 7F, $P=0.0177$, $t=7.144$, $df=2$, paired t test, two-tailed). Incubation of synaptosomes with eADPR (1 mmol/L, 60 min) caused the appearance of 5.13 ± 1.04 nmol/mg protein ($n=2$) eADPR, suggesting that some ADPR that is formed in the synaptic cleft can also be taken up by synaptosomes.

Neuronal Ca²⁺ dynamics

To determine whether β -NAD⁺ affects target neurons, the effects of locally applied β -NAD⁺ were examined on cultured rat cortical neurons. [Ca²⁺]_i levels were assessed in neurons loaded with the high-affinity Ca²⁺ indicator Fura-2. Localized application of β -NAD⁺ caused a rapid, concentration-dependent [Ca²⁺]_i transients (Fig. 8A, Movie in Supplemental materials). β -NAD⁺ (50 mmol/L loaded in the picospritz pipette) produced ratio changes of 0.344 ± 0.027 ($n=9$) which equated to a $59.1 \pm 7.7\%$ increase in Ca²⁺ relative to responses to 40 mmol/L KCl applied at the end of each experiment (Fig. 7D; $n=9$ cultures). Almost all neurons responded to β -NAD⁺. On average 93.2% of neurons (identified by both morphology and sustained [Ca²⁺]_i elevations to KCl) located in front of the β -NAD⁺

application electrode responded with a stimulus-locked $[Ca^{2+}]_i$ transient (9 cultures, 148 total neurons). Control studies with identical localized pressure applications (500 ms) of either 1) solutions of matched osmolarity (NaCl) or 2) low pH HEPES buffer (pH 3) showed no change in $[Ca^{2+}]_i$ levels (Figs. 1S and 2S, Supplemental materials), indicating that the Ca^{2+} transients were not triggered by activation of mechanosensitive channels or pH changes, but by the action of β -NAD⁺. The rapid time course of Ca^{2+} transients suggested that transmembrane Ca^{2+} influx might be involved. To test this hypothesis, neurons were challenged with β -NAD⁺ in Ca^{2+} -free buffer. Reproducible β -NAD⁺ responses were first generated in normal buffer (containing 2 mmol/L Ca^{2+}) at 10 min intervals and then, after 10 min superfusion in nominally Ca^{2+} -free buffer, responses to β -NAD⁺ were abolished (Fig. 8B, Movie in Supplemental materials). A partial recovery was observed after reintroduction of Ca^{2+} -containing solution (not shown). These results are consistent with β -NAD⁺ stimulation of Ca^{2+} influx, rather than mobilization of Ca^{2+} from intracellular stores.

The approach of localized application of millimolar concentrations of β -NAD⁺ has been utilized in other preparations, to mimic aspects of accumulation at sites of transmitter release (Mutafova-Yambolieva et al., 2007, Hwang et al., 2011). We next sought to determine whether localized application of another purine nucleotide, ATP, elicits comparable responses in rat brain neurons. As shown in Fig. 9A similar Ca^{2+} transients were observed with ATP challenges, using identical pipette concentrations and delivery pulses. Although individual neurons could respond preferentially to either ATP or β -NAD⁺, the population responses demonstrated rapid onset Ca^{2+} transients with no significant difference in initial rise times. A full pharmacological characterization of the receptor type(s) involved in β -NAD⁺ responses is outside the scope of the present study. However, it is noted that activation of some P2X receptors is unlikely to explain the postsynaptic Ca^{2+} increases observed here. Thus, bath exposures to $\alpha\beta$ -MeATP at concentrations sufficient to fully desensitize P2X receptors (Evans et al., 1992) reduced but did not abolish β -NAD⁺ responses (Fig. 9B). Finally, we determined whether the direct metabolite of β -NAD⁺, ADPR (Lee, 2001, De Flora et al., 2004), could also stimulate postsynaptic neurons. As shown in Fig. 9C, robust Ca^{2+} transients were generated by localized ADPR applications. Like β -NAD⁺ responses, signals recovered quickly to baseline levels after termination of the stimulus, and the average peak amplitude of transients (Fura-2 ratio changes of 0.35 ± 0.018 ; $n=4$ cultures), was very similar to those reported above for β -NAD⁺ (0.34 ± 0.027 ; $n=9$ cultures). The rates of initial increase of Ca^{2+} signals in response to ADPR and β -NAD⁺ in Fig. 9C were 95.66 ± 16.57 and 122.14 ± 14.19 (percent of peak response/second), respectively ($P=0.167$, $t=1.616$, $df=5$, paired t test, two tailed).

Discussion

Of major importance in this study are the findings that β -NAD⁺, a ubiquitous intracellular constituent, can be released by brain synaptosomes upon membrane depolarization via vesicle exocytosis and can also directly stimulate cortical neurons. β -NAD⁺ was previously suggested as a novel neurotransmitter in the peripheral nervous system (i.e., Mutafova-Yambolieva et al., 2007, Hwang et al., 2011) and was shown to be released by cultured astrocytes (Verderio et al., 2001), but it has never been considered as a candidate for a neurotransmitter in brain nerve terminals. In the present study the high- K^+ -evoked release of β -NAD⁺ in isolated forebrain synaptosomes required intact synaptic vesicle exocytosis machinery. Mechanisms for removal of extracellular β -NAD⁺, including enzymatic degradation and neuronal uptake, were also present. Localized application of β -NAD⁺ produced Ca^{2+} transients in cortical neurons, suggesting that when released from presynaptic neurons, endogenous β -NAD⁺ could activate postsynaptic neurons. Taken together, these observations suggest that β -NAD⁺ qualifies as a *candidate* neurotransmitter in the brain.

Several lines of evidence in the present study support the notion that, like classical neurotransmitters, β -NAD⁺ is released upon membrane depolarization via vesicle exocytosis. Several lines of evidence in the present study support this notion: 1) membrane depolarization with high-K⁺ solutions caused overflow of β -NAD⁺ from viable synaptosomes used within 4–6 hours of fractionation (Gomez-Villafuertes et al., 2007), 2) the amount of released β -NAD⁺ appeared proportional to the membrane depolarization, 3) the high-K⁺-evoked release of β -NAD⁺ was abolished by inhibition of neuronal N-type voltage-dependent Ca²⁺ channels, 4) the evoked release of β -NAD⁺ was inhibited after disruption of the proton gradient in synaptic vesicles, and 5) cleavage of the SNARE protein SNAP-25 inhibited the high-K⁺-evoked release of β -NAD⁺. Taken together these observations suggest that vesicle exocytosis was the primary mechanism of β -NAD⁺ release in response to depolarization of the synaptosomal membrane.

After release of a neurotransmitter, mechanisms for recovery or local degradation of the substance are needed to terminate neural signals. β -NAD⁺ is the primary purine nucleotide in the β -NAD⁺+ADPR+cADPR mixture in synaptosomal superfusates, but yet, a significant formation of ADPR also occurred. Therefore, membrane-bound β -NAD⁺-degrading enzyme(s) are likely present on synaptosomal membranes. In agreement with previous studies in brain tissue (White, 1977, Sperlagh et al., 1998) we found that ATP was released upon high-K⁺-stimulation. The samples collected before and during stimulation also contained the direct ATP metabolite ADP, as well as AMP and adenosine as previously shown (Zimmermann, 1996). As depicted in Fig. 2, AMP and adenosine are degradation products of both β -NAD⁺ and ATP. Because the changes in total purines followed the changes in the β -NAD⁺ overflow better than those of the ATP overflow, it can be assumed that β -NAD⁺ is the primary source of AMP and adenosine in brain synaptosomes. Also, two approaches for evaluation of enzyme activities (i.e., small-chamber superfusion and at-rest-incubation) demonstrated that mechanisms for local degradation of β -NAD⁺ are available in brain synaptosomes. Finally, CD38, a type II integral membrane glycoprotein that exhibits ADP-ribosyl cyclase, cADPR hydrolase and NAD glycohydrolase activities (Graeff et al., 1998, Lee, 2001, De Flora et al., 2004) was present on synaptosomal membranes and in the intrasynaptosomal compartment. This is in agreement with previous observations showing that CD38 is expressed on plasma membranes and cell organelles of neurons and astrocytes in rat brain (Yamada et al., 1997). The catalytic site of membrane-bound CD38 faces the extracellular space (Lee et al., 1993, Munshi et al., 2000), making this enzyme suitable as a regulator of extracellular β -NAD⁺ levels (Billington et al., 2006). Therefore, once released β -NAD⁺ can quickly be converted into ADPR and cADPR in the extracellular space.

Efficient control of synaptic transmission and rapid termination of the action of neurotransmitters may be achieved not only by the degradation of the neurotransmitter substances but also by their uptake into neural cells by specific transporters (reviewed in Amara and Kuhar, 1993). Neurotransmitter uptake may also be an essential component in the recycling of neurotransmitters released during neural activity. To our knowledge the possibility that β -NAD⁺ can be imported across the plasma membrane to replenish intracellular or vesicle β -NAD⁺ pools in synaptosomes has not been addressed. However, information in other systems suggests that this may occur. For example, high concentrations of extracellularly applied NAD increase intracellular NAD levels in NIH3T3 fibroblasts, cultured murine astrocytes, human neuroblastoma SH-SY5Y, and HeLa cell types (Bruzzone et al., 2001b, Ying et al., 2003, Billington et al., 2008). NADH, a close “relative” of β -NAD⁺, can be transported across the plasma membranes of astrocytes by a P2X7R-mediated mechanism (Lu et al., 2007). Bidirectional NAD transport in isolated connexin 43-expressing murine 3T3 fibroblasts has also been reported (Bruzzone et al., 2001a). In the present study we demonstrate that incubation of synaptosomes with the fluorescent analogue eNAD caused appearance of this substance in the intra-synaptosomal compartment, which

otherwise does not contain eNAD. Simultaneous incubation with 10-fold higher concentrations of authentic β -NAD⁺ inhibited the appearance of eNAD in the intracellular compartment, suggesting that β -NAD and eNAD compete for the same transport mechanism(s). Inhibition of connexin 43 with Gap 27 did not significantly affect intrasynaptosomal uptake of β -NAD⁺, consistent with the notion that other transport mechanisms may be involved in rat brain synaptosomes. Defining the exact nature of β -NAD⁺ transporters warrants further studies. It is also possible that some amount of the β -NAD⁺ metabolites ADPR and ADO can be taken up directly through the synaptosomal membrane.

We demonstrated that, if released, exogenous β -NAD⁺ could affect neuronal functions. A subpopulation of CNS neurons responded to β -NAD⁺ with rapid Ca²⁺ transients, suggesting that extracellular β -NAD⁺ could indeed participate in neuron-to-neuron communication. Picospritzing β -NAD⁺ close to target neurons, instead of superfusing the substance, was meant to simulate rapid direct application and avoid some of the factors that might dampen responses. Concentrations of β -NAD⁺ in synaptic vesicles or in the synaptic cleft have not been reported. However the concentrations of β -NAD⁺ applied with picospritz pipettes were likely comparable to concentrations of neurotransmitters achieved near postsynaptic neurons following exocytosis, since in analogy with ATP and other neurotransmitters (Van der Kloot, 2003, Borycz et al., 2005, Fields and Burnstock, 2006, Zimmermann, 2008) the concentration of β -NAD⁺ in synaptic vesicles could likely reach up to 150–1000 mmol/L. By analogy glutamate concentrations within the synaptic cleft rise to millimolar levels when glutamate is released from a presynaptic vesicle (Clements et al., 1992). Thus, localized application of very small volumes and rapid dilution of millimolar concentrations of exogenous neurotransmitter substances appears to be a reasonable approach to determine whether exogenous β -NAD⁺ applications can indeed cause significant postsynaptic activation of central neurons. As shown in the present study equimolar concentrations of ATP produced Ca²⁺ responses with very similar amplitudes as observed for β -NAD in the same cell population. β -NAD⁺ failed to elicit Ca²⁺ transients in the absence of extracellular Ca²⁺ implying that the rapid neuronal responses are due to influx of Ca²⁺ from the extracellular space. These results differ from a recent report in rat cultured neurons (Fischer et al., 2009) which has demonstrated more modest Ca²⁺ increases following much longer exposures (10 seconds) to lower P2 receptor agonist concentrations (i.e., pressure application of 100 μ mol/L ATP). In that prior study, only metabotropic responses were detected, and it is likely that the robust Ca²⁺ influx responses observed in the present study with greater local concentrations of β -NAD⁺ or ATP were missed. It is also possible that during the longer period of ATP delivery some degradation of ATP might have occurred. Therefore, some of the observed effects in this prior study may in fact be due to bioactive ATP metabolites (e.g. ADP).

Localized application of ADPR, a purine nucleotide that could be formed by the NAD-glycohydrolase-mediated catabolism of β -NAD⁺, produced Ca²⁺ transients with similar rates of onset in neurons that also responded to β -NAD⁺. ADPR might be generated after release of β -NAD⁺ from brain neurons, or ADPR could be stored and released as a primary neurotransmitter. At present we cannot clearly distinguish between these possibilities, however our data demonstrate that both β -NAD⁺ and ADPR are bioactive in cortical neurons and suggests that multiple purines could contribute to postsynaptic responses in brain neurons. The dynamics of the responses to β -NAD⁺ and ADPR appear comparable and β -NAD⁺ and ADPR likely function in concert in brain neurons. α , β -Me-ATP-sensitive receptors (i.e., P2X1 and P2X3 types, North, 2002) appeared to be only partially responsible for the Ca²⁺ transients in response to locally applied β -NAD⁺. Future studies should determine the mechanisms underlying the evoked influx of extracellular Ca²⁺ including possible activation of ionotropic receptors, non-selective cation channels or voltage-operated

Ca^{2+} channels. Likewise, defining the subpopulation of $\beta\text{-NAD}^+$ -sensitive neurons should shed more light on the functional significance of $\beta\text{-NAD}^+$ release in the brain.

In conclusion, our data provide the first evidence for the release of $\beta\text{-NAD}^+$ by membrane depolarization of brain synaptosomes, for extracellular degradation and synaptosomal uptake of $\beta\text{-NAD}^+$, as well as for $\beta\text{-NAD}^+$ and ADPR-induced rapid activation of cortical neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADO	adenosine
ADP	adenosine 5'-diphosphate
ADPR	adenosine 5'-diphosphate ribose
$\alpha,\beta\text{MeATP}$	α,β -methylene-ATP
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BoNT/A	botulinum neurotoxin A
$\beta\text{-NAD}^+$	beta-nicotinamide adenine dinucleotide
CNS	central nervous system
$\omega\text{-Ctx GVIA}$	ω -conotoxin GVIA
cADPR	cyclic ADPR
cGDPR	cyclic guanosine diphosphate-ribose
eADO	1, N^6 -etheno-ADO
eADP	1, N^6 -etheno-ADP
eADPR	1, N^6 -etheno-ADPR
eAMP	1, N^6 -etheno-AMP
eATP	1, N^6 -etheno-ATP
eNAD	1, N^6 -etheno-NAD
HPLC	high-pressure liquid chromatography
HPLC-FLD	high-pressure liquid chromatography with fluorescence detection
KBH	Krebs-bicarbonate-HEPES
NGD	nicotinamide guanine dinucleotide
SNAP-25	the 25-kDa synaptosomal associated protein

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Figure 1. Protocol for isolation of synaptosomes from rat forebrain

Different velocities of cold ultracentrifugation and Percoll gradient centrifugation were used to obtain intact synaptosomes (P3). In some experiments synaptosomal membranes were hypotonically disrupted and supernatant (S5) was used as intrasyntosomal content as described in Methods.

Figure 2. Diagram of major enzymatic pathways for the degradation of β -NAD⁺ and ATP
 CD38 exhibits NAD glycohydrolase (NADase), ADP-ribosyl cyclase and cADPR hydrolase activities and degrades β -NAD⁺ to ADPR and cADPR. ADPR is degraded to AMP by nucleotide pyrophosphatases (NPPs). AMP in turn is degraded to ADO by 5'-nucleotidase. ATP is degraded by a family of ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) to ADP and AMP, and then to ADO by 5'-nucleotidases.

Figure 3. Spontaneous release and high K^+ -evoked release of adenine purines

KCl 25 mmol/L (closed circles, $n=7$) or 65 mmol/L (open circles, $n=13$) evoked overflow of ATP (A), ADP (B), AMP (C), ADO (D), and β -NAD+ADPR+cADPR (E) in rat isolated forebrain synaptosomes. The sum of all purines (i.e., ATP+ADP+AMP+ β -NAD+ADPR+cADPR+ADO) is also shown (F). Data are averaged for each individual purine as well as for the sum of all purines (total purines) and are expressed as the mean $\pm$ SE. * $P<0.05$ vs. controls at 0 min. One-way ANOVA followed by *post-hoc* Bonferroni's multiple comparison tests. Note the sustained pattern of the overflow of β -NAD $^+$ and total purines.

Figure 4. Effects of botulinum neurotoxin A (BoNT/A), bafilomycin A1 and ω -conotoxin GVIA (ω -Ctx GVIA,) on spontaneous and high- K^+ -evoked release of adenine purines
 (AD) Original chromatograms of superfusates from synaptosomes pretreated with either KBH solution (controls, n=13), BoNT/A (300 nmol/L for 2 h, n=6), bafilomycin (0.1 μ mol/L for 30 min, n=6) or ω -Ctx GVIA (20–50 nmol/L for 30 min, n=6) and superfused with 25 mmol/L K^+ for 5 minutes. Superfusate samples were collected at 0, 1, 2, and 5 minute of superfusion with high K^+ -solution. The peak eluting at ~11.5 minute is formed by β -NAD⁺+ADPR+cADPR; this peak is labeled as β -NAD⁺ to reflect the primary compound in the β -NAD⁺+ADPR+cADPR mixture. Scales apply to each groups of chromatograms. LU, luminescence units. (E, F) Data are averaged for β -NAD⁺ and total purines and are expressed as the mean $\pm$ SE. In contrast to controls the high K^+ solution failed to evoke an increased release of purine nucleotides in the presence of BoNT/A, bafilomycin A1 or ω -Ctx GVIA. *P<0.05 vs. controls at 0 minutes. One-way ANOVA followed by *post-hoc* Bonferroni's multiple comparison tests.

Figure 5. Degradation of exogenous purine substrates during superfusion of synaptosomes
(A) Ecto-ATPase activity. Left panel: Original chromatograms of 50 nmol/L eATP in the absence of synaptosomes (–s) and after 1-minute contact with synaptosomes (+s); LU, luminescence units. Note the decrease in eATP and the increase in eADP, eAMP, and eADO in the (+s) samples. Right panel: Graphic representation of e-product (eADP+eAMP+eADO) formation in superfusate samples collected before (–s) and during 1-min perfusion of synaptosomes with eATP (+s). **(B) NAD glycohydrolase activity.** Left panel: Original chromatograms of 0.2 μmol/L eNAD in the absence (–s) and presence of synaptosomes (+s). Note the decrease in eNAD and the increase in eADPR formation in the (+s) samples. Right panel: Graphic representation of eADPR formation in superfusate samples collected in the absence (–s) or presence (+s) of synaptosomes. **(C) ADP-ribosyl cyclase activity.** Left panel: Original chromatograms of 0.2 mmol/L NGD in the absence (–s) and presence of synaptosomes (+s). Note the increase in cGDPR formation in the (+s)-samples. Right panel: Graphic representation of cGDPR formation in superfusate samples collected in the absence (–s) or presence (+s) of synaptosomes. *P<0.05, **P<0.01. Data represent mean ± SE from 4 experiments with each substrate, paired Student's t-test.

Figure 6. Degradation of eNAD and expression of CD38

(**A**) NAD glycohydrolase activity determined by a 60-minute incubation of synaptosomes with 1 mmol/L eNAD. Original chromatograms of eNAD in the absence (-s) and presence of synaptosomes (+s); LU, luminescence units. Note the increased formation of eADPR and eADO in contact with synaptosomes. (**B**) Western analysis showed expression of CD38 (45 kDa) in intact synaptosomes and in the intrasynaptosomal compartment. Intrasynaptosomal compartment also expressed the synaptic vesicle markers synaptophysin (42 kDa) and chromogranin B (78 kDa).

Figure 7. Uptake of eNAD

Original chromatograms of intra-synaptosomal content of eNAD after 0, 1, 5, and 60-minute incubation of intact synaptosomes with 1 mmol/L eNAD alone (**A**) and in the presence of Gap 27 (100 μ mol/L, $n=2$) (**B**). Incubation with eNAD caused appearance of eNAD and its metabolites eADPR and eADO in the intra-synaptosomal compartment. Gap 27 had no effect on the intrasynaptosomal levels of eNAD or eNAD⁺+eADPR+eADO (**C**, **D**). Simultaneous incubation of β -NAD⁺ (10 mmol/L) and eNAD (1 mmol/L) abolished the appearance of eNAD in the intrasynaptosomal compartment and reduced the intrasynaptosomal levels of eNAD+eADPR+eADO after 60-minute incubation, $n=3$ (**E**, **F**). Data represent mean $\pm$ SE, * $P<0.05$, unpaired Student's *t*-test.

Figure 8. Intracellular Ca²⁺ increases in a cultured neurons following localized applications of β -NAD⁺

(A) Rapid Ca²⁺ increases in 6 neurons identified by number in the culture illustrated in the *inset*. β -NAD⁺ was applied via a brief (500 ms) pressure pulse from a microelectrode (E). Two consecutive responses are shown (2 min interstimulus interval), showing that responses in individual neurons are reproducible. (B) After 10 min of exposure to Ca²⁺-free buffer, responses to the same β -NAD⁺ stimuli were abolished. (C) For comparison with β -NAD⁺ responses, Ca²⁺ elevations are shown in the same neurons after restoration of extracellular Ca²⁺, and bath application of 40 mmol/L KCl. Scale bar: 30 μ m.

Figure 9. Similar neuronal $[Ca^{2+}]_i$ transients with ADPR and ATP

(A) $[Ca^{2+}]_i$ increases in the same neurons, following sequential applications of β -NAD⁺ and ATP. The delivery micropipette was changed between β -NAD⁺ and ATP trials, during a 10 min interval indicated by the axis break. (B) Desensitization of P2X receptors by pre-exposure to α , β -methylene ATP (50 μ mol/L, 10 min) reduced, but did not abolish β -NAD⁺ responses. (C) Comparison of responses to β -NAD⁺ and its metabolite ADPR in the same neurons. As in A, the delivery micropipette was changed between challenges with the two agonists. Identical pipette concentrations (i.e., 50 mmol/L) and delivery pulses (500 ms) were used for β -NAD⁺, ATP and ADPR.

Table 1

Intra-synaptosomal content and spontaneous overflow of purines in rat brain synaptosomes (pmol/mg protein).

	ATP	ADP	AMP	ADO	β -NAD+ADPR+cADPR	Total purines
Intra- synaptosomal content (n=37)	100.3 $\pm$ 15.5	191.2 $\pm$ 33.4	588.5 $\pm$ 99.8	2176 $\pm$ 268.8	4485 $\pm$ 602	7542 $\pm$ 934
Spontaneous overflow (n=65)	0.17 $\pm$ 0.027	0.96 $\pm$ 0.07	1.50 $\pm$ 0.11	3.84 $\pm$ 0.82	8.76 $\pm$ 0.82	15.19 $\pm$ 1.15